BIOLUMINESCENCE IN FOCUS – A COLLECTION OF ILLUMINATING ESSAYS

EDITOR
VICTOR BENNO MEYER-ROCHOW

RESEARCH SIGNPOST

Bioluminescence in Focus
A Collection of Illuminating Essays
2009

Editor
Victor Benno Meyer-Rochow

Faculty of Engineering and
Science, Jacobs University
D-28759 Bremen
Germany

Department of Biology
University of Oulu
SF-90014 Oulu
Finland

Bioluminescence in Focus
A Collection of Illuminating Essays
2009

Published by Research Signpost

Research Signpost
T.C. 37/661(2), Fort P.O.,
Trivandrum-695 023, Kerala, India

Editor
Victor Benno Meyer-Rochow

Managing Editor
S.G. Pandalai

Publication Manager
A. Gayathri

ISBN: 978-81-308-0357-9

Contents

Preface

A book like this one is the product of teamwork. But how to select the right team and the right topics, so that the book does indeed become a valuable item of support in our struggle to advance knowledge and understanding of bioluminescent phenomena and in getting more scientists interested in at least one of the many branches of bioluminescence research? Three main thoughts occupied my mind when planning to produce this book. First, there was the question of the target group. Whom are we writing the book for, who actually is it we want the book to read and from where should the book be available? Ideally it ought to be of interest to all broad-minded scientists, but specifically to biologists and more particularly to bioluminescence enthusiasts (or those who want to become some). Therefore, the book should be available in universities, libraries, perhaps high schools, and of course book stores. But should the book address only professionals? Shouldn't a good book also appeal to non-specialists? Of course it should.

Obviously, whether potential buyers find a book attractive and readable depends on the chapters within it and, naturally, the authors, who wrote the articles. Thus, my second thought revolved around the question whom I could ask to participate in this venture. I needed to enlist the help of people, who really had something to contribute to the book, had something important to "say" like presenting new results, concepts, and ideas or critically discussing old results and examining them from different angles, in a novel way, looking at and presenting them in a new light.

The third problem I faced when planning this book concerned the scope and the extent to which it was meant to cover the various and somewhat disjunct aspects of bioluminescence research as well as the widespread occurrences of bioluminescence in the living world. For instance, a book solely dealing with the biochemistry of bioluminescence reactions or focusing entirely on fireflies or dealing exclusively with new molecular results might be of considerable interest to a few highly specialized researchers, but would hardly be a "winner" when it came to the wider audience. On the other hand a book with plenty of beautiful colour photographs and little text, reporting only the most exciting and amazing cases of bioluminescence, a book like that might well be an "eye-catching" tome and entertaining, but not exactly useful for those who would want to pick up a book to learn something from and to gain some deeper insights into what lay behind bioluminescent phenomena. Afterall, new discoveries,

reported almost monthly in international scientific journals, are constantly being made and appear all the time. Nobody wishes to be left behind, but how to keep up with the pace of discoveries?. The internet is not always the answer, especially when it comes to summaries, reviews, and integrated treatises. Thus, there still is a need for a book like this, which contains in a single volume a diverse range of up-to-date reports, all stemming from recent results in the field of bioluminescence research.

In the end, I think, not only did I win the support of a wonderful team of contributors, experts in their own fields of bioluminescence research, but also succeeded in obtaining an exciting blend of articles of a wide range of bioluminescent phenomena. I truly believe that the diversity of 'our' field could not have been covered in any better way in a single book of this size than what this book actually does. We have chapters dealing with luminescent sharks and other light-producing creatures of the sea; there are articles that occupy themselves with morphological aspects of luminescence in freshwater invertebrates; other chapters tackle biochemical and genetic questions of beetle bioluminescence, in particular those related to fireflies, while a couple of essays provide exceptionally detailed analyses of the ecology and ethology of terrestrial and cavernicolous light-producers like glowworms. Furthermore we have chapters that deal with bacterial luminescence and applications of it; even luminescent earthworms in the soil have a chapter devoted to them. And finally, the reader will find an expert review scrutinizing the still poorly understood phenomenon of biophoton emissions, a branch of bioluminescence that has begun to assert itself only recently as a separate and very exciting field of scientific inquiry.

Although layout and format of the individual chapters conform to the standards set by the publisher of this series of educational science books, I deliberately abstained from too strict an editorial control and wanted each author to retain her or his own unique style of writing. I am convinced that this adds to the 'flavour' of a book like this and makes more interesting reading as if each chapter were written in the same uniform way.

Diversity of styles, chapter lengths, and illustrations, thus, mirror the multifarious nature, the many facets, of our subject of inquiry: Bioluminescence. I have, of course, tried my best to iron out typing errors, ambiguities, and unnecessary or erroneous statements before they made it into the printed chapters, but ultimately the responsibility for the chapters, and especially the references to the works of others, remains with the authors, to whom I express my sincere gratitude. Each author has worked diligently and with enthusiasm to make this book a success. They were a brilliant team to work with. I also acknowledge the support received from the publisher and in particular Dr Pandalai and Mrs. Gayathri. Furthermore I have to thank my

friends, colleagues, and students - too many persons to mention individually- for the stimulating discussions I've had with them, for having provided interesting ideas and for having made a variety of valuable suggestions, often unintentionally and unprovoked. Last but not least I am deeply grateful to Sulochana, Yamuna, Susmita and Ayu for their constant encouragement and support, for having been so wonderfully patient and tolerant all the time, and for having accepted without a single word of complaint that I often failed to spend as much time with them as they had expected me to do.

I hope this book will fill a gap and find a readership, curious and keen to learn about bioluminescence, keen to dive into the fascinating world of living lights, and ready to emerge thereafter a little bit brighter than before!

V.B. Meyer-Rochow

Contributors

BAJPAI, Rajendra P.
Sophisticated Analytical Instruments Facility, North Eastern Hill University
Shillong 793022, India. E-mail: rpbajpai@nehu.ac.in

BAKER, Claire
GPO Box 267, Yungaburra, QLD 4884, Australia
E-mail: bugsyclaire@yahoo.com.au

BALLANTYNE, Lesley
School of Agricultural and Veterinary Sciences, Charles Sturt University
PO Box 588 Wagga Wagga 2678, Australia
E-mail: rballant@optusnet.com.au

BECHARA, Etelvino J. H.
Departamento de Bioquimica, Instituto de Quimica, CP 26077
Universidade de São Paulo, 05513-970 São Paulo, and Departamento
de Ciências Exatas e da Terra Universidade Federal de São Paulo
09972-270 Diadema, SP, Brazil. E-mail: ebechara@iq.usp.br

BROADLEY, R. Adam
Australian Quarantine & Inspection Service (AQIS) – Entomology
P.O. Box 1006, Tullamarine, Victoria 3043, Australia
E-mail: adam.broadley@aqis.gov.au

CLAES, Julien M.
Laboratory of Marine Biology, Catholic University of Louvain
3 Place Croix du Sud Kellner Building, B-1348 Louvain-la-Neuve
Belgium. E-mail: julien.m.claes@uclouvain.be

CLARKE, Arthur K.
School of Zoology, University of Tasmania, Hobart, Tas 7001, Australia
E-mail: a.clarke@utas.edu.au

COSTA, Cleide
Museu de Zoologia, Universidade de São Paulo, CP. 42494,04218-970
São Paulo, SP, Brazil. E-mail: cleideco@usp.br

CRONIN, Thomas W.

Department of Biological Sciences, University of Maryland, Catonsville MD 21228, U.S.A. E-mail: cronin@umbc.edu

DAY, John

Centre for Ecology and Hydrology (CEH) Oxford, Mansfield Road, Oxford OX1 3SR, England, U.K. E-mail: jcda@ceh.ac.uk

DE COCK, Raphaël

Associate Researcher, Evolutionary Ecology Group, University of Antwerp B-2610 Antwerp, Belgium. E-mail: rdecock@hotmail.com

FU, Xinhua

Department of Plant Science and Technology
Hua Zhong Agricultural University, Wuhan, 430070, China
E-mail: fuxinhua2001@yahoo.com

KUZNETSOV, Alexander M.

Institute of Biophysics, Siberian Division, Russian Academy of Sciences
Akademgorodok, 50, Krasnoyarsk, 660036, Russia
E-mail: ccibso@ibp.ru

LALL, Abner B.

Department of Biology, Howard University, Washington, DC 20059, USA
E-mail: alall@howard.edu

LEWIS, Sara M.

Department of Biology, Tufts University, Medford MA 02155, USA
E-mail: Sara.Lewis@tufts.edu

MALLEFET, Jérôme

Laboratory of Marine Biology, Catholic University of Louvain, 3 Place Croix du Sud, Kellner Building, B-1348 Louvain-la-Neuve, Belgium
E-mail: jerome.mallefet@uclouvain.be

MEDVEDEVA, Svetlana. E.

Institute of Biophysics, Siberian Division, Russian Academy of Sciences
Akademgorodok, 50, Krasnoyarsk, 660036 Russia
E-mail: ccibso@ibp.ru

MERRITT, David, J.

School of Integrative Biology, The University of Queensland, Brisbane, Qld 4072, Australia. E-mail: d.merritt@uq.edu.au

MEYER-ROCHOW, Victor Benno

Faculty of Engineering and Science, Jacobs University, D-28759 Bremen Germany. E-mail: b.meyer-rochow@jacobs-university.de
and
Department of Biology, University of Oulu, SF-90014 Oulu, Finland
E-mail: vmr@cc.oulu.fi

MOGILNAYA, A. O.

Institute of Biophysics, Siberian Division, Russian Academy of Sciences Akademgorodok, 50, Krasnoyarsk, 660036 Russia
E-mail: ccibso@ibp.ru

MOORE, Stephen

Landcare Research, Tamaki Campus, University of Auckland, Private Bag 92170 Auckland, New Zealand. E-mail: moores@landcareresearch.co.nz

OBA, Yuichi

Graduate School of Bioagricultural Sciences, Nagoya University, 464-8601 Nagoya Japan. E-mail: oba@agr.nagoya-u.ac.jp

OHBA, Nobuyoshi

« The Ohba Firefly Institute », 4-1-12-204 Maborikaigan, Yokosuka City, 239-0801 Japan. E-mail : qgb00523@nifty.ne.jp

POPOVA, L. Yu

International Center for Research of Extreme States of Organisms, Krasnoyarsk Scientific Center SB, RAS, Akademgorodok, 50, Krasnoyarsk, 660036 Russia.
E-mail: ccibso@ibp.ru

RODICHEVA, Emma K.

Institute of Biophysics, Siberian Division, Russian Academy of Sciences Akademgorodok, 50, Krasnoyarsk, 660036, Russia. E-mail: ccibso@ibp.ru

ROSENBERG, Joerg

Central Animal Laboratory, University Duisburg-Essen Medical School D-45122 Essen, Germany. E-mail: sommerhaus-rosenberg@t-online.de

ROTA, Emilia

Department of Environmental Sciences, University of Siena, I-53100 Siena
Italy. E-mail: rota@unisi.it

SHIMOYAMA, Ayu

c/o Meyer-Rochow Research Group, Eläinmuseo, University of Oulu
SF-90014 Oulu, Finland. E-mail: ayu.shimoyama@gmail.com

STRINGER, Ian A. N.

Science & Research Unit, Department of Conservation, P.O. Box 10420
Wellington, New Zealand. E-mail: istringer@doc.govt.nz

TYULKOVA, Nataliya A.

Institute of Biophysics, Siberian Division, Russian Academy of Sciences
Akademgorodok, 50, Krasnoyarsk, 660036, Russia. E-mail: ccibso@ibp.ru

VIVIANI, Vadim R.

Laboratorio de Bioluminescencia e Biotecnologia, Universidade Federal de Sao Carlos
Campus de Sorocaba, Sorocaba, SP, 08060-070 São Paulo, SP, Brazil
E-mail: viviani@ufscar.br

Research signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 1-8
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

1 Bioluminescence through the ages: A brief, but enlightening survey

V.B. Meyer-Rochow
Faculty of Engineering and Science, Jacobs University, D-28759 Bremen Germany; Department of Biology, University of Oulu, SF-90014 Oulu Finland

Abstract

The phenomenon of living lights has fascinated humans for ages. First seen merely as something inexplicable and mysterious, something to be either fearful or cheerful of, bioluminescence gradually began to be investigated scientifically and from a variety of angles. Although we are still far from having discovered and described all of the biological phenomena in which light is produced, we are beginning to enter a new phase. The future will see applications of bioluminescence in a variety of fields;

Correspondence/Reprint request: Prof. Dr. V.B. Meyer-Rochow, Faculty of Engineering and Science, Jacobs University, D-28759 Bremen, Germany. E-mail: b.meyer-rochow@jacobs-university.de

we shall possess a better understanding of the bioluminescence reactions than ever before and will routinely be able to manipulate the genes responsible for the reactions. What will not change, however, is the awe-inspiring beauty of the living lights and our quest to understand more and more of it and to probe deeper and deeper into bioluminescent phenomena.

1. First observations

Bioluminescence has come a long way. Not only were bioluminescent bacteria most likely present already at the "dawn of life" more than 900 million years ago, when oxygen was a highly toxic, dangerously reactive element that could be rendered less damaging by involving it in a chemical reaction that resulted in the emission of light [1, 2]; no, the phenomenon of light-producing organisms also attracted the attention of humans from the beginning of mankind. Mentioned in writing for the first time about 3,000 years ago in the Shi Jing, known as the 'Book of Songs' or 'Book of Odes', it says there "I-yao hsiao-hsing", which can be translated as 'glowing intermittently are the fireflies' [3]. Another early and interesting mention of the phenomenon of living lights comes from the Upanishads, close to 2,000 years ago:

> "Fog, smoke, sun, fire, wind,
> Fireflies, lightning, a crystal, a moon –
> These are the preliminary appearances,
> Which produce the manifestation of Brahma"
> (Svestasvatara Upanishad 2.8-15, *cf.* Wilson [4])

Nagarjuna, a sage also from ancient India, compares Buddha to firefly and sun and in Europe's antiquity the Greek philosophers Thales, Anaximenes, and Aristotle are credited with having been the first to observe and describe occurrences of marine bioluminescence [5].

In the New World the Mayans in particular must have been fond of fireflies, for they attributed to them supernatural powers, which gave these insects spiritual, symbolic meaning. Often Mayan deities contained elements of stars and were represented as fireflies [6, 7]. Coe [8] even suggested that in Mayan mythology fireflies were associated with the underworld. Traditionally, references to cigarette smoking employed the word 'firefly' and it is thought smoke and fire could have been associated with firefly lights. Other linguistic constructs from Mayan cosmology based on or related to firefly luminescence are known [9]. Mayan art frequently depicts fireflies, often with an exaggerated and enlarged abdomen, presumably highlighting the region from which the light appears. Such art can be found in temples, on vases, utensils of every day use, etc. [10].

2. Wondrous lights

Mysterious lights in the forests have been reported, described, and commented on from various parts around the world. Fairytales, making reference to 'foxfires', 'will-o-the-wisp', corpse-lights and the like, link the luminescent phenomena to goblins, ghosts, human and non-human spirits, etc. and even Sir Isaac Newton (1704, reprinted 1952 [11]) in his "Opus Opticks" mentioned the "*ignis fatuus*" (Latin for 'living fire'). What all these observations most likely have in common and are based on, are small luminescent fungi, growing on rotting wood in damp places. I, myself, have collected a small greenish light-emitting fungus from a dead tree trunk in the Papua New Guinea jungle (Fig. 1) and can testify to the eerie glow radiating from the tree trunk at a distance and I can well believe that shamans could have used wooden pillars with growth of luminescent fungi on the sides of a doorway to light the front of their living space [12, 13].

Of course, luminescent fungi, whose light has been thought to either attract small positively phototactic insects to help dispersing the fungal spores or, on the contrary, to attract parasitic wasps to specifically attack the spore-eating insects, need not necessarily have been the only reason for the 'forest-light folklore'. As has been convincingly argued by Callahan and Mankin [14], atmospheric electrical discharges could lie behind the phenomenon

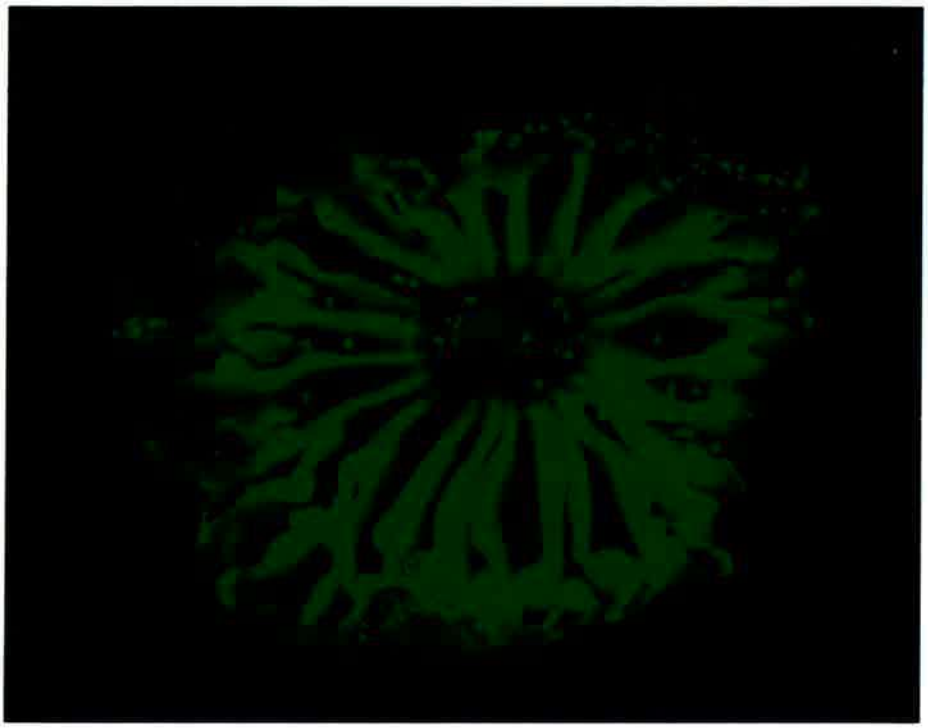


Figure 1. Unidentified luminescent fungus (computer-enhanced) from Kaibola Island (Papua New Guinea), collected by V.B. Meyer-Rochow in 2004.

of small insects glowing under thunderheads and being surrounded by a bright luminescent halo: the bluish light seen under these conditions could be a manifestation of the famous "St Elmo's fire". Naturally, ordinary people would not distinguish between these purely physical and truly bioluminescent phenomena and in northern countries even auroral lights could have played a role.

In northern Finland, the inhabitants (often still being referred to as Lapps or Sami) describe the northern lights as 'revontulet', which translates into 'fox fire'. In Lapland, in the land of the Sami, there lies a huge lake, known as "Lake Inari". Interestingly, in Japan mysterious lights in the woods are associated with 'Inari', the God of grain and rice, and the spirit of a fox, termed 'kitsune'. Foxes are considered to possess supernatural abilities and thought to be able to produce light with their tails, even guiding lost persons to their home [15]. Could it be that ancient beliefs on luminescence were kept alive into our days, linking far-flung and seemingly unrelated nordic cultures? Even the biblical story of the burning bush showing Moses the way, according to Schaechter [13], could have had its origin in bioluminescence, for it is known that in Scandinavia pieces of glowing oak bark were left at critical places in the woods to allow foresters to find their way home in the dark.

3. Solving luminous mysteries

When I was living in Jamaica and serving as the Director of the Electron Microscopy Unit, I frequently took visitors to a place known as "Glistening Waters". There, at night, one could witness fish leaving a comet-like trail of light behind or one could swim oneself in a sea of luminescence (Fig. 2) and bathe in liquid light [16]. A fantastic experience, caused by billions of bioluminescent dinoflagellates that seem to find ideal growth conditions in this and a few other bays of the West Indies. Yet, while the luminescence of these areas and the causes of its luminescence (as well as other forms of marine luminescence [17]) have been studied relatively well, there are luminescent phenomena of the sea that still defy explanation: the so-called "Milky Seas". Herring and Watson [18] quote from the log book of Captain Kingman in 1865:

"The whole appearance of the ocean was like a plain covered with snow. There was scarce a cloud in the heavens, yet the sky....appeared as black as if a storm was raging. The scene was one awful grandeur; the sea having turned to phosphorus, and the heavens being hung in blackness, and the stars going out, seemed to indicate that all nature was preparing for the last grand conflagration, which we are taught to believe is to annihilate this material world".

Perhaps the great Jules Verne knew about Kingman's log or had been given similar information by some other seaman, for in his 1870 classic "20,000

Figure 2. Photograph taken at night with the natural light of the luminescent dinoflagellates, probably *Pyrodinium* sp., of a person floating in Jamaica's "Glistening Waters", Falmouth.

Leagues under the Sea" he narrates travelling through the milky sea of the Bay of Bengal for about 40 miles in January and mentions uncountable numbers of microorganisms. Nealson and Hastings [19] then report of the "eureka moment", which Miller et al. [20] must have experienced, when they discovered the incredible agreement between Jules Verne's account and the milky sea sighting, logged by the British merchant ship *S.S. Lima*, transiting that same area on the night of 25 January 1995.

Another relatively recent quote comes from August 13 th, 1986 and, once again, refers to the Northwest Indian Ocean: "The entire sea surface took an intense white glow, which was not unlike viewing the negative of a photograph." In fact, since 1915, there have been 235 documented cases of similar observations at the British Meteorological Office's Bioluminescence Database [21]. What could be behind these strange luminescent phenomena of the seas?

According to Miller et al. [20] more than 70% of the reported cases involved the northwestern Indian Ocean, while 17% came from Java, Indonesia. The phenomenon, moreover, seemed to have a seasonal component as most occurrences took place in the months of January and August. Furthermore, distance from land and depth of the water seemed to be

important. The 'milky light' was generally reported to last from several hours up to several days and appeared to be independent of the wind speed. Herring and Watson [18] noted that if bacteria were the cause, then a tremendous amount of such bacteria was needed to produce the amount of light associated with the milky seas.

Satellite pictures, published in the Proceedings of the National Academy of Sciences of the USA in 2005, show a region in the Indian Ocean, the size of Connecticut, glowing in the typical "milky sea" way and since only bacteria, and not dinoflagellates, can glow continuously, such observations from space strengthen the hypothesis that the causal agents behind the fascinating light emissions of the milky seas are, indeed, the smallest of the luminescing organisms: bacteria. Miller et al. [20] estimate the number of bacteria involved to have been around 40 trillion.

4. A look ahead

And where do we stand today? The enigma of the milky seas is only half-solved, but hundreds of bioluminescent species, from marine, terrestrial and freshwater habitats, have over time become known to science. The chemical nature of a variety of bioluminescent reactions has been elucidated, the biological function of the emitted light is known for a certain number of species, for more and more bioluminescent species we possess the genetic information, and biotechnological applications of bioluminescence are being pursued. Tests and experimental trials how best to use and further develop the new technology are being conducted in very diverse fields, but chiefly in the medical disciplines (pathology), animal husbandry, horticulture, etc.

Yet, despite some of the scientific unveiling of the mysteries that were associated for millennia with the living lights, bioluminescence never fails to inspire, still holds some of its age-old magic, and still creates a great deal of scientific interest. The role of bioluminescent phenomena in the lore of traditional peoples and different cultures has scarcely been touched and one could be reminded of the, until recently largely neglected, role that insects played in human societies [22]. Simple questions like those that Meyer-Rochow [16] posed ("why has not a single bioluminescent species of spider been discovered and why are there some bioluminescent *diurnal* fireflies?) continue to baffle and occupy researchers even to this day. Other questions of a more general character for which there is still no consensus address the problem of the origin of bioluminescence in bacteria and the various phyla of eucaryotes. Ever more sophisticated methods have resulted in the discovery of light emissions by living organisms that are so weak that the organisms themselves can probably not see them. They are the so-called biophoton emissions [23, 24] and have addedd yet another dimension to the field of

Bioluminescence Research. Very poorly investigated is also the phenomenon of pathological luminescence, in which individuals of an otherwise non-luminescent species begin to shine and emit light as a consequence of an infection with luminescent bacteria [25].

Thus, as we have seen, lots of open questions remain, in fact abound, and exciting new and often surprising discoveries are still being made at an astonishing rate. A school teacher by the name of G. East from Christchurch New Zealand, only a few years ago, wrote to me that he had collected brightly luminescent springtails from his garden. Until his observation, the occurrence of luminescent springtails from New Zealand was totally unknown. The description of the luminescent males of a species of South American cockroach by Zompro and Fritzsche [26], the investigation of the causes of luminescent termite mounds in Brazil by Bechara [27], the finding that the red-light-emitting deep sea fish *Malacosteus niger* contains a chlorophyll-like photosensitizer in its eye, allowing it to approach prey that is insensitive to red-light [28] as well as those numerous recent reports of an 'invasion' of the tiny luminescent oligochaete *Microscolex phosphoreus* on European golf courses, all of these were unexpected, thought-provoking, new discoveries, providing incentives for further study into this phenomenon of never-ending fascination: Bioluminescence.

Acknowledgement

I wish to thank Ms Emanuela Kozhushkova for her help in locating some interesting and relevant ethnobiological literature and Ms Seija Leskela of Oulu University for data processing.

References

1. Hastings, J.W. 1978, Bacterial and dinoflagellate luminescent systems. In Bioluminescence in action, P. Herring (Ed.). Academic Press, New York, 129-170.
2. Nealson, K.H., and Hastings, J.W. 1979, Microbiol. Rev., 43, 496-518.
3. Harvey, E.N. 1952, Bioluminescence, Academic Press, New York.
4. Wilson, A. 1991, World's scripture: a comparative anthology of sacred texts. International Religious Foundation, Paragon House Publishers, St Paul (Minnesota) and New York.
5. Lloyd, J.E. 1971, Ann. Rev. Entomol.,16, 97-122.
6. Milbrah, S. 1999, Star gods of the Maya. University of Texas Press, Austin.
7. Lopes, L. 2004, http://www.mesoweb.com/features/lopes/Fireflies.pdf.
8. Coe, M.D. 1973, The Maya scribe and his world. Grolier World, New York.
9. Watanabe, J.M. 1983, Man NS, 18, 710-728.
10. Robicsek, F., and Hales, D.M. 1981, The Maya book of the dead: the ceramic codex. University of Oklahoma Press, Norman.

11. Newton, I. 1952, Opus opticks, or a treatise of the reflections, refractions, inflections and colours of light. Dover Publications, New York.
12. Glawe, A., and Solberg, W.U. 1989, Mycologia, 81, 296-299.
13. Schaechter, M. 1998, In the company of mushrooms. Harvard University Press, Cambridge.
14. Callahan, P.S., and Mankin, R.W. 1978, Appl. Optics, 17, 3355-3360.
15. Opler, M.E., and Hashima, E.S. 1964, Am. Anthropologist, NS 48, 43-53.
16. Meyer-Rochow, V.B. 2001, The Biologist, 48, 163-167.
17. Widder, E.A. 1999, Bioluminescence. In Adaptive mechanisms in the ecology of vision, S.N. Archer, M.B.A. Djamgoz, J.C. Loew, J.C. Partridge, and S. Vallenga, (Eds.). Kluwer, Dordrecht, 555-581.
18. Herring, P.J. and Watson, M. 1993, Mar. Observer, 63, 22-30.
19. Nealson, K.H., and Hastings, J.W. 2006, Appl. Environ. Microbiol., 72, 2295-2297.
20. Miller, S.D., Haddock, S.H.D., Elvidge, C.D., and Lee, T.F. 2005, Proc. Nat. Acad. Sci., 102, 14181-14184.
21. Watson, M., and Herring, P.J. 1992, Mar. Observer, 62, 182-183.
22. Meyer-Rochow, V.B., Nonaka, K., and Boulidam, S. 2008, Entomologie Heute, 20, 3-25.
23. Kobayashi, M., and Inaba, H. 2000, Appl. Optics, 39, 183-192.
24. Wijk, R.V., and Wijk, E.P. 2005, Forsch. Komplementarmed. Klass Naturheilkd., 12, 77-83.
25. Pfeiffer, H., and Stammer, H.J. 1930. Z Morphol. Oekol. Tiere 20, 136-171.
26. Zompro, O., and Fritzsche, I. 1999, Amazoniana, 15: 211-219.
27. Bechara, J.H.E. 1988, Adv. Oxygenat. Proc., 1, 123-178.
28. Douglas, R.H., Partridge, J.C., Dulai, K.S., Hunt, D.M., Mullineaus, C.W., and Hynninen, P.H. 1999, Vision Res., 39, 2817-2832.

Research signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 9-26
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

2 Heterogeneity of natural populations of marine luminescent bacteria

S. E. Medvedeva[1], O. A. Mogilnaya[1] and L. Yu. Popova[2]
[1]Institute of Biophysics, Siberian Division, Russian Academy of Sciences Akademgorodok, 50, Krasnoyarsk, 660036, Russia; [2]International Center for Research of Extremal States of Organisms, Krasnoyarsk Scientific Center SB RAS, Akademgorodok, 50, Krasnoyarsk, 660036, Russia

Abstract

Population variability is characteristic for the majority of microorganisms. We have been investigating heterogeneity in natural populations of the following marine luminescent bacteria: Photobacterium phosphoreum, P. leiognathi, P. sp., Vibrio harveyi, V. fischeri. As usual, the culture was a binary population of cells with various electronic densities of cytoplasm, with different store inclusions or intracellular membrane structures, but one cell morphotype was dominated in the populations of these

Correspondence/Reprint request: Dr. S. E. Medvedeva, Institute of Biophysics, Siberian Division, Russian Academy of Sciences, Akademgorodok, 50, Krasnoyarsk, 660036, Russia. E-mail: ccibso@ibp.ru

luminous bacteria. An accumulation of electron-transparent inclusions (poly-β-oxybutyrate – POB) in the cells was connected with the luminescence intensity level as well as dependent on growth phase and medium composition. Some certain luminous strains (str.54, 208, 213 P. leiognathi) had cells with electron-dense inclusions of a diameter of about 30 nm in the nucleoid area. Among psychrophilic P. sp. and mesophilic P. leiognathi, strains with greater heterogeneity in the population were found. Populations of psychrophilic luminous bacteria exhibited a certain cycle of development, but at each stage had no less than two morphotypes. Psychrophilic strains changed cell wall thickness due to the expense of microcapsule formation during their growth. The investigated isogenic variants of the luminescent bacteria P. leiognathi 54 were pleiotropic and inherited fairly stable morphological characteristics, colony architectonics, level of luminescence, and activity of some enzymes; variants with reduced bioluminescence formed colonies of the S type. Stable, bright variants with S- and R-type colonies appeared both in the initial strain population and in the dark variant population, but with smaller frequency. Populations of the bright variant with R-type colonies were most heterogeneous; this can be determined by the lack of glucose repression of the bioluminescence in contrast to other investigated inherited variants of P. leiognathi. The decrease or increase of the expression level of bioluminescence could be caused by changes in lux-operon regulation.

1. Introduction

Population variability in the phenotypic manifestation of gene expression is characteristic of the majority of microorganisms [1,2]. The expression of many genes can alter with changes in the substrate [3]; this is particularly important for the maintenance of collections of microorganisms with specific characteristics. The heterogeneity of microbial populations is caused by a wide variety of bacteriophages and also by migratory genetic elements, transposons, integrons, and plasmids [4,5]. Adaptive mutations and regulatory mechanisms play a special role in the manifestation of heterogeneity: they cause cumulative effects in the phenotypic variability of microbial populations [6-8]. Such cumulative effects caused by the common regulation of several operons are known for marine luminescent bacteria [9-11]. For instance, coordinated regulation of the genes of riboflavin synthesis and of *lux* genes occurs in the cells of *Photobacterium phosphoreum* [12]. Genetic polymorphism was revealed in symbiotic luminescent bacteria of *P. leiognathi* [13] and in the luminescent bacteria of the genus *Vibrio* [14]. With the change of their habitat, luminescent bacteria

lose their usual physicochemical environment and nutrient sources [15]. At the present time there is information that the bacterial population is heterogeneous in terms of its cell ultrastructure with prevalence of one or two morphotypes. The cell types determine the basic properties of the culture, as well as the cytomorphological variety of the population and affect the survival of the bacteria under varying environment conditions. Hence, when the metabolic activity of the cells changes, it can result in decreases or increases of the bioluminescent signal.

At cultivation the luminous bacteria produce not only dark and dim, but also brightly luminous variants with increased enzyme activity. These variants can transform from one into another with high frequency [16,17]. Intertransition of variants is not an independent process. Knowledge of the tendency of formation of variants with various expression of the luciferase gene is important for understanding the mechanism of luminescent system functioning of luminous bacteria and for maintaining a stable luminescent population in museum and other scientific collections.

The collection of cultures of marine luminescent bacteria (CCIBSO 836), isolated from different parts of the World Oceans, has been maintained for many years in the Institute of Biophysics, Siberian Division, Russian Academy of Sciences (IBP SB RAS). Variants with different intensity of bioluminescence (dark, dim, and bright) appear in the populations of luminescent bacteria during re-plating and cultivation [9, 18]. The heterogeneity of populations of luminescent bacteria is primarily assessed as the visual heterogeneity of luminescence of the colonies and colony morphology on plates with solid nutrient medium. The application of electron microscopy to investigate morphologic changes makes it possible to assess in detail the structure of the population, the processes of cell differentiation, and dissociation into subpopulations. Such information is particularly important for the understanding of the reasons for the appearance of variants with reduced activity of the bioluminescent system in the initial culture of luminescent bacteria.

The heterogeneity of population of marine luminescent bacteria of different species that are stored in CCIBSO, in particularly *P. leiognathi* strain 54, depending on the conditions of cultivation and the functional activity of luciferase-producing strains, are discussed.

2. Heterogeneity of the manifestation of bioluminescence in luminescent bacteria

Bacterial bioluminescence is a chemiluminescent reaction, which involves reduced flavin mononucleotide, oxygen, long-chain aldehyde, and

the enzyme luciferase; its end products are fatty acid, water, and visible light [9, 19,20]:

$$FMN.H_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + \text{light}$$

The dynamics of the manifestation of bioluminescence in luminescent bacteria is rather variable, depending on their growth conditions. In batch mode of cultivation, the intensity of cell luminescence changes in parallel with increasing quantity of enzymes and substrates participating in the reaction of the bioluminescence. The concentration of the synthesized enzymes and substrates depends on a number of regulatory mechanisms, which involve the protein repressor of *lux* operon and the corresponding inducer of the homoserine lactone class. It also depends on the complex of the catabolism-activating protein (CAP) with cAMP, which ensures the positive regulation of the action of bacterial operons [20]. The bioluminescence inhibition in media with glucose occurs due to the last mechanism; the repression of the *lux* operon in minimal medium is rapid (Fig. 1). In minimal media, the synthesis of regulatory proteins is limited due to lack of nutrients, and the specific intensity of the luminescence per cell is always higher than that in rich media at high growth rates. In media with peptone and, particularly, with fish extract, the dynamics of luminescence depends on the phase of bacterial growth to a greater extent than in minimal media. The relationship between the bioluminescence and various metabolic processes in the cell explains such differences in the manifestation of the

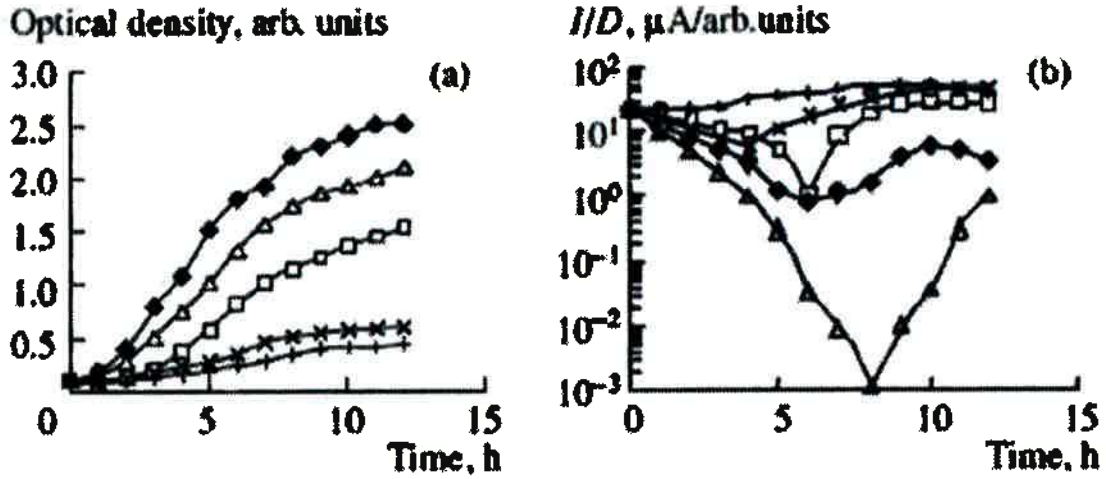


Figure 1. The dynamics of the growth (a) and the luminescence (b) of luminescent bacteria *P. leiognathi* 54 in batch culture on different media: with glucose (x Mglu), glycerol (|, Mglyc), glucose and peptone (Δ, Pglu), glycerol and peptone (□, Pglyc), peptone, and glycerol and fish extract (♦, PglycFE).

dynamics of luminescence intensity depending on the media composition. Thus, changes in *lux* operon expression may depend not only on mutations in its genes or on the medium composition but on the changes of regulator control that can result in both increase and decrease in the synthesis of the enzymes responsible for the bioluminescence reaction [21]. If the regulation is common to the cells, heterogeneity in the populations of luminescent bacteria may be pleiotropic.

K variants (spontaneously arising variants with decreased bioluminescence) do not entirely lose their ability to emit light; this is evidence of changes in the regulation of *lux* operon expression. These variants reveal weak light emission caused by the low content of luciferase and by all or several substrates of the luciferase system (Fig. 2). K variants are rare in the cultures maintained under conditions of fast growth and intense aeration, while limited oxygen supply in dense cultures and high temperature are favorable for the emergence of variant forms. However, the reasons for the origin of this phenomenon, which is common among luminescent bacteria, are still obscure. For instance, the bright luminescent variant of the marine strain *P. leiognathi* 54 was maintained on the standard medium with peptone and preserved a high level of luminescence for many years, but exhibited practically no population heterogeneity in this respect. However, rapid accumulation of dark and dim variants in the population of *P. leiognathi* 54 occurred after passages on rich media (supplemented with fish extract) or after storage under petrolatum in semiliquid agar (oxygen limitation) (Fig. 2, phenotypes 2, 3). On the contrary, cultivation of *P. leiognathi* 54 in poor medium without peptone resulted in the emergence of variants with constitutive expression of *lux* operon in the population (Fig. 2, phenotypes 4, 5). Variants with intermediate bioluminescence intensity were also isolated.

Investigation of the bioluminescence dynamics of dark, dim, and bright variants in batch culture on media of different composition revealed the regulatory character of such manifestations of population heterogeneity that determines by the metabolic activity of the cells. The data presented in Figs. 1 and 2 demonstrate that the bioluminescence of the initial phenotype of the strain *P. leiognathi* 54 (phenotype 1) depended on the presence of glucose in the medium (catabolite repression of the *lux* operon). Under these conditions, the bioluminescence level did not depend on the addition of an exogenous substrate (C_{14} aldehyde) to the cell suspension. The addition of the supernatant of a bright cell culture with the autoinducer for the *lux* operon reduced the duration of the latent period in the bioluminescence dynamics in phenotypes 1–3 (Fig. 2, curve *3*) in comparison with the control (Fig. 2, curve *1*).

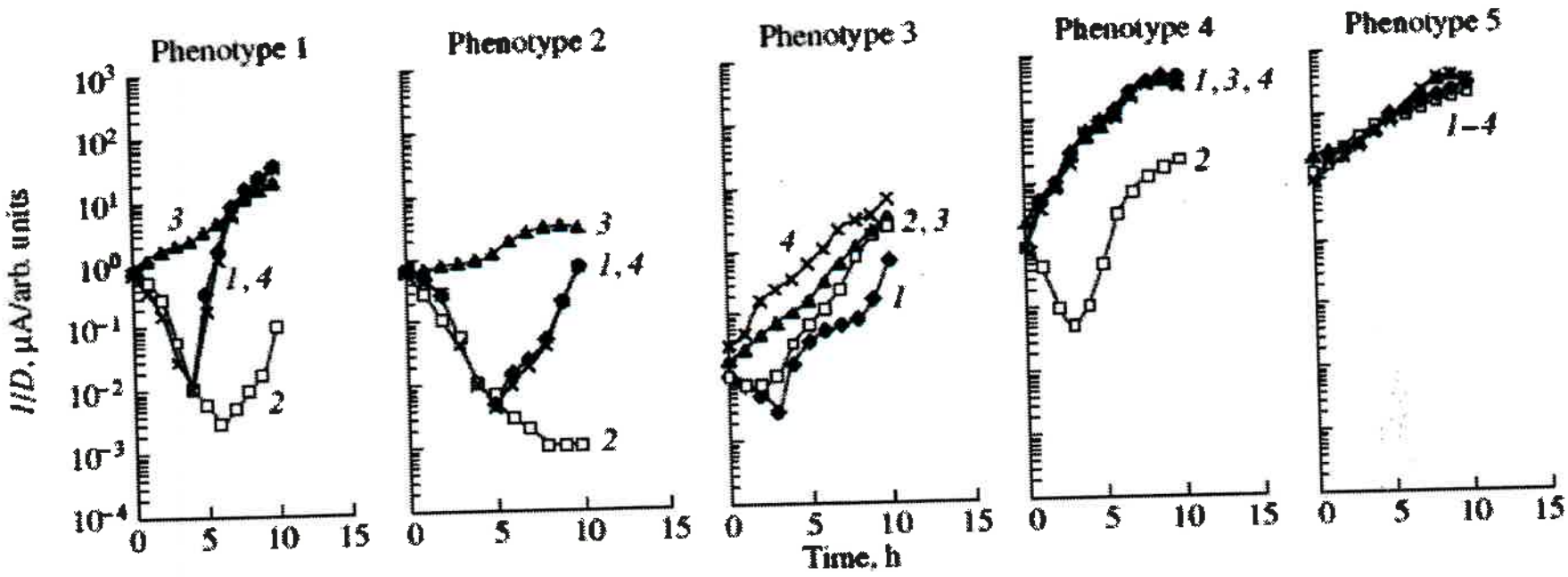


Figure 2. Specific luminescence of isogenic variants emerging in the population of luminescent bacteria *P. leiognathi* 54 during longterm storage. Phenotype 1, Rep+Ind+Cap+Ald+ (original wild type phenotype); phenotype 2, Rep++Ind–Cap++Ald+ (the variant with amplified control of *lux* genes expression); phenotype 3, Rep+Ind+–Cap–Ald– (the variant with impaired catabolite control of *lux* genes expression and with bioluminescence depending on exogenous aldehyde); phenotype 4, Rep–Ind++Cap+Ald+ (the variant with impaired control of *lux* genes expression visible in constitutive synthesis of the *lux* operon enzymes, with but conserved catabolite control of bioluminescence); phenotype 5, Rep–Ind++Cap–Ald+ (the variant with completely impaired control of *lux* genes expression, constitutive synthesis of the *lux* operon enzymes in the absence of catabolite control of bioluminescence). 1, specific luminescence on the medium with peptone and glycerol; 2, specific luminescence on the medium with peptone and glucose; 3, specific luminescence on the medium with peptone, glycerol, and additional inducer; 4, specific luminescence on the medium with peptone and glycerol after addition of exogenous myristic aldehyde.

The cells with low bioluminescence level that appear in the population of *P. leiognathi* 54 preserve their basic parameters in the bioluminescence dynamic, although they are more dependent on additional autoinducer (phenotype 2) or on exogenous myristic aldehyde (phenotype 3). The catabolic control may be impaired in the cells of certain dark variants (for instance, in the variant with phenotype 3, Fig. 2). Impairments of the catabolic control is more pronounced in those cells with constitutive expressions of the *lux* operon (phenotype 4 and phenotype 5). However, the dependence of the bioluminescence dynamics on the additional autoinducer or on exogenous aldehyde was not detected in the cells with such an unregulated phenotype of *lux* operon expression.

The knowledge of the tendency of formation of dark variants in luminescent bacteria populations and of the factors that determine the reversible transformation between bright and dark forms is necessary for an understanding of the ecology of luminescent bacteria. More profound investigations of the properties of the variants with changed luminescence expression can expand the idea of the role of the luminescent system in the metabolism of luminescent bacteria and determine the relationship between growth and bioluminescence.

3. Cytomorphological characteristics of mesophilic luminous bacteria

The various strains under investigation were cultivated on solidified and in liquid nutrient media, in order to examine the cytomorphological heterogeneity of the populations. For example, the *P. leiognathi* population contained cells with similar form and size at the beginning of cultivation in a liquid medium (Fig. 3 A). Then, by comparison with the initial cells, cells with electron-transparent inclusions became larger and more dense, dominating the stationary phase of the growth (Fig. 3 B). After inoculation of such cells on solid media it was found that all grown colonies emitted light and did not exhibit any obvious differences in form and consistence. Both kinds of cell possessed very similar ultrastructural organization and differed only in size and quantity of their electron-transparent inclusions in them. In some experiments *V. harveyi* cells formed pili under cultivation with deviations from the optimal conditions (Fig. 3 C).

The population of luminous bacteria can contain a few different cell types. Morphotype 1 had diffuse nucleoid, localized in the centre of the cell; numerous ribosomes and electron-transparent areas of poly-oxybutyrate (POB) in the cytoplasm (Fig.4, A, B, E).

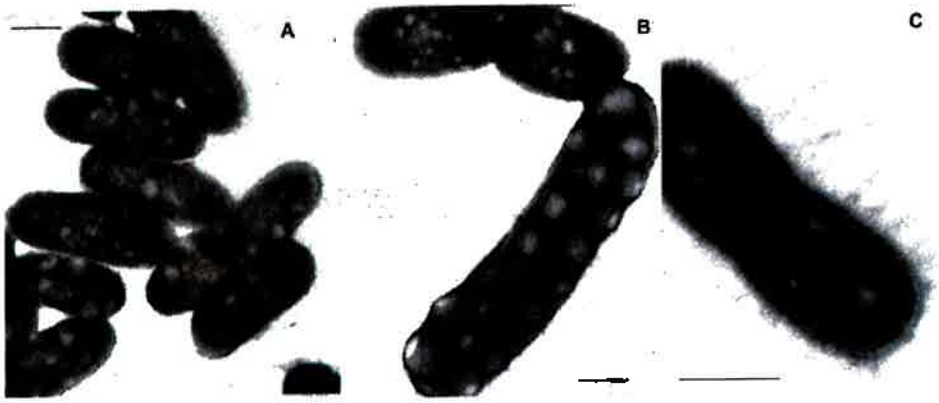


Figure 3. Homogeneous (A) and heterogeneous (B) populations of *P. leiognathi* under cultivation in mode chemostat and *V. harveyi* cells with pili (C.) Scale bar, 0.5 μm.

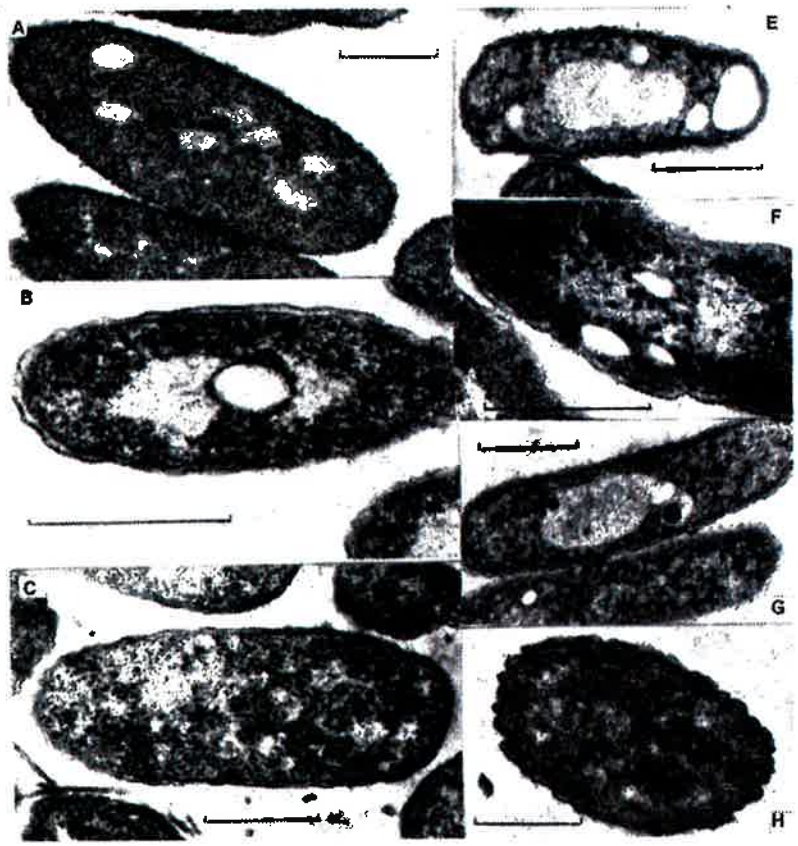


Figure 4. Cell ultrastructure of luminous bacteria *P.phosphoreum* (A, H), *P.leiognathi* (E, F, G), *V.harveyi* (B). and *V.fischeri* (C). Scale bar, 0.5 μm.

Morphotype 2, due to close packing of ribosomes, was characterized by high electron density of the cytoplasm, with cell shapes being of the usual form or of pleomorphic appearance (Fig.4, C, H). It is interesting that morphotype 1 dominated in *V. harveyi* str. 44 under cultivation in flasks on a shaker and in the chemostat mode. But colony populations of *V. harveyi*

(str. 1212) and *V. fischeri* (str. 1231) contained cells that were typical for the given species (Fig.4 B) and pleomorphic cells. Electron-transparent inclusions were found in all of the studied luminous species, even in *V. harveyi* cells (Fig 3C, 4B), which was unusual for the given species [9]. Yet, later additional research revealed electron-transparent inclusions (POB) in *V. harveyi* cells [22]. Moreover, non-luminous *P. leiognathi* strain *5* had cells with electron-transparent (POB) inclusions (Fig. 4G).

Osmiophilic granules in the cytoplasm (e.g., in *V. harveyi* cells) and small electron-dense inclusions of a size of about 30 nm, located in the nucleoid zone (in *P. leiognathi* 54, 213, 208) were revealed under cultivation in several experiments (Fig. 4 F). They were observed in the control medium [17] and in the medium with hydrolyzate from hydrogen bacteria as a substitute of peptone [23]. Probably, the presence of electron-dense inclusions in the nucleoid of luminous bacteria cells is a strain-specific attribute, arising as a result of secondary metabolic changes accompanying luminescence. The nature of these inclusions is still an open question.

The colony morphology and ultrastructure of *P. phosphoreum* bacteria were investigated simultaneously with the registration of its luminescence intensity. The bacterial population was distinguished as bright, dim and dark variants following long-term cultivation in the chemostat. Accumulation of dim and dark variants was observed during cultivation that was accompanied by a reduction of luminescence intensity in the chemostat culture [24]. Bacterial clones grown from separated colonies exhinited different luminescence levels (bright, dim, dark) and bath cultures of these clones of the luminous bacteria had heterogeneous populations, containing cells with different ultrastructures. The first type of cells prevailed in brightly luminescent populations, but dense pleiomorphic cells of the second type prevailed in the dark population, while both cell types were found in the dim population. Numerous cells with moderate electronic density of their cytoplasm contained intracellular membranes. We can possibly assume that the luminescence of the dim variant is less than the one of the brightly luminous culture, because the 1 cell type was not prevailing in dim variant.

4. Cytomorphological characteristics of psychrophilic *Photobacterium spp.*

A more complex structure of a population was observed in the culture of the psychrophilic luminous bacteria *P. spp.* 125 and 151. Upon growth on RPA these cultures dissociated to S- and the R-forms, in which the cells changed their form from coccus-like to rod- and egg-shape during development. The cells of the R-variant were non-uniform with regard to

electronic density. Chains and conglomerates covered with slime were quite often formed and pleiomorphic cells appeared. However, at each stage of development the population consisted mainly of one morphotype with a few cells from the following stages (Fig. 5). The ultrastructure of cells from the S-colony also varied during growth, and 2 morphotypes were found in the population (Fig. 6). One cell type, prevailing and similar to the first type, has been described above for *P. phosphoreum*. They formed the electron-transparent inclusions during a stationary phase of growth. Lamellar and ring-shaped membrane structures were observed in the initial phases of growth and then disappeared thereafter. In a logarithmic phase the majority of the cells had thinner cellular envelopes than in the initial phase. It was found that an additional outside layer as well as slime were tightly adjoined to an outside membrane (Fig. 6), derived from acid mucopolysaccharides [25]. Another morphotype appeared to be a combination of the usual pleiomorphic cell type with cytoplasm of greater density not unlike that of the second type of *P. phosphoreum*. It is interesting to note that this type of cell also possessed thick cell walls in the logarithmic phase of growth. Probably, ultrastructural changes are common for the two morphotypes and testify to an identical orientation of metabolism in these cells.

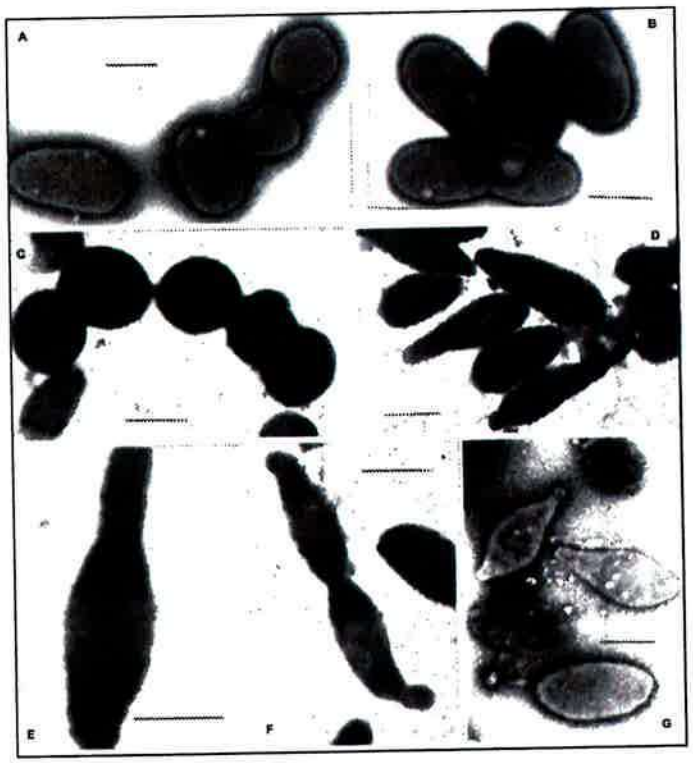


Figure 5. Morphological changes of cells during population development of psychrophilic luminous bacteria *P. sp.*: A – lag-phase; B, C – logarithmic phase; D, E, F, G – stationary phase. Scale bar, 0.5 μm.

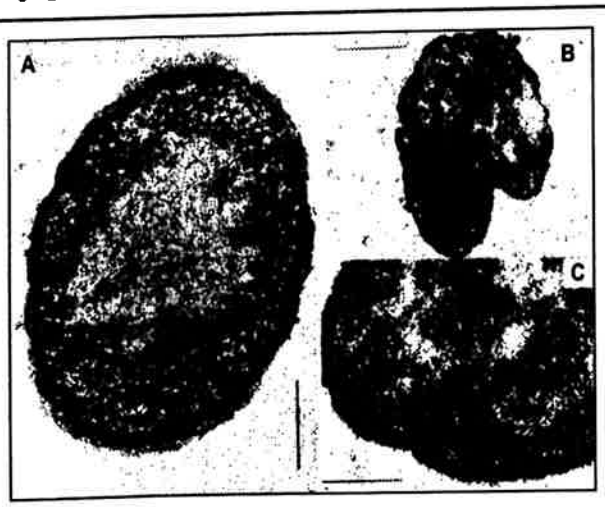


Figure 6. Cell structure of psychrophilic luminous bacteria *P. sp.*125. A –crosssection of a cell; B – staining of an additional layer of a cellular wall by ruthenium red (culture from a logarithmic growth phase); C – structure of the cellular wall in a logarithmic growth phase. Scale bar 0.2 μm.

5. Structure of colonies *P. leiognathi* 54

The investigation of the dynamics of inherited variability of *P. leiognathi* 54 revealed several inherited variants that appeared with a frequency of 10^{-3} -10^{-5} and which exhibited differences in luminescence intensities (Fig. 7) as

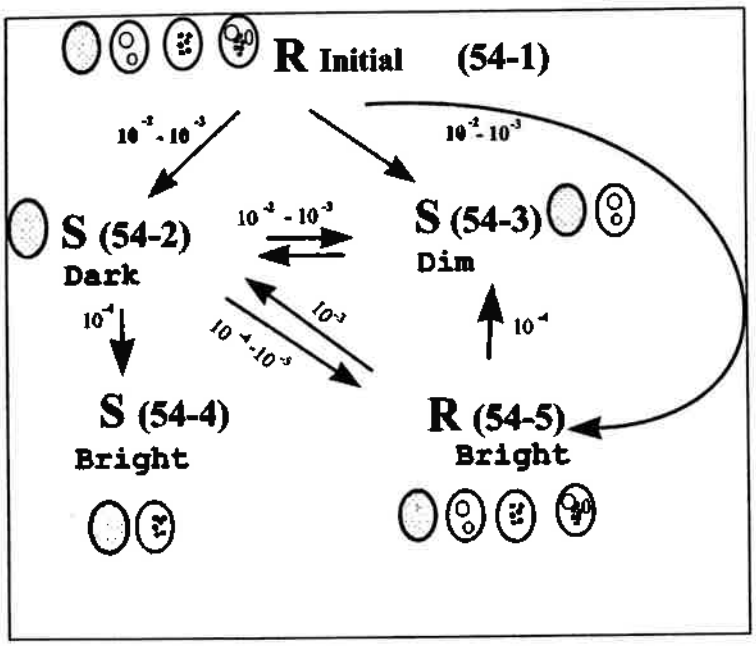


Figure 7. Scheme of the mutual conversions of isogenic variants in the population of *P. leiognathi* 54. The morphologic types of colonies (R, S), ultrastructural differences of cells in these colonies (see more detailed descriptions in text) and frequency of the variants' mutual conversions are shown.

well as physiological and biochemical properties. All of the selected variants showed a fairly stable inheritance of the investigated morphological characteristics at colony level, with regard to luminescence characteristics, and in terms of the activity of *lux* operon enzymes.

Three-day colonies of the investigated variants of the luminescent bacteria *P. leiognathi* 54 had distinct morphological characteristics. They differed in their surface geometry, density, color, and luminescence intensity. Electron microscopy of the structure of three-day colonies revealed that they consisted of cells of different ultrastructure and with different localization within the colony (Fig. 8–10). On the basis of the revealed differences, the observed morphological features were classified in accordance with the cytoplasmic condition and the presence and character of the inclusions. The first type of cells was coccobacilli; the outer membrane of the cell wall had a slightly wavy profile, the cytoplasm was homogeneous and filled with ribosomes, and the thin fibrillar nucleoid was located in the central zone (Fig. 8 A). The cells of the other types can be considered derivatives of the first cell type. The second type was characterized by the presence of electron-

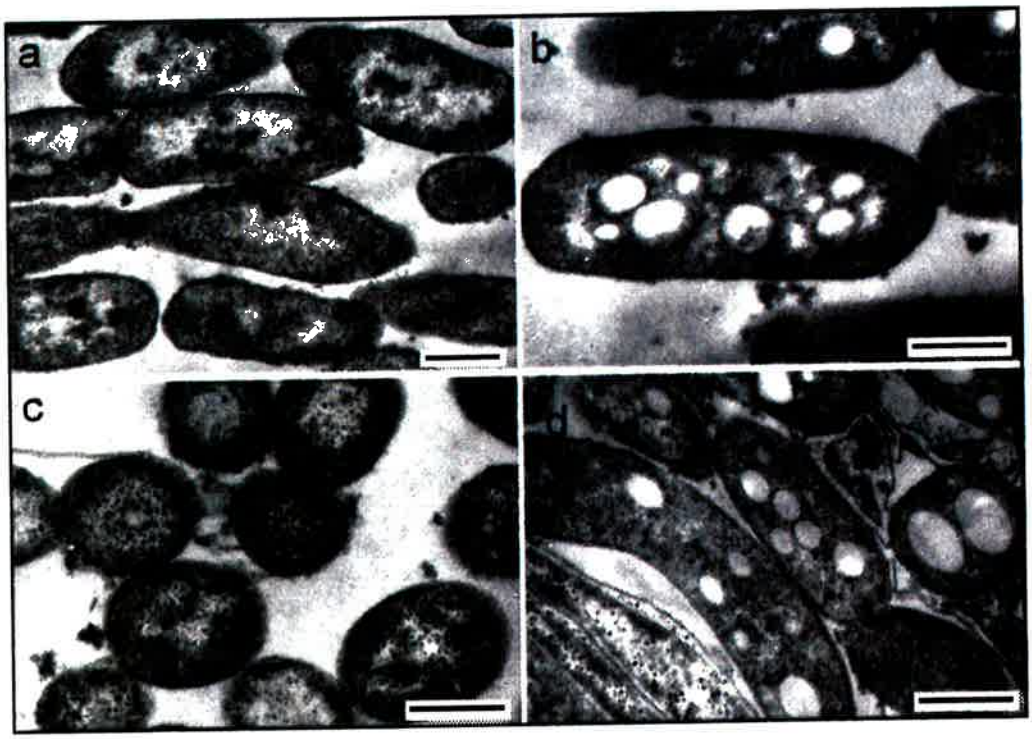


Figure 8. Ultrastructure of the cells from 3-day colonies of isogenic variants of *P. leiognathi* 54: a - cells in smooth (S) colonies of dark variant 54-2 (phenotype 2); b - cells with electron-transparent inclusions in S-colonies of dim variant 54-3 (phenotype 3); c - homogeneous population of cells with electron-dense granules in S-colonies of luminescent variant 54-4 (phenotype 4); d - heterogeneous population in rough (R) colonies of bright variant 54-5 (phenotype 5). Scale bar, 0.5 μm.

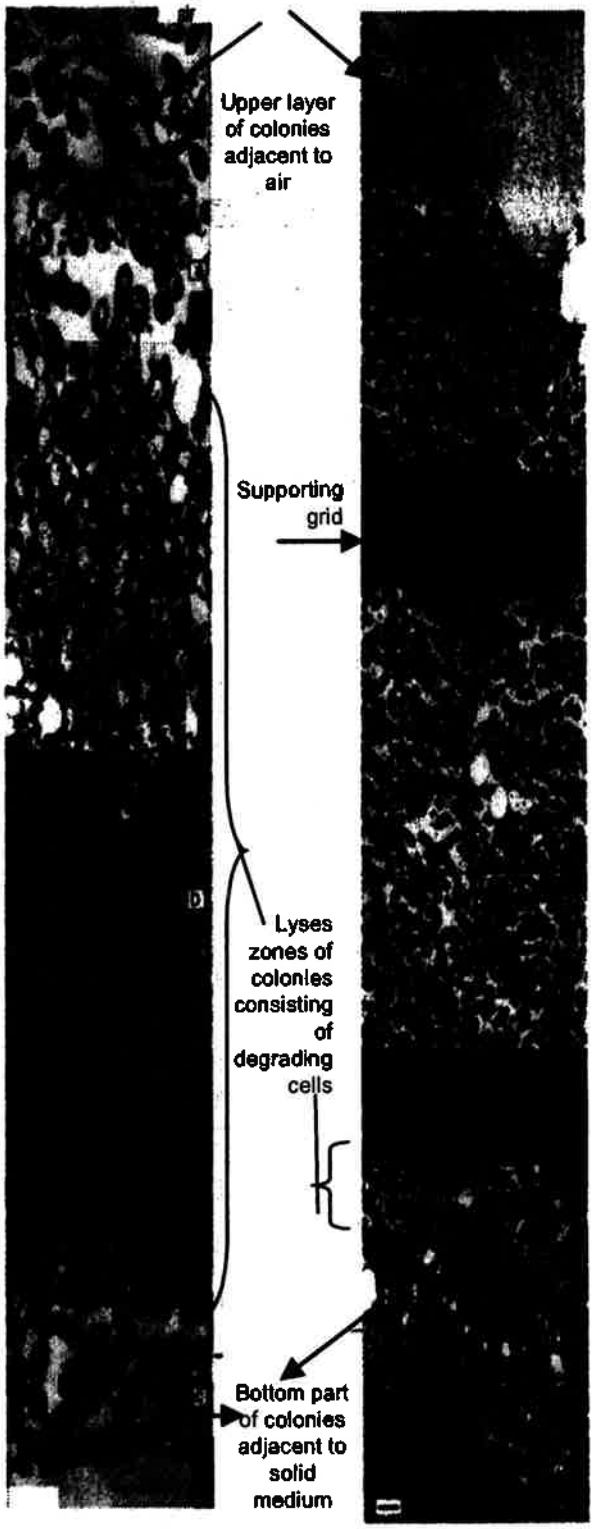


Figure 9. Ultrathin vertical sections of *P.leiognathi* 54 7-day colonies.

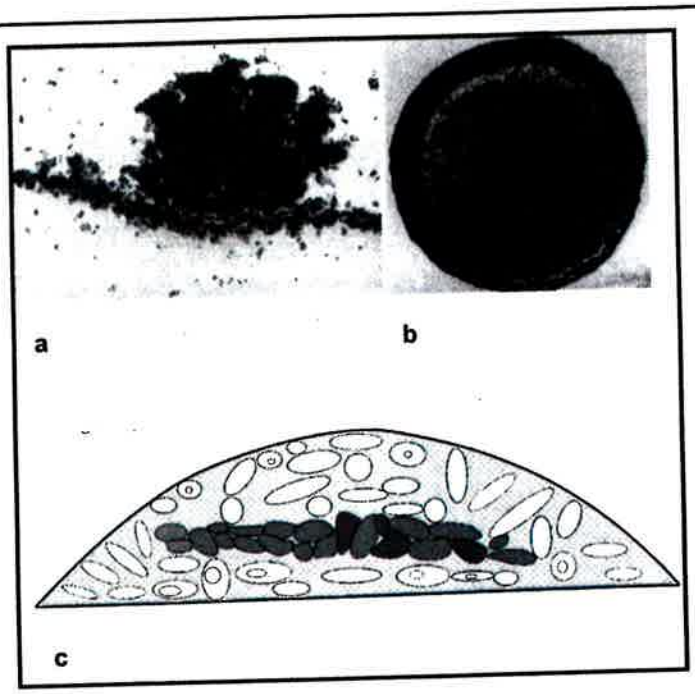


Figure 10. Microcolony formation (a) and mature colony (b) of *P. leiognathi* 54, and schematic presentation of the location of active (light) and lysed (dark) cells in colonies of *P. leiognathi* variants (c).

Table 1. Morphological features and cell composition of the colonies of variants arising in *P. leiognathi st.* 54.

Variants	Morphological features of colonies	Number of cell types in colony		
		1-day colonies	3-day colonies	7-day colonies
54-1	Rough, semitransparent, whitish, luminescent	5 types	5 types	5 types
54-2	Smooth, transparent, yellowy, dark	1 type	2 types	2 types
54-3	Smooth, dense, whitish, dim	1 type	3 types	4 types
54-4	Smooth, dense, whitish, bright	2 types	4 types	2 types
54-5	Rough, semitransparent, whitish, very bright	5 types	7 types	6 types

transparent inclusions of poly-β-hydroxybutyric acid in the cytoplasm (Fig. 8 B). In the nucleoid zone of the cells of the third type, electron-dense granules with diameters of up to 30 nm were present (Fig 8 C) and in the fourth type, both types of inclusions were present (Fig. 8 D).

Degrading cells with an ultrastructure corresponding to that of the above four types, but with a different cytoplasmic condition also occurred. Thus, 8 types of cells, resulting from different combinations of characteristics were

identified. It turned out that the colonies of each variant contained a selection of these 8 morphological types. Each type of colony is characterized by its typical set (Table 1).

A distinct correlation between the cell types of the colony and its morphological characteristics was revealed. The following pattern can be noted upon the analysis of the cytomorphological characteristics of the isogenic variants of *P. leiognathi* 54, of the process of colonies formation, and of their physiological and biochemical characteristics and mutual transformations. The bright luminescent variant 54-5 (Fig. 2), forming rough colonies, displays a stable luminescence, but its cellular composition is extremely heterogeneous (Table, Fig. 8D). The dark variant 54-2 (Table, Fig. 2, 8A), with smooth colonies, possesses a homogenous morphological type of cell, but emits unstable luminescence, because clones with different luminescence levels (i.e., other variants) are prone to appear in its population (Fig. 7).

The colonies of the dark variant 54-2 *P. leiognathi* consist of one type of cell with homogeneous cytoplasm without inclusions (Fig. 8A). Importantly, this bacterial type along with others occurred in all isolated variants. Furthermore, only such cells are 54 and 54-5 with the most heterogeneous composition (initial phenotype 1 and phenotype 5, respectively; Fig. 2), when the colonies can already be visually present and detected in young colonies (18 h of growth) of the brightly luminescent variants, but do not yet glow. Liquid batch culture at the beginning of the exponential phase of growth also consists of cells of the first type. This type of cell is probably the original one, and other types differentiate during colony growth (Fig. 8). The descriptions of the colonies of freshly isolated luminous bacteria indicate that the dark S variant is probably the original one under environmental conditions and prevails there. The dark S variant composed of "undifferentiated" cells is probably important for the preservation and stabilization of the species in natural conditions. The R variants with high bioluminescence level and a more varied cell composition are probably important for the adaptation of the species to different environmental conditions.

Electron microscopy of the colonies of the investigated variants of *P. leiognathi* 54 revealed that their architectonics corresponded to classical representations (Fig. 9, 10). Vertical section of the colonies demonstrated the basic tendencial characteristics of cells of all variants: the actively operational organisms formed layers located at the periphery of the colony while degrading cells were located in the central part of the colony in the form of a local layer that could reach a third or more of the colony height (Fig. 9). The cell layer closest to the surface of nutrient agar usually occupied, a smaller volume than the same cells from the outer portion of the colony. In the lysis

zone, the cells were impacted, whereas at the periphery they were located fairly loosely although maintaining contact with each other. A zone of cells in lysis was shown to occur in the colonies of *P. leiognathi* 54 as early as a day after transfer. The investigated variants of the photobacteria differed in many characteristics, affecting mainly features of the intracellular metabolism. In particular, the change in the fatty acid composition of the membranes, namely, the higher unsaturation coefficient of the smooth variants, led to a change in membrane permeability and decreased sensitivity to antibiotics and other toxic substances [18,26]. There is, accordingly, reason to believe that the lowering of luminescence intensity is correlated with changes in cell ultrastructure. In every case (batch or continous cultivation) the prevailing morphotype determined the level of luminescence intensity.

The analysis of the population structure of *P. phosphoreum* under continuous cultivation has shown, that the reduction of luminescence intensity is caused by replacement of cells of type 1, prevailing at the beginning of cultivation, by type 2 of the pleiomorphic cells, which is characteristic and typical for a dark population at the end of cultivation. Highly electron-dense, pleiomorphic cells had membrane structures of unusual configuration and, making up about half of the population, represented a condition that was never met in cells of other kinds of luminous bacteria.

6. Conclusion

The extensive and long evolution of marine luminous bacteria resulted in the production of a strong system of regulation of *lux* gene expression that can cause wide-ranging variations of luminescence levels. The heterogeneity of populations of luminescent bacteria is primarily assessed on the basis of the visual heterogeneity of luminescence of the colonies and colony morphology on plates with solid nutrient medium. Thus, the ability of cells to form subpopulations with a reduced expression of *lux* genes, but greater resistance to environmental toxic compounds, is of ecological importance for the survival of the populations of luminescent bacteria. The existence of stable variants may indicate their adaptation to the habitat. The variety of hereditary variants indicates the existence of several regulators. The presence or absence of these regulators leads to significant reconstructions of the cell metabolism and, accordingly, to the emergence of certain cell clones with different types of bioluminescence dynamics and the formation of different types of colonies. The bright variant with homogeneous cell composition, which forms colonies of a smooth type, exhibits the constitutive type of bioluminescence dynamics and retains catabolite regulation by glucose; it is

the most preferable variant for long-term storage in collections. Furthermore, the knowledge of the characteristics of population heterogeneity, particularly, of the cytomorphological characteristics, is necessary for a complete description of the available strains of luminescent bacteria in order to fill the databank of this microbial group maintained in the IBSO Collection of cultures (CCIBSO 836, http://bl.ibp.ru).

Acknowledgments

This work was supported by project no. 38 of the Program of Basic Research of the Siberia Division of the Russian Academy of Sciences.

References

1. Mil'ko, E.S., and Egorov, N.S. 1991 Bacterial Population Heterogeneity and the Process of Dissociation: Coryneform and Nocardioform Bacteria, Moscow: Mosk. Gos. Univ.
2. Shapiro, J.A. 1998, Annu. Rev. Microbiol., 52, 81-104.
3. Brandt, B.W., Kelpin, F.D., Van Leeuwen, I.M., and Kooijman, S.A. 2004, Water Res., 38 (4), 1003-1013.
4. Bennett, P.M. 2004, Meth. Mol. Biol, 266, 71-114.
5. Hayes, F. 2003, Ann. Rev. Genet., 37, 3-29.
6. DeLong, E. 2002, Curr. Opin. Microbiol., 5 (5), 520.
7. Hersh, M.N., Ponder, R.G., Hastings, P.J., and Rosenberg, S.M. 2004, Res. Microbiol., 155 (5), 352–359.
8. Scanlan, D.J. 2003, Adv. Microb. Physiol., 47, 1-64.
9. Gitelson J.I., Rodicheva E.K., Medvedeva S.E. et al. 1984, The Luminous Bacteria. Nauka, Moscow (in Russian).
10. Hurlbert, R.E., Jumin, X.U., and Christopher, L.S. 1989, Appl. Environ. Microbiol., 55 (5), 1136-1143.
11. Akhurst, R.J., Mourant, R.G., Baud, L., and Boemare, N.E. 1996, Int. J. Syst. Bacteriol., 46, 1034-1041.
12. Sung, N.D. and Lee, C.Y. 2004, J. Microbiol., 42 (3), 194-199.
13. Dunlap, P.V., Jiemjit, A., Ast, J.C., Pearce, M.M., Marques, R.R., and Lavilla-Pitogo, C.R. 2004, Environ. Microbiol, 6 (2), 145-158.
14. Wolfe, A.J., Millikan, D.S., Campbell, J.M., and Visick, K.L. 2004, Appl. Environ. Microbiol., 70 (4), 2520-2524.
15. Aertsen, A. and Michiels, C.W. 2004, Crit. Rev. Microbiol., 30 (4), 263-273.
16. Lutskaya, N.I. 1990, PhD thesis, Institute of Biophysics SB RAS, Krasnoyarsk.
17. Medvedeva, S.E., Mogil'naya, O.A., and Popova, L.Yu. 2006, Microbiology, 75 (3), 292-299.
18. Shenderov, A.N., Videlets, I.Yu., Lutskaya, N.I., Gurevich, V.B., and Svetlakov, A.V. 1989, Mikrobiologiya, 58 (6), 1000-1006.
19. Hastings, J.W. 1968, Ann. Rev. Biochem., 37, 597-608.
20. Hastings, J.W, and Johnson, C.H. 2003, Methods Enzymol., 360, 75-104.

21. Popova, L.Yu., and Shenderov, A.N. 1983, Biokhimiya, 48 (6), 983-990.
22. Sun, W., Teng, K., and Meighen, E. 1995, Can.J.Microbiol., 41 (1). 131-137.
23. Rodicheva, E.K., Trubachev, T.I., Medvedeva, S.E., Egorova, O.I., and Shitova, L.Yu. 1993, J. Biolum.Chemilum., 6, 293-298.
24. Vysotski, E.S., Zavoruev, V.V., Mezhevikin, V.V., Primakova, G.A., Rodicheva, E.K., and Shcherbakova, G.Y. 1982, In: Bioluminescence in the Pacific Ocean, J.W. Hastings and J.I. Gitelson (Eds.), IP SB USSR, Krasnoyarsk, 324-336.
25. Primakova, G.A., Vorobjova, T.I., Medvedeva, S.E., and Fish, A.M. 1981, Mikrobiologia, 50 (3), 487-493.
26. Popova, L.Yu., Kalacheva, G.S., Mogil'naya, O.A., Medvedeva, S.E., and Pechurkin, N.S. 1994, Prikl. Biokhim. Mikrobiol., 30 (4/5), 650-656.

Research signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 27-49
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

3 Bacterial bioluminescence and its applications

Nataliya A.Tyulkova, Svetlana E. Medvedeva, Emma K. Rodicheva and Alexander M. Kuznetsov
Institute of Biophysics SB RAS, Krasnoyarsk, 660036, Russia

Abstract

Bioluminescent analyses are one of the most promising express methods for biologically monitoring the environment because the luminescent system is highly sensitive to even micro quantities of toxicants. Bioassays based on luminous bacteria give an integral estimation of toxicity and frequently surpass other known bioassays in speed, accuracy, sensitivity and simplicity. The enzymes of bacterial luminescent system are used in developing highly sensitive analytical methods for practical purposes. This paper considers questions about the development and use of bioluminescent bioassays, and the influence of analyzed substances on bacterial luminescence.

Correspondence/Reprint request: Dr. S. E. Medvedeva, Institute of Biophysics, Siberian Division, Russian Academy of Sciences, Akademgorodok, 50, Krasnoyarsk, 660036, Russia. E-mail: ccibso@ibp.ru

1. Introduction

The ability to emit light in the visible spectrum range can be found in representative living organisms, belonging to almost every branch of the evolutionary tree. Yet, it is in the aquatic ecosystems, where the majority of the luminescent organisms can be found. The biological meaning of luminescence for many of these organisms is still unclear. However, bioluminescence has attracted the attention of scientists for a long time. Our particular interest is the possibility of using this trait for solving various basic and applied problems.

Bioluminescence is the parameter of the vital functions that is used in bioluminescent assays. Luminescent bacteria - producers of luciferase and oxidoreductase - are widely used to make different preparations for biotesting of various substances. Bioassays based on luminous bacteria can quantify toxicity and are often quicker, simpler, more precise and sensitive than other bioassays based on ciliates, daphnia, algae, and fish [1-6]; they can be used to monitor a large number of toxicants simultaneously. Bacteria of two natural marine genera, *Photobacterium* and *Vibrio* (*P.phosphoreum, P.leiognathi, V.fischeri and V.harveyi*) are widely used for this purpose. At present more than a hundred various bioluminescent tests have been developed which make use of both bacteria containing genes of bioluminescent systems of natural luminous bacteria and enzyme-substrate systems isolated from them.

2. Bacterial bioluminescence

Bioluminescence in the visible spectrum area is a mysterious natural phenomenon. This is a feature of living organisms occupying various branches of the evolutionary tree; the overwhelming majority of luminous organisms can be found in aquatic ecosystems. The process of emitting light by living organisms is based on chemiluminescence resulting from the formation of an electronically excited intermediate in the course of enzyme reaction. Reversion of this intermediate into the ground state is accompanied by the emission of visible light with certain wavelength, intensity and duration. The participation of highly specific protein biocatalysts (enzymes - luciferases) results in an effectively increased probability of forming an electronically excited intermediate. The quantum yield of bioluminescence reactions is very high and amounts to 0.1 - 1.0. Luciferases of luminous bacteria, being flavin dependent monooxygenases, catalyze the oxidation of the long-chain aliphatic aldehyde (RCHO) and reduced flavin mononucleotide ($FMNH_2$), involving molecular oxygen, to a corresponding fatty acid and oxidized flavin mononucleotide (FMN). This process is accompanied by emission of light in the visible range [7, 8]:

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + \text{light}$$

Even the most primitive luminescent organisms - bacteria and protozoa - evolved special mechanisms to regulate this function. The luminescence of bacteria in favourable conditions is rather bright; in a slightly blacked-out room it is well visible to the naked eye. Colonies of photobacteria grown on a solid nutrient medium emit cold greenish-blue light, reminiscent of the light reflected by a snow-covered field during a frosty and moonlit night or coming from the flickering stars of the night sky. Usually, bacteria emit greenish-blue light (max ~ 490nm), but some of the strains of *V. fischeri* have been found to emit yellow light (max ~ 545nm).

Luminescence is closely connected with the cell metabolism. DNA sequences, coding proteins of a luminescent system, are called *lux* genes. The bacterial luciferase is a heterodimer consisting of two different polypeptides called α- and β-subunits (with a molecular mass being 40 kDa and 37 kDa, respectively), coded by *lux*A and *lux*B genes. The α-subunit has the active site. When the β-subunit is absent the α-subunit gives a low level of light intensity. Based on the available crystal structure of *V. harveyi* luciferase it has been assumed that the β-subunit plays a supportive role in the conformational change of the subunit in the catalysis [9].

Permanent light emission of luminescent bacteria is provided by different enzymes, which constantly produce components necessary for the bioluminescent reaction. To provide bioluminescence expression, the DNA fragment of size 9000 p.n. is sufficient to code for the following genes: *lux*R (repressor), *lux*I (inducer), *lux*C, *lux*D *lux*E (aldehyde), *lux*A, *lux*B (luciferase) [10, 11]. The sequence of structure genes *lux*C, *lux*D, *lux*E, coding the synthesis of the aldehyde factor, and luciferase genes (*lux*A and *lux*B), was the same in all bacterial species, indicating the similarity of the luminescent systems. At the same time the regulator side of *lux* systems diverged remarkably; therefore, several regulator genes *lux* (*lux*I, *lux*R) found in *V. fischeri* did not reveal any analogs in *V. harveyi.* Other genes including *lux*F, *lux*G, and *lux*H, whose functions are not clearly identified yet and whose role for bioluminescence is not clear, are also located in *lux* operon.

Differences in bioluminescence intensity are conditioned by metabolism activity, governed by a common and specific regulation of bacterial operon expression. Light emission of luminous bacteria depends on growth and environmental conditions. In the laboratory luminous bacteria, growing in liquid media at low cell density, emit minimum light, because of the weak expression of the *lux*CDABE genes and a deficiency of precursors of bacterial luciferase reaction. From the middle till the end of the exponential growth phase the intensity of the light emission increases abruptly due to the

quick accumulation of synthesized substrates and enzymes for the activation of *lux*CDABE gene expression. Density-dependent regulation of gene expression (autoinduction), or "quorum-sensing" (quorum effect), first described in *V. fischeri* and *V. harveyi*, [11, 12], occurs in bacteria of many species, pathogenic and symbiotic, gram-negative and gram-positive. The bioluminescence appears at threshold concentration of autoinducer N-(3-oxohexanoyl) homoserine lactone (3-oxo-C6-HSL) in the environment, when the density of the culture reaches 10^7 cells/mL [12, 13]. Light emission by bacterial cells is closely connected with their general metabolism and depends on the stage of culture growth and environmental conditions. Therefore, unfavourable influences, damaging cells, result in decreasing or increasing luminescence. The mechanisms underlying the toxic effects of chemicals in these assays are both varied and complex [14]. For example, toxicity may involve interactions with cell surface receptors, disruption of cell membrane function, and chemical reactions with cellular components or inhibition/competition of enzyme systems [15, 16]. Antagonistic and synergistic interactions with other compounds are complicating factors that can dramatically affect toxic responses both inside and outside the cell and within the cell membranes.

3. Bioluminescent analysis

3.1. Basic principles for developing bioluminescent bioassay

The high sensitivity of the luminescent system to even micro-quantities of toxicants, the rapid results, and the exact quantification of changes in the luminescence level make bioluminescent analyses suitable for quick biological monitoring of certain environmental conditions [17-23]. Creating bioluminescent bioassays usually consists of three stages: 1) preparing the test bacterial culture; 2) measuring bacterial luminosity in the presence or absence of the analyzed substances; and 3) establishing the relationship between luminosity parameters and the quantitative characteristics of the medium's toxicity.

3.1.1. Preparing the test bacterial culture

Bacterial cultures are usually standardized in two ways: either by using a continuous culture of luminous bacteria or by producing reagents based on freeze-dried or immobilized bacteria. In continuous luminostat cultivation, a bacterial culture can be maintained in a certain physiological state with constant luminescence intensity by controlling the nutrient solution flow [17]. A new portion of media is added when bacterial luminescence exceeds the predetermined level of luminosity. The supply of nutrient solution is stopped

when bacterial luminescence falls to the target intensity. The luminescence then continues to fall due to a reduction in specific luminescence, but this is counterbalanced by the increase in the number of bacterial cells, which increases the intensity of the luminescence. Luminostat significantly increases the measurement precision, but the assay sensitivity is almost 10 times lower than in the case of a batch culture. Another drawback of continuous cultivation is its technical complexity and great expenditure of nutrient solution.

The optimized conditions for continuous cultivation of the luminous bacterium, *Vibrio fischeri* NRRL-B-11177 in a fermenter has been reported by Scheerer S. and co-workers [24]. The system provided a reliable long-term (more than 1 month) continuous culture facility for the reproducible measurement of perturbation of *V. fischeri* metabolism by monitoring changes in its luminescence. The other system for continuous tracking toxicity of the water, which fixed the response at once from several recombinant strains with a *lux*-gene, is developed by scientists from South Korea [25].

Lyophilization of luminous bacteria is most frequently used to produce standardized and stabile bioluminescent assays to analyze some luminosity-inhibiting substances [26-29]. Some researchers have proposed a bioassay using immobilized luminous bacteria [30-32].

Luminescent genetically-modified microorganisms (*Pseudomonas, E. coli,* etc.) with *lux* genes from marine *Vibrio* and *Photobacterium* species or from *Photorhabdus luminescens* are widely used as test-objects in bioluminescent bioassays to determine the presence of heavy metals, phenols and other substances in water and soil samples [22, 33-47]. The possibility of using *lux* genes as markers of gene expression is important for studying pathogenicity, virulence, adaptation and secondary metabolism [40, 48-, 49]. The bioluminescent reporter strains were created to investigate and control the survivability of bacterial species in freshwater or different host cells [50-52]. The strain *Salmonella hadar,* which had a complete set of the luminescent system (*lux*CDABE) from *P. phosphoreum,* was used to study the bacteria's ability to restore their original properties after stress action [53]. Bioassays based on recombinant bioluminescent strains are being developed to determine the presence and effectiveness of various antibiotics [54-58] and narcotics [59, 60].

Strains were constructed in which the luminescence increased after the toxicant's action. Some examples were used to determine the presence of mercury [61, 62] and some additional metals [63, 64]. Other *lux*-fusions were constructed to monitor the expression of catabolite genes, including those for degradating isopropylbenzene [65] and toluene [66], mixtures of naphthalenes [67], expression of heat shock genes [68-70] and oxidative stress [44, 71]. In

these cases, *lux*-fusions had basic plasmids and were constructed by placing the interested promoter opposite the non-promoter *lux* genes from *V. fischeri* contained in pUCDG15 [72]. Applegate et al [66] constructed a strain by using *Pseudomonas putida F1* with a complete *lux*-cassette (*lux*CDABE) to investigate the induction of tod-operon after exposition with benzene, toluene, ethylbenzene and xylene compounds. The tod-*lux*-reporter was very sensitive and allowed bacterial luminescence in whole cells to be measured without added aldehyde substrate. In addition, the constructed strain was sensitive to benzene, ethylbenzene, m- and p-xylene, and may be used as a reporter to estimate hydrocarbon fuel pollution that contains these substances.

The use of recombinant strains of *E. coli* with a cloned luciferase gene is successful in developing a bioluminescent bioassay that can be used in analyses of fresh waters. Scientists have already shown how genetically engineered strains of *E. coli* with a complete set of luminescent system (*lux*CDABE) from the luminescent bacterium *V. fischeri* can be used to identify 23 out of 25 investigated toxicants in mixtures of various compositions and to assess their degree of toxicity [73]. Several reporter strains with a high specificity towards specific toxic agents were obtained using this approach. Different bioluminescent reporters were designed to register heavy metals and phenols in water and soil samples [36, 39, 54, 74-78], and mutagenic pollution of the environment [79, 80].

Methods based on biochemiluminescent reactions play an important role not only in environmental research, but also in biomedical studies and clinical medicine [42, 56, 57, 81, 82], as well as in immunology [83]. The new generation of bioreporters for *in vivo* monitoring and diagnostics technology was developed to investigate the expression of the bacterial luciferase system in mammalian cells [84]. The bioluminescent method was suggested to determine the sensitivity of microbe cells from septated blood to different antibiotics [85]. The toxicity of pharmaceutical products and their metabolites and the ability of luminous bacteria in decomposing them was investigated [86].

3.1.2. Effect of different substances on bacterial luminescence

The bioluminescence index ($BI=I_e/I_c$ where I_c – luminescence intensity in control cuvette, I_e - luminescence intensity in experimental cuvette) was used to estimate the effect of different substances on bacterial luminescence. Comparing the effect of model substances on Microbiosensor B17-677F and Microbiosensor ECK revealed that the luminescence level dependence on the substance's concentration was similar in the concentration ranges studied. The analysis of these results demonstrates that the luminescence sensitivity of Microbiosensor ECK to phenol compounds was higher than that for

Microbiosensor B17-677F (Fig. 1). It was shown that parabenzoquinone was the most toxic, and maximal inhibition of luminescence was revealed at a concentration of 10^{-5} – 1 mg/mL.

The effective concentration (EC_{50}) was $6x10^{-5}$ mg/ml after 5 min of action. For hydroquinone the effective concentration (EC_{50}) was $2x10^{-4}$ mg/ml. EC_{50} for pyrocatechin after 5 min of action was 10^{-2} mg/ml. This phenol's toxicity row for Microbiosensor ECK corresponds to the phenol's toxicity row determined on the intact cells of the luminescent bacterium *Photobacterium phosphoreum* and various hydrobionts [87].

Electron microscopy was employed to study the actions of various phenols, quinone, and heavy metals on bacterial cells. The solutions of easily oxidized diphenols (pyrocatechin, hydroquinone) and the products of their oxidation (o,p-benzoquinone), metaphenols (resorcinol) were used in the concentration, which caused 50% quenching of luminescence. The tested compounds were shown to produce different effects on the luminous bacteria cells. Cadmium chloride had the greatest effect, resulting in cell damage (Fig.2a). Then followed resorcinol, which caused considerable damage of the cell ultrastructure (Fig. 2b). During a prolonged treatment cleavages in the cell wall were seen more clearly. Hydroquinone and p-benzoquinone had a

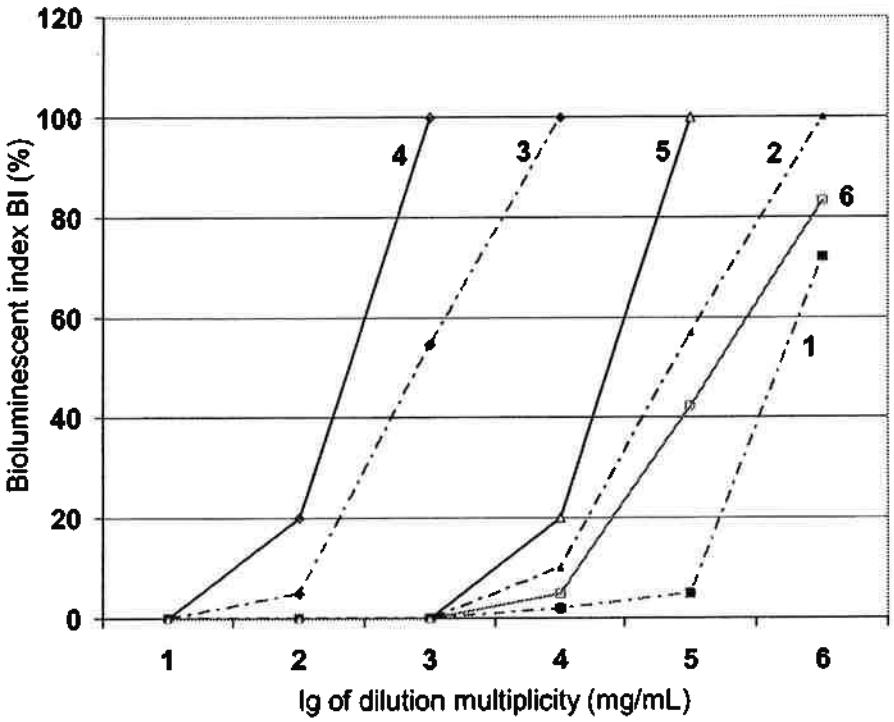


Figure 1. Microbiosensor bioassay's Bioluminescent Index vs. concentration of phenol compounds. Parabenzoquinone (**1**- *E. coli*, **6** - *P. phosphoreum*); hydroquinone (**2** – *E. coli*, **5** – *P. phosphoreum*); catechol (**3** – *E. coli*, **4** – *P. phosphoreum*). BI norm = 80-120%.

more specific effect on the luminescent system, but did not cause severe structural changes (Fig. 2c). Simultaneously the number of living cells in the population and their viability were evaluated. Cadmium chloride had the greatest effect, killing nearly half of the population. Then followed resorcinol, p-benzoquinone, hydroquinone, mercury chloride, and pyrocatechin.

The combined effects of heavy metal salts on luminescence were also investigated (Fig.3). Any individual salt (curves 1, 2) inhibited the luminescence intensity, but the sum of the coupled effect of luminescence inhibition (curve 3) was less than the theoretical additive effect (curve 4). Thus, the non-additiveness of the effects of these salts was ascertained.

Creating an assay based on luminous bacteria often involves increasing the sensitivity of the bacterial cells to low concentrations of the toxic compounds. This can be achieved by varying the cultivation conditions and the treatment procedure with a toxicant to increase cell membrane permeability [88] as well as by using specific sensitive mutant strains. The high concentration of EDTA and toluene decreased the luminescence and caused the condensation of DNA-fibrils and cell damage after long-term and short-term actions. The low concentration of EDTA and toluene did not decrease the bacterial luminescence, and the noticeable damage of cell membranes did not take place during short-term treatment (Fig.4). However, the long action of these substances changed the membrane permeability, resulting in increased sensitivity of bacterial luminescence to some toxic substances (Fig.5).

We used several approaches to devise an assay system that determined various phenols and their derivatives, and hexachlorancyclohexane (HCCG) [87, 89, 90]. There is a bioluminescent assay for detecting soil toxicity, based on getting water extracts from the soil and quantitatively assessing toxicants in them. A biosensor was used to estimate the degree of luminescence inhibition by all toxicants contained in soil samples [39, 45, 78, 91]. The information about investigations of the effect of low-level α-radiation on

Figure 2. Action of cadmium chloride (a), resorcinol (b) and p-benzoquinone (c) on luminous bacteria.

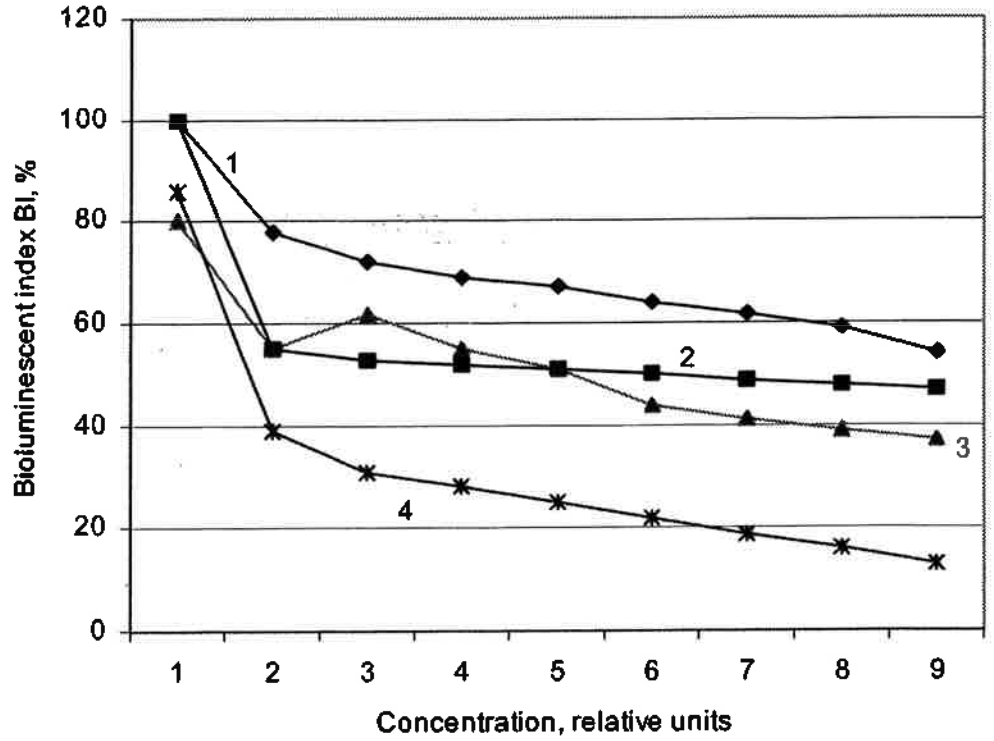


Figure 3. Combined action of Co-, and Cu-salts on bacterial bioluminescence. 1 - Cu-salt, 2 - Co-salt, 3 - mixture of Co- and Cu-salts, 4 - theoretical curve of additive action of Cu- and Co-salts mixture.

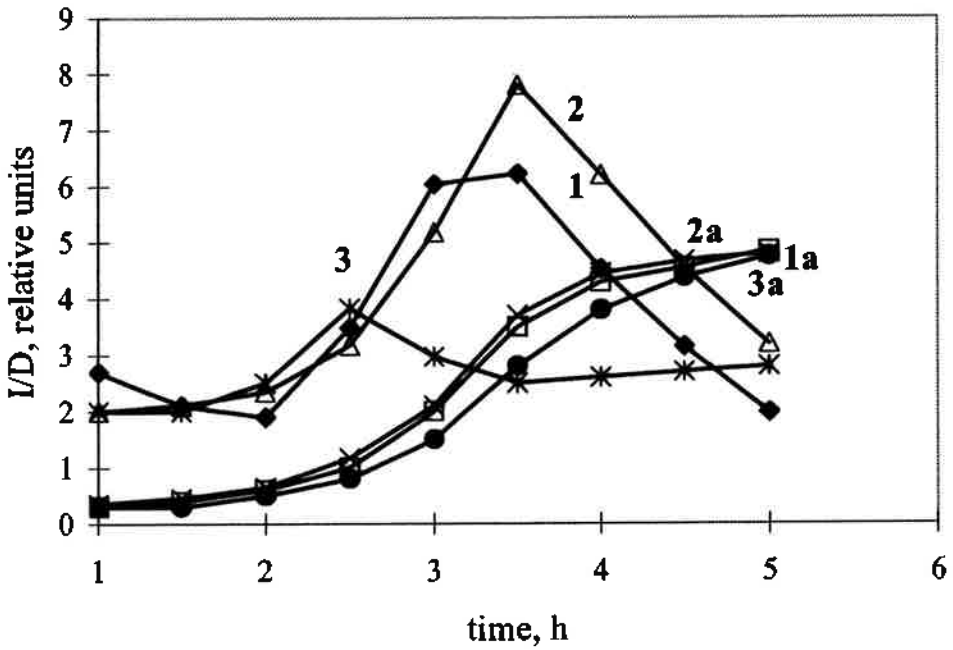


Figure 4. Prolonged action of EDTA on growth (1a,2a,3a) and luminescence (1,2,3) of *P. leiognathi* 213: 1,1a -control, 2,2a - 10^{-5} mol/L EDTA, 3,3a -10^{-4} mol/L EDTA.

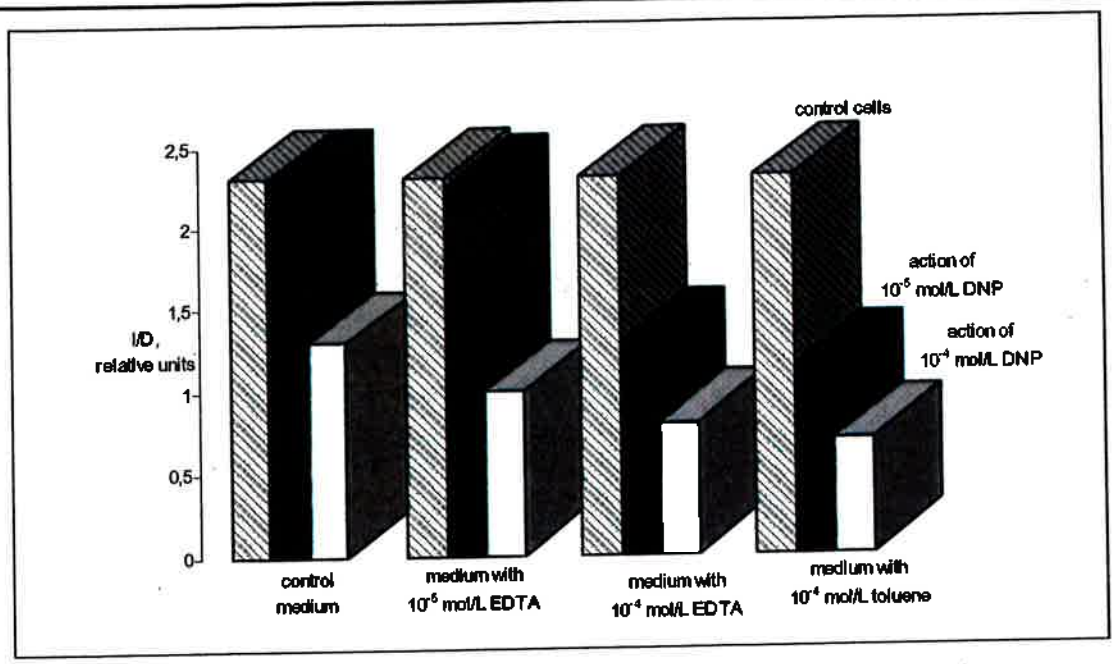


Figure 5. *P. leiognathi* cells with maximal luminescence after cultivation in control medium, medium with 10^{-5} mol/L EDTA, medium with 10^{-4} mol/L EDTA and medium with 10^{-4}-mol/L toluene. The long-time treatment by these substances increased cell sensitivity to 10^{-5} mol/L 2,4-DNP (dark column) and 10^{-4} mol/L 2,4-DNP (white column) in comparison with cells grown in control medium (the shaded column). The first (shaded) column of the control cells' luminescence is presented with other data to make more evident the action of substances on each medium.

bioluminescent assay systems of various complexity [92] permits the development of new approaches for environmental monitoring.

3.1.3. Applying of bioluminescent bioassays in environmental monitoring

Toxicity research has several basic directions: 1) toxicity studies of separate substances, in particular, phenols, indoles, nitrobenzenes, insecticides, pesticides, heavy metals, etc, 2) toxicity studies of mixtures with a large amount of specific substances, and 3) environmental studies to determine the integral toxic effect of all pollutants in a sample.

Bioluminescent analyses are based on the change in the luminescence intensity of the bioassays after the action of an analyzed substance and fulfil modern requirements for estimating substance toxicity. Analyses of this kind can be used to determine such toxicologically accepted parameters as: efficient concentration (EC_{50}) – the substance concentration that inhibits the luminescence function by 50%, and threshold concentration (TC_0), or biologically safe dilution level (BSDL) – the substance concentration (dilution) at which the luminescence level of analyzed solutions is equal to the luminescence intensity in the control sample. The most important

toxicological parameter (EC_{50}) is the substance concentration that reduces bacterial luminescence by 50% [93].

Microbiosensor-B17-677F bioassay (based on luminous lyophilized *Photobacterium phosphoreum* bacteria from the Culture Collection IBSO) and Microbiosensor-ECK (based on *E.coli Z905* carrying pHL1 plasmid from *Photobacterium leiognathi* kindly provided by Dr. B.A. Illarionov) were developed at the Institute of Biophysics of SB RAS [26, 94]. One flask of Microbiosensor can be used to measure 100 experimental samples. Their activity is stable for 6 months in a refrigerator stored at +5-10° C and more than 1 year at –18°C. Bioluminescent bioassays based on luminous bacteria – Microtox, ToxAlert, LUMIStox, SOS-LUX-TOXICITY-Test, etc. – are already in use in different countries [18, 20, 22, 28, 95-97].

The most common is Microtox, which is widely used in laboratory and field experiments to monitor the quality of industrial and natural waters and to determine the degree of toxicity of newly devised chemical compounds and pharmaceutical preparations. Microtox is used to test micro-quantities of aromatic hydrocarbons, heavy metal salts, pesticides, respiration poisons, and other water contaminants [18, 20, 23, 98-101]. It is also used in medical investigations [56, 59]. Bioassays can be employed successfully in many ways: in continuous on-the-spot monitoring of the environment in industrial areas and nature-management systems, in monitoring harmful industrial discharges, in estimating the efficiency of the methods used in environmental detoxication and the operation of purification facilities, and in environmental certifications of industrial facilities and regions [1, 27, 87, 94, 102, 103].

One of the sources of phenol compounds and heavy metals in water bodies is industrial waste. We used the Microbiosensor ECK (lyophilized recombinant strain *E.coli* with *lux* gene) to test the wastewaters of Krasnoyarsk's pulp-and-paper plants. Our investigations showed that 50% inhibition of luminescence (EC_{50}) was reached by dilution of 100 times and that the waste waters of the PPP became non-toxic only when diluted a 1000 times.

The underground and surface waters of the Altai Territory were studied with the bioluminescent assay Microbiosensor B17-677F [103]. It was shown that the waters of the Katun and Biya Rivers were uncontaminated and those of the Alei and Choumych Rivers were slightly toxic. The presence of organic substances in water samples could cause marked stimulation of bioassay luminescence (Fig.6 curves 1, 2). There were deviations from the norm in most water samples taken from village wells; the assay luminescence was inhibited. Surface water pollution of the Bolshoye Ostrovnoye Lake was demonstrated by the inhibition of bioluminescence in all water samples (Fig.6, curve 3). This data correlated with optical and hydrobiological estimates of water quality, as well as with the effect of sample water on the

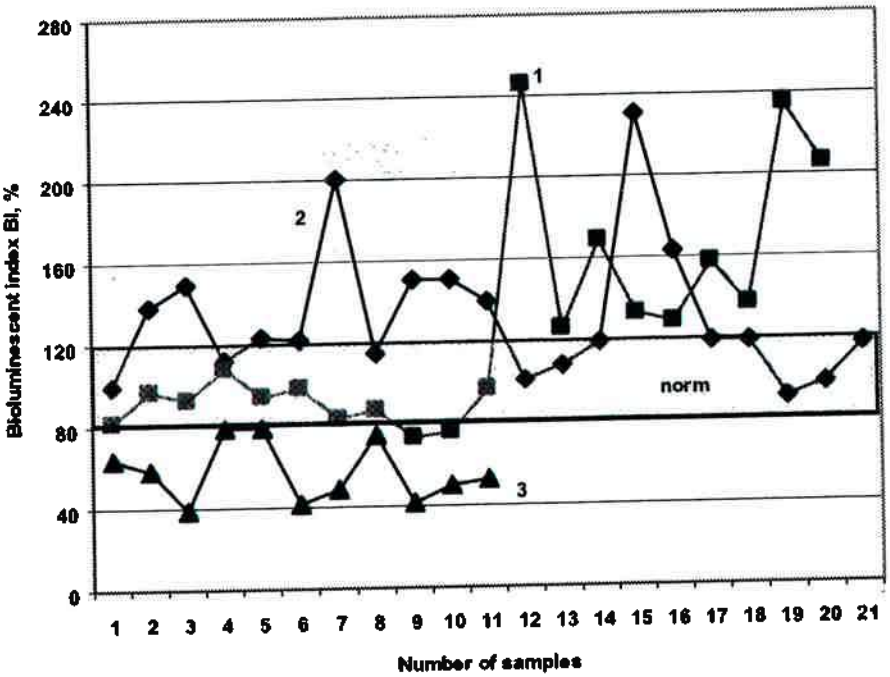


Figure 6. Zones of different water pollution in Alei (1) and Choumysh (2) Rivers and Bolshoye Ostrovnoye Lake (3) detected by bioluminescent bioassay Microbiosensor B17- 677 F. BI norm = 80-120%.

ultrastructure of intact luminous bacteria cells. It was shown that a high poison concentration damaged the cell structure, while a low poison concentration changed the membrane penetrability. The lyophilized luminous bacterial bioassays were also applied to a pollution test of the Yenisei River. Figure 7 shows the zones of water pollution of the Yenisei River observed in 1998. These zones were located 300 km downstream of Krasnoyarsk.

Results showed that the genetically modified *E.coli* strain was more sensitive to the pollutants presented in the medium than was the *P. phosphoreum*'s bioassay. This can be explained by: 1) the higher permeability of the *E. coli* cell wall to inhibiting substances, 2) the salting-out of toxic agents (decrease in the concentration of inhibiting substances after sodium chloride was added to the solution) in bioassays using the marine luminous *P. phosphoreum* bacteria.

Monitoring waters from the Angara River by bioluminescent bioassay showed that its water was nontoxic at the studied points, because no inhibition of bioluminescence was revealed (Fig. 8). However, the water cannot be considered completely clean many samples stimulated bioassay luminescence, sometimes very considerably. This testifies to the presence of numerous organic substances of both natural and anthropogenic origin in the samples.

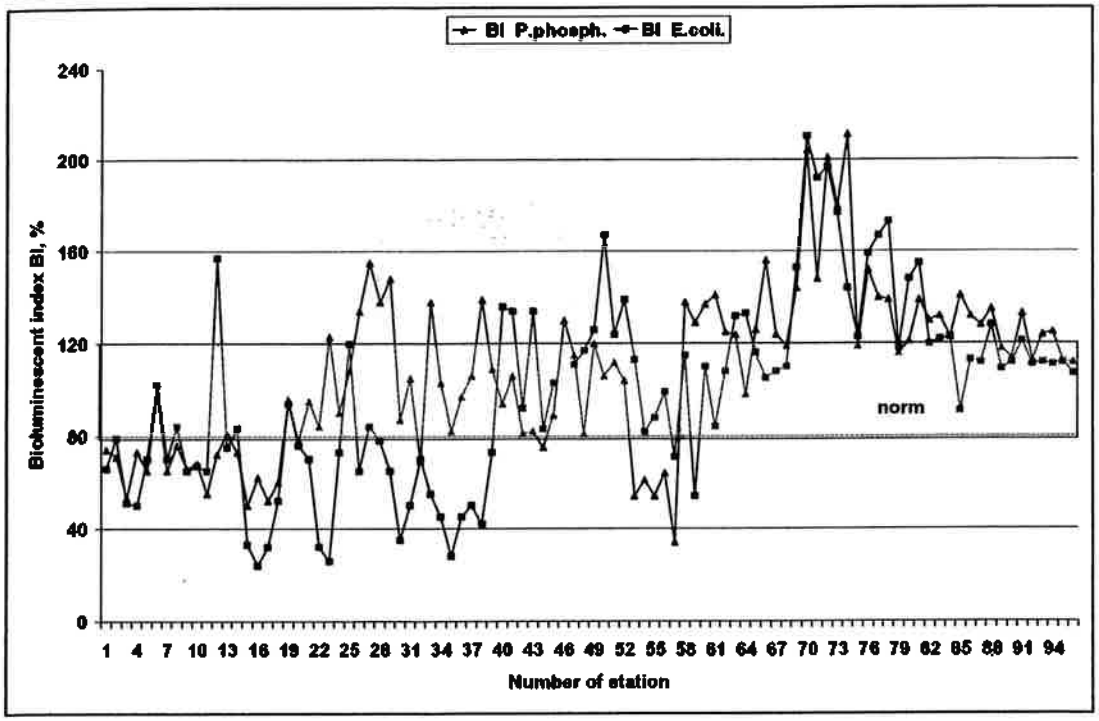


Figure 7. Zones of different water pollution in the Yenisei River detected by bioluminescent bioassays Microbiosensor B17- 677F and Microbiosensor ECK. BI norm = 80-120%.

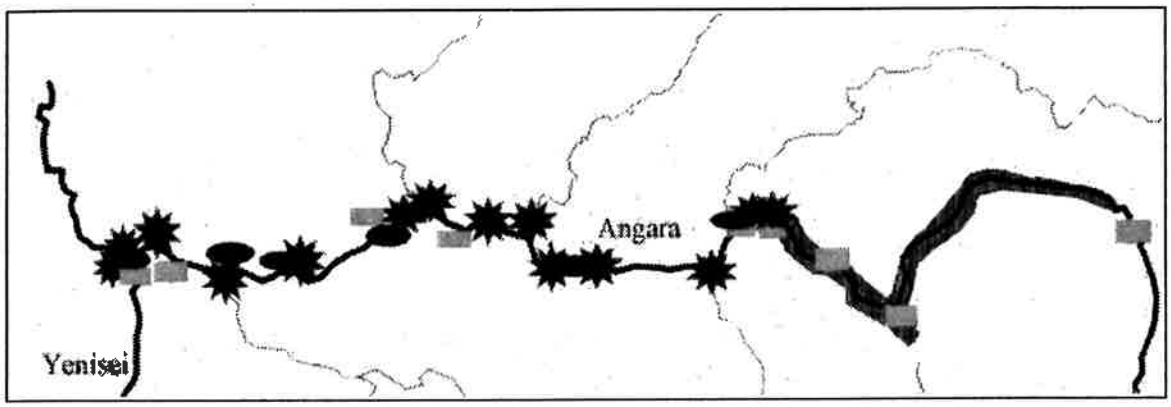


Figure 8. Pollution testing water in the Angara River detected by bioassay Microbiosensor B17- 677F: Rectangular – BI is norm; Oval – BI is near norm (deviation not more than 20% from norm); Star – BI is increased by 1,5-2,5 times in comparison with norm.

3.2. Enzyme bioluminescent analysis

The enzyme bioluminescent analysis is based on measuring the intensity of the light emission, resulting from two conjugated reactions. The reduced of FMNH-H substrate for luciferase reaction is provided by the action of NAD(P)H:FMN-oxidoreductase. The fermentative reduction of FMN by

NAD(P)H-FMN – oxidoreductase produce the prolonged bioluminescence in luciferase reaction:

$$NAD(P)H + FMN + H^+ \xrightarrow{\text{reductase}} NAD(P)^+ + FMNH_2$$

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + light$$

At the Institute of Biophysics reagent sets for bioluminescent analyses have been developed. The luciferase is isolated from the recombinant strain of *Echerichia coli* SL60 carrying *lux*A and *lux*B genes of *Photobacterium leiognathi* luminous bacteria from the Culture Collection of the Institute of Biophysics SB RAS. The enzyme is purified by ion-exchange chromatography on DEAE-sepharose column and on blue agarose. The purity of luciferase preparations is evaluated by electrophoresis in the polyacrylamide gel in the presence of DS-NA according to Laemmli is 90-95% [104]. Two kinds of NAD(P)H:FMN-oxidoreductase are extracted and purified from *Vibrio fischeri* and *V. harveyi* with specific activity to NADH and NAD(P)H. To produce a set of reagents the flavin reductase from the *Vibrio fischeri* species is used, which employs NADH and NAD(P)H with similar efficiencies. The enzyme is purified by ion-exchange chromatography on the DEAE-sepharose column and in the FPLC-system. The luciferase and NAD(P)H:FMN-oxidoreductase are mixed in the necessary proportion, which ensures the intensity of the produced light to be proportional to the concentration of the reduced NAD(P)H. Bi-enzyme reaction provides the bioluminescent analytical methods to be widely used while maintaining a stable sensitivity to NADH up to 0.01 pycomol (Fig.9). One kit flask can be used to measure 400 experimental samples. Their activity is stable for 6 months in the fridge stored at +5-10° C and more than 1 year at –18°C.

Bioluminescent methods, developed for enzymes isolated from luminous bacteria can conveniently be divided into the following groups: 1) analysis of substrates of luminescence reaction; 2) analysis of the activity of enzymes and their substrates in multielement chains of coupling; 3) analysis of bioluminescence inhibitors. The set for a bioluminescent analysis can be used in applied microbiology and environmental monitoring, in biochemistry, clinical laboratory tests for the determination of the activity of NAD-dependent dehydrogenases, their substrates, proteases and antiproteases in biological objects.

Bi-enzyme luminescent reactions have been used as an analytical tool for quantifying the activity of NAD(P)H-dependent enzymes. The bioluminescent method of NADH analysis possesses a 25,000 times higher sensitivity in comparison with spectrophotometric methods. The high sensitivity of the method

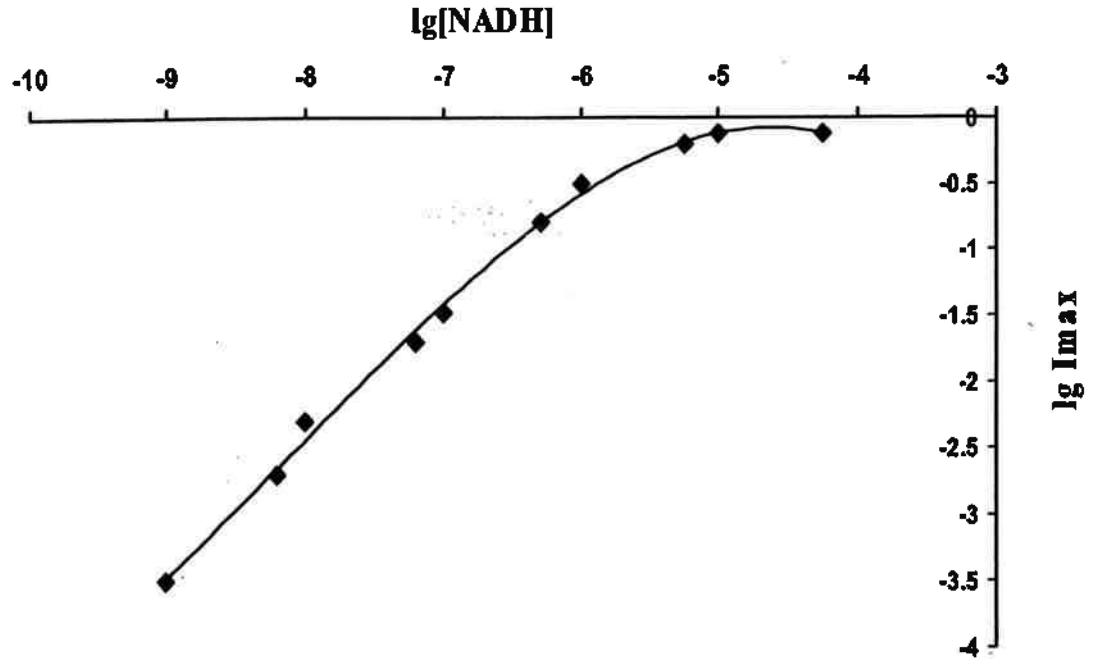


Figure 9. The correlation between the light intensity and concentration of NADH in bi-enzyme reaction.

allows carrying out research in microsamples of biological material with a minimum quantity of reactants. The wide spectrum of NAD-and NADP-dependent dehydrogenase activities in blood lymphocytes of the big circle of patients with various autoimmune diseases have been studied in medical and biological researches. Methods of an estimation of functional parameters of lymphocytes which have diagnostic and forecasting importance are being developed [105, 106].

Comparisons of the effects of great amounts of various pollutants on the bioluminescence of bacterial cells and enzyme systems isolated from them have shown that there is a correlation between the toxicity level of pollutants and the change of luminescence parameters of both systems. The sensitivity of the bi-enzyme system is usually 100-1000 times higher. Correlations between changes of kinetic parameters of bi-enzyme reactions and physical-chemical peculiarities of pollutants have been found [107-111]. All the parameters of the luciferase reaction, namely maximum luminescence intensity (I_m), total amount of resulting light quanta (Q) and constant rate of luminescence decay after maximum (k) change due to the influence of various anthropogenic pollutants. Herein, I_m is a determining parameter since it changes in all cases of the substance action, while Q and k change depending only on I_m. Therefore, in monoenzyme reactions the integral action of the substances can be traced with regard to the value of I_m. In

bi-enzyme reactions the reaction chain of NADP, catalyzed successively by FMN-oxidoreductase and luciferase, includes more components and, thus, more "targets" of the toxicant's action. Correspondingly, various substances influence differently the time course of the luminescence curve in the bi-enzyme system, i.e., one can observe different changes of the parameters of the conjugated system: maximum luminescence intensity (I_m), time of reaching luminescence maximum(t_m) and time of retarding luminescence from starting the reaction (T). The most complex was the change of luminescence dynamics upon 2,4-dinitrophenylfluoride (DNPF) action. Not only the decrease of luminescence intensity (I), but the increase of time of reaching luminescence maximum (t_m) and the gradual formation of the second peak have been observed. Such changes of bioluminescence dynamics, when influenced by DNPF, are conditioned by the separation of the FMN reduction reaction and light emission due to competitive inhibition of the active centre for $FMNH_2$ by DNPF [112].

Inhibition constants (K) were used to estimate the effects of the quinones and phenols on the test systems. They were calculated using the formula $I/I_m = e^{-KC}$, where I is the intensity of the bioluminescence signal in the presence of a quencher (quinone or phenol) of the concentration C (mol/liter) and I_m is the intensity of the bioluminescence signal in the absence of quenchers. Basic approaches using bi-enzyme systems are carried out

Table 1. Inhibition Constants K of Organic Compounds in the Bioluminescence Systems.

N	Substance	1 ($K\ 10^{-4}$, mol/liter)	2 ($K\ 10^{-4}$, mol/liter)
1	1,4-Benzoquinone	40 ±10	400 ±90
2	**Toluquinone**	340 ± 50	280 ±6
3	Thymoquinone	66 ±4	270 ±15
4	1,4-Naphthoquinone	7±3	140 ±6
5	1,4-Anthraquinone	1.3 + 0.4	70+1
6	9,10-Anthraquinone	<0.1	15+1
7	9,10-Anthraquinone-2-monosulfo acid	0.80 + 0.03	17 + 2
8	Anthron	31 + 5	145 ±6
9	Hydroquinone	0.09 + 0.02	0.2 ± 0.03
10	Resorcine	0.03 + 0.01	0.01 + 0.001
11	Pyrocatechol	0.8 + 0.1	0.002 ± 0.0002
12	Br-hydroquinone	16 ±2	0.02 + 0.002
13	1,4-Anthracendiol (95%)	0.4 ± 0.2	0.6 + 0.1
14	9,10-Anthracendiol-2-monosulfo acid (60%)	0.36 ± 0.03	1.2 + 0.3

Note. 1, bi-enzyme system NADH:FMN-oxidoreductase-luciferase; 2, luminescent bacteria *Photobacterium phosphoreum* (113).

by mixing luciferase, NAD(P)H:FMN-oxidoreductase and their substrates. The variants of these methods differ with regard to the time of the introduction of the analyzed substances: before initiating the reaction by adding NADP or after reaching the constant luminescence level.

For ecological monitoring in water reservoirs with a high content of redox-active compounds the use of the bioluminescent three-enzymes system - NADH:FMN-oxidoreductase – luciferase with alcoholdehydrogenase, lactatedehydrogenase and trypsin has been demonstrated [114, 115]. Influences of a model range of pollutants (fluorescent organic molecules of different spectral-luminescent peculiarities, molecules containing atoms of different atomic mass, metal salts, whose cations differ by the affinity for an electron, groups of redox-active compounds with different redox-potentials) on enzyme systems (luciferase, HADH- and HAD-dependent dehydrogenases, proteases etc.) have been studied [113, 116, 117]. Thus, the light-emitting system is particularly attractive for use in bioassays as it is readily available and sensitive to a wide variety of different biological compounds.

4. Conclusion

Bioluminescent assays can be used without hesitation for detecting the toxicity of certain chemical compounds, because the toxicants directly affect the luminescent system. Bioluminescent bioassays can be recommended for: a) continuous express-monitoring of the environmental conditions of industrial regions and natural-economic complexes, b) checking the release of volleys of pollutants by factories, c) estimating the degree of well fresh water cleaning in connection with industrial enterprises and d) methods used in environmental detoxification and to certify enterprises and regions as ecologically sound. Bioassays are standardizable to be used to detect and compare the integral toxicity of different water samples; there is no need for cultivating and maintaining bacterial cultures with the marker *lux* gene. Microbiosensor ECK (based on *E. coli* bearing *lux* gene from *P. leiognathi*) and Microbiosensor B17 677F (based on *P. phosphoreum*) are successfully being used for defining effluent toxicity and purifying installations in different cities of Russia and the CIS. Bioluminescent methods using bacterial luminous cells and, isolated from them, luminescent systems, were approved by experts in ecology, recommended as an additional method for ecological monitoring, and under the name of Microbiosensor got a certificate from the Federal Agency on Technical Regulation and Metrology (№ 224.01.13.151/2007). The sensitivity of the biosensors developed in the IBP SB RAS is comparable to that of the foreign analogues Microtox, ToxAlert, Lumistox, etc. Bioluminescent assays using bacteria and enzyme

systems are mutually complementary in ecological research approaches. The simultaneous use of bioassays allows for an expansion of the range of analyzed toxicants.

The Culture Collection at the Institute of Biophysics, Siberian Branch, Russian Academy of Sciences, operating under the acronym CCIBSO 836, contains the marine luminous bacteria *P. phosphoreum, P. leiognathi, V. harveyi,* and *V. fischeri,* and genetically modified strains of *Escherichia coli,* bearing *lux* genes from the luminescent cells of *P. leiognathi*. The collection not only provides many possibilities for perfecting bioassays and finding strains that are sensitive to certain toxicants, but also finds the know-how behind it for producing kit reagents for bioluminescent analyses based on isolated bacterial luminescent systems. Information in connection with numerous problems of bioluminescence and bioluminescent analyses is available at the Web-portal "Bioluminescence and luminous organisms" (http://bl.ibp.ru), which is the result of the development by scientists of the Culture Collection IBSO.

Acknowledgment

This work is supported by project no. 38 of the Program of Basic Research of the Siberia Division of the Russian Academy of Sciences.

References

1. Kaiser, K.L. 1998, Environ. Health Perspect., Suppl 2, 583-591.
2. Hemming, J. M., Turner, P.K., Brooks, B.W., Waller, W.T. and La Point, T.W. 2002, Arch. Environ. Contam. Toxicol., 42, 9-16.
3. Kim, B.C., Park, K.S., Kim, S.D., and Gu, M.B. 2003, Biosens. Bioelectron., 18 (5/6), 821-826.
4. Roda, A., Pasini, P., Mirasoli, M., Michelini, E., and Guardigli, M. 2004, Trends Biotechnol., 22 (6), 295-303.
5. Deheyn, D.D., Bencheikh-Latmani, R., and Latz, M.I. 2004, Environ. Toxicol., 19(3), 161-178.
6. Jos, A., Repetto, G., Rios, J.C., Peso, A.D., Salguero, M., Hazen, M.J., Molero, M.L., Fernandez-Freire, P., Perez-Martin, J.M., Labrador, V., and Camean, A. 2005, Aquat. Toxicol., 71 (2), 183-192.
7. Hastings, J.W. 1968, Ann. Rev. Biochem., 37, 597-608.
8. Hastings, J.W, and Johnson, C.H. 2003, Methods Enzymol., 360, 75-104.
9. Fisher, A.J, Thompson, T.B., Thoden, J.B., Baldwin, T.O., and Rayment, I. 1996, J. Biol. Chem, 271 (36), 21956-21968.
10. Engebrecht, J., and Silverman, M. 1984, Proc. Natl. Acad. Sci. USA, 81, 4154-4158.
11. Meighen, E. A. 1994, Annu. Rev. Genet., 28, 117-139.
12. Meighen, E. A. 1991, Microbiol.Rev., 55, 123-142.

13. Eberhard, A., Burlingame, A. L., Kenyon, G. L., and Nealson K. H., and Oppenheimer N. J. 1981, Biochemistry, 20, 2444-2449.
14. Cronin, M. T. D., and Schultz, T. W. 1998, Ecotoxicol. Environ. Saf., 39 (1), 65-69.
15. Sixt, S., Altschuh, J., and Bruggeman, R. 1995, Chemosphere, 30 (12), 2397-2414.
16. Gustavson, K. E., Svenson, A., and Harbin, J. M. 1998, Environ. Toxicol. Chem., 17(10), 1917-1921.
17. Gitelson, J.I., Rodicheva, E.K., Medvedeva, S.E., et al. 1984,The Luminous Bacteria. Nauka, M. (in Russian).
18. Wolska, L, and Polkowska, Z. 2001, Bull. Environ. Contam. Toxicol., 67 (1), 52-58.
19. Ulitzur, S., Lahav, T., and Ulitzur, N. 2002, Environ. Toxicol., 17 (3), 291-296.
20. Wang, C., Yediler, A., Lienert, D., Wang, Z., and Kettrup, A. 2002, Chemosphere, 46 (2), 339-344.
21. Parvez, S., Venkataraman, C., and Mukherji, S. 2006, Environ. Int., 32 (2), 265-268.
22. Grande, R., Di Pietro, S., Di Campli, E., Di Bartolomeo, S., Filareto, B., and Cellini, L. 2007, J. Environ. Sci. Health. A Tox. Hazard Subst. Environ. Eng., 42 (1), 33-38.
23. Girotti, S., and Maiolini, E.E. 2008, Anal. Chim. Acta., 608, 2-29.
24. Scheerer, S., Gomez, F., and Lloyd, D., 2006, J. Microbiol. Methods., 67, 321-329.
25. Lee, J.H., and Gu, M.B. 2005, Biosens. Bioelectron., 20 (9), 1744-1749.
26. Kuznetsov, A., Primakova, G., and Fish, A. 1990, Biological Bioluminescence. B.Jezowska-Trzebiatowska, B.Kochel, J.Slawinski, W. Strek (Edts), World Scientific. 559-563.
27. Kuznetsov, A.M., Rodicheva, E.K., and Medvedeva, S.E. 1998, Field Analyt. Chem. Technol., 2 (5), 267-275.
28. Jennings, V.L.K., Rayner-Brandes, M.H., and Bird, D.J. 2001, Water Res., 35 (14), 3448-3456.
29. Hyung, C.S., and Gu, M.B. 2002, Biosens. Bioelectron., 17, 433-440.
30. Schreiter, P.P., Gillor, O., Post, A., Belkin, S., Schmid, R.D., and Bachmann, T.T. 2001, Biosens. Bioelectron., 16, 811-818.
31. Lee, H.J., Villaume, J., Cullen, D.C., Kim, B.C., and Gu, M.B. 2003, Biosens. Bioelectron., 18 (5/6), 571-577.
32. Park, K.S., Baumstark-Khan, C., Rettberg, P., Horneck, G., Rabbow, E., and Gu, M.B. 2005, Radiat. Environ. Biophys., 44 (1), 69-71.
33. Gu, M.B., and Choi, S.H. 2001, Water Sci. Technol., 43 (2), 147-154.
34. Abd-El-Haleem, D., Ripp, S., Scott, C., Sayler, G.S. 2002, J. Ind. Microbiol. Biotechnol., 29 (5), 233-237.
35. Abd-El-Haleem, D., Zaki, S., Abulhamd, A., Elbery, H., and Abu-Elreesh, G. 2006, J. Basic Microbiol., 46 (5), 339-347.
36. Bechor, O., Smulski, D.R., Van Dyk, T.K., La Rossa, R.A., and Belkin, S. 2002, J. Biotechnol., 94 (1), 125–132.
37. Ren, S., and Frymier, P.D. 2003, Water Environ. Res., 75 (1), 21-29.
38. Ren, S., and Frymier, P.D. 2005, Chemosphere, 58 (5), 543-550.

39. Ivask, A., Francois, M., Kahru, A., Dubourguier, H.C., Virta, M., and Douay, F. 2004, Chemosphere, 55 (2), 147-156.
40. Lovanh, N., and Alvarez, P.J. 2004, Biotechnol. Bioeng., 86 (7), 801-808.
41. Berno, E, Pereira Marcondes, D.F., Ricci Gamalero, S., and Eandi, M. 2004, Ecotoxicol. Environ. Saf., 57 (2), 118-122.
42. Lang, T., Goyard, S., Lebastard, M., and Milon, G. 2005, Cell. Microbiol., 7 (3), 383-392.
43. Lee, J.H., Mitchell, R.J., Kim, B.C., Cullen, D.C., and Gu, M.B. 2005, Biosens. Bioelectron., 21 (3), 500-507.
44. Lee, J.H., Youn, C.H., Kim, B.C, and Gu, M.B. 2007, Biosens. Bioelectron., 22 (9/10), 2223-2229.
45. Toba, F.A., and Hay, A.G. 2005, J. Microbiol. Methods., 62 (2), 135-143.
46. Wiles, S., Lilley, A.K., Philp, J.C., Bailey, M.J., and Whiteley, A.S. 2005, Environ. Microbiol., 7 (2), 260-269.
47. Chinalia, F.A., Paton, G.I., and Killham, K.S. 2008, Bioresour. Technol., 99 (4), 714-721.
48. Van Dyk, T.K. 1998, Methods Mol. Biol., 102, 153-160.
49. Qazi, S.N., Harrison, S.E., Self, T., Williams, P., and Hill, P.J. 2004, J. Bacteriol., 186 (4), 1065-1077.
50. Francis, K. P., Joh, D., Bellinger-Kawahara, C., Hawkinson, M.J., Purchio, T.F., and Contag, P.R. 2000, Infection & Immunity, 68, 3594-3600.
51. Van Dyk, T.K. 2001, J. Bacteriol., 183 (19), 5496-5505.
52. Gillor, O., Harush, A., Hadas, O., Post, A.F., and Belkin, S. 2003, Appl. Environ. Microbiol., 69 (3), 1465-1474.
53. Bautista, D.A., Chen, J., Barbut, S., and Griffiths, M.W. 1998, J. Food Prot., 61(11), 1439-1445.
54. Korpela, M.T., Kurittu, J.S., Karvinen, J.T., and Karp, M.T. 1998, Anal. Chem., 70 (21), 4457-4462.
55. Kurittu, J., Karp, M., and Korpela, M. 2000, Luminescence., 15, 291-297.
56. Simon, L., Fremaux, C., Cenatiempo, Y., and Berjeaud, J.-M. 2001, Appl. Microbiol. Biotechnol., 57, 757-763.
57. Kuklin, N.A., Pancari, G.D., Tobery, T.W., Cope, L., Jackson, J., Gill, C., Overbye, K., Francis, K.P., Yu, J., Montgomery, D., Anderson, A.S., McClements, W., and Jansen, K.U. 2003, Antimicrob. Agents Chemother., 47 (9), 2740-2748.
58. Vesterlund, S., Paltta, J., Laukova, A., Karp, M., and Ouwehand, A.C. 2004, J. Microbiol. Methods, 57 (1), 23-31.
59. Pellinen, T., Bylund, G., Virta, M., Niemi, A., and Karp, M. 2002, J. Agric. Food Chem., 50 (17), 4812-4815.
60. Valtonen, S.J., Kurittu, J.S., and Karp, M.T. 2002, J. Biomol. Screen., 7 (2), 127-134.
61. Stewart, G. S.A.B., and Williams P. J. 1992, Gen. Microbiol., 138, 1289-1300.
62. Selifonova, O., Burlage, R., and Barkay, T. 1993, Appl. Environ. Microbiol., 59, 3083-3090.
63. Riether, K.B., Dollard, M.A., and Billard, P. 2001, Appl. Microbiol. Biotechnol., 57 (5/6), 712-716.

64. Fulladosa, E., Murat, J.C., and Villaescusa, I. 2005, Arch. Environ. Contam. Toxicol., 49 (3), 299-306.
65. Selifonova, O. V., and Eaton, R. W. 1996, Appl. Environ. Microbiol., 62, 778-783.
66. Applegate, B. M., Kehrmeyer, S. R., and Sayler, G. S. 1998, Appl. Environ. Microbiol., 64 (7), 2730-2735.
67. Ford, C.Z., Sayler, G.S., and Burlage, R.S. 1999, Appl. Microbiol. Biotechnol., 51 (3), 397-400.
68. Van Dyk, T.K., Majarian, W.R., Konstantinov, K.B., Young, R.M., Dhurjati, P.S., and La Rossa, R.A. 1994, Appl. Environ. Microbiol., 60, 414-1420.
69. Van Dyk, T.K., Smulski, D.R., Reed T.R., Belkin, S., Vollmer, A.C., and La Rossa, R.A. 1995, Appl. Environ. Microbiol., 61 (11), 4124-4127.
70. Rupani, S.P.,and Gu, M.B., Konstantinov, K.B., Dhurjati, P.S., Van Dyk, T.K., and La Rossa, R.A. 1996, Biotechnol. Prog., 12 (3), 387-92.
71. Belkin, S., Smulski, D. R., Dadon, S., Vollmer, A. C., Van Dyk, T. K., and LaRossa, R.A. 1997, Water Res., 31, 3009-3016.
72. Rogowsky, P.M., Close, T.J., Chimera, J.A., Shaw, J.J., and Kado, C.I. 1987, J. Bacteriol., 169 (11), 5101-5112.
73. Ben-Israel, O., Ben-Israel, H., and Ulitzur, S. 1998, Appl. Environ. Microb., 64 (1), 4346-4352.
74. Stoyanov, J.V., Magnani, D., and Solioz, M. 2003, FEBS Lett., 546 (2/3), 391-394.
75. Kahru, A., Ivask, A., Kasemets, K., Pollumaa, L., Kurvet, I., Francois, M., and Dubourguier, H.C. 2005, Environ. Toxicol. Chem., 24 (11), 2973-2982.
76. Alkorta, I., Epelde, L., Mijangos, I., Amezaga, I., and Garbisu, C. 2006, Rev. Environ. Health., 21 (2), 139-152.
77. Dawson, J.J., Campbell, C.D., Towers, W., Cameron, C.M., and Paton, G.I. 2006, Environ. Pollut., 42 (3), 493-500.
78. Trott, D., Dawson, J.J., Killham, K.S., Miah, M.R., Wilson, M.J., and Paton, G.I. 2007, J. Environ. Monit., 9 (1), 44-50.
79. Czyz, A., Szpilewska, H., Dutkiewicz, R., Kowalska, W., Biniewska-Godlewska, A., and Wegrzyn, G. 2002, Mutat. Res., 519 (1/2), 67-74.
80. Guzzella, L., Di Caterino, F., Monarca, S., Zani, C., Feretti, D., Zerbini, I., Nardi, G., Buschini, A., Poli, P., and Rossi, C. 2006, Mutat Res., 608 (1), 72-81.
81. Rathinam, K., and Mohanan, P.V. 1998, J. Biomater. Appl., 13 (2), 166-171.
82. Gad, F., Zahra, T., Francis, K.P., Hasan, T., and Hamblin, M.R. 2004, Photochem. Photobiol. Sci., 3 (5), 451-458.
83. Edinger, M., Cao, Y.A., Verneris, M.R., Bachmann, M.H., Contag, C.H., and Negrin, R.S. 2003, Blood., 101 (2), 640-648.
84. Patterson, S.S., Dionisi, H.M., Gupta, R.K., and Sayler, G.S. 2005, J. Ind. Microbiol. Biotechnol., 32 (3), 115-123.
85. Frundzhian, V.G., Brovko, L.Iu., Karabasova, M.A., and Ugarova, N.N. 1997, Prikl. Biokhim. Mikrobiol., 33 (4), 455-460.
86. Henschel, K.P., Wenzel, A., Diedrich, M., and Fliedner, A. 1997, Regul. Toxicol. Pharmacol., 25 (3), 220-225.

87. Stom, D.I., Geel, T.A., Balayan, A.E., Shachova, G.I., Kuznetsov, A.M., and Medvedeva, S,E. 1992, Arch. Environ. Contam. Toxicol. 2, 203-208.
88. Mariscal, A., Peinado, M.T., Carnero-Varo, M., and Fernandez-Crehuet, J. 2003, Chemosphere, 50 (3), 349-354.
89. Popova, L.Yu., Kalacheva, G.S., Mogil'naya, O.A., Medvedeva, S.E., and Pechurkin, N.S. 1994, Prikl. Biokhim. Mikrobiol., 30 (4/5), 650-656.
90. Medvedeva, S.E. 1999, Luminescence., 14 (5), 267-270.
91. Leitgib, L., Kalman, J., and Gruiz, K. 2006, Chemosphere., 66 (3), 428-434.
92. Rozhko, T.V., Kudryasheva, N.S., Kuznetsov, A.M., Vydryakova, G.A., Bondareva, L.G., and Bolsunovsky, A.Ya. 2007, Photochem. Photobiol. Sci., 6, 67-70.
93. Drzyzga, O., Gorontzy T., Schmitl A., and Blotevogel, K.H. 1995, Arch. Environ. Contam.Toxicol., 28 (2), 229-235.
94. Kuznetsov, A.M., Rodicheva, E.K., and Medvedeva, S.E. 2000, Probl. Environ. Nat. Resourc., 10, 67-73.
95. Loibner, A.P., Szolar, O.H., Braun, R., and Hirmann, D. 2004, Environ. Toxicol. Chem., 23 (3), 557-564.
96. Micevska, T., Warne, M.S., Pablo, F., and Patra, R. 2006, Arch. Environ. Contam. Toxicol., 50 (2), 205-212.
97. Rabbow, E., Stojicic, N., Walrafen, D., Baumstark-Khan, C., Rettberg, P., Schulze-Varnholt, D., Franz, M., and Reitz, G. 2006, Res. Microbiol., 157 (1), 30-36.
98. Ritchie, J. M., Cresser M., and Cotter-Howells, J. 2001, Environ. Pollut., 114, 129-136.
99. Fernández-Alba, A.R., Hernando Guil, M.D., D´ýaz López, G., and Chisti, Yu. 2002, Analytica Chimica Acta, 451, 195-202.
100. Kim, B.C., Gu, M.B., 2005, Environ. Monit. Assess, 109 (1-3), 123-133.
101. Phyu, Y.L., Warne, M.S., and Lim, R.P. 2005, Water Res., 39, 2738-2746.
102. Kuznetsov, A.M., Rodicheva, E.K., and Medvedeva, S.E. 1999, Luminescence, 14 (5), 263-265.
103. Rodicheva, E.K., Kuznetsov, A.M., and Medvedeva, S.E. 2004, The Bulletin OGU, 5, 96-100.
104. Illarionov, B.A.,and Tyulkova, N.A. 1997, *Escherichia coli* bacterial strain SL-60 - producer of bacterial luciferase. Invention Patent N 2073714.
105. Inzhevatkin, E.V., Fomenko, E. Yu., Slepov E. V., and Savchenko, A. A. 2007, Biol. Bull., 34 (3), 310-313.
106. Kolenchukova, O.A., Savchenko, A.A., Borisov, A.G., Kouznetsov, S.A., Borodina, N.A., and Otto, V.S. 2008, Sibirsky Medizinsky Journal, 23 (3), 23-26.
107. Kudryasheva, N.S., Kratasyuk, V.A., and Belobrov, P.I. 1994, Anal. Letter, 27 (15), 2931-2947.
108. Kudryasheva, N.S., Shalayeva, E.E., Zadorozhnaya, E.N., Kratasyuk, V.A., Stom, D.I., and Balayan, A.E. 1994, Biofisika, 38 (3), 455-464.
109. Middleton, A.J., and Smith, E.B. 1976, Proc. Roy. Soc. Lond. B, 193 (4), 159-171.
110. Middleton, A.J., and Smith, E.B. 1976, Proc. Roy. Soc. Lond. B, 193 (4), 173-190.

111. Kratasyuk, V.A., Makurina, V.I., Kuznetsov, A.M., Kudryasheva, N.S., Plotnikova N.B., Medvedeva, S.E., Gritsenko M.S., and Chernykh, V.P. 1991, Appl. Biochem. Microbiol., 27 (1), 127-133.
112. Kratasyuk, V.A., and Fish, A.M. 1980, Biochemistry, 45 (7), 1175-1182.
113. Kudryasheva, N., Vetrova, E., Kuznetsov, A., Kratasyuk, V., and Stom, D. 2002, Ecotoxicology Environ. Safety, 53 (3), 198-203.
114. Kudryasheva, N.S., Kudinova, I.Y., Esimbekova, E.N., Kratasyuk, V.A., and 0Stom, D.I. 1998, Chemosphere, 38 (4), 751-758.
115. Kudryasheva, N.S., Kratasyuk, V.A., Esimbekova, E.N., Vetrova, A., and Kudinova, I.Y. 1998, Field Analyt. Chem. Technol., 2 (5), P.277-280.
116. Petushkov, V.N., Kratasyuk, G.A., Rodionova, N.S., Fish, A.M., and Belobrov.P.I. 1984, Biochemistry, 49 (4), 699-709.
117. Kudryasheva, N.S. 2006, J. Photochem. Photobiol., 1, 77-86.

Research signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 51-65
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

4 Bioluminescence of sharks: First synthesis

J. M. Claes and J. Mallefet
Laboratory of Marine Biology, Catholic University of Louvain, Place Croix du Sud, Kellner Building, B-1348 Louvain-la-Neuve, Belgium

Abstract

This work gives a state-of-the-art synthesis on shark luminescence. We present information on the different luminescent sharks, their luminous patterns, the histological organisation of their photogenic organs, their specialised squamation, and the characteristics and putative functions of their bioluminescence. We illustrate differences between the two families of luminescent sharks and give a hypothesis for the bioluminescence acquisition in these two groups. We also highlight the limitations of our current knowledge of these luminous organisms; finally, recent discoveries on one model species, the velvet belly lantern shark (Etmopterus spinax), are being presented.

Correspondence/Reprint request: J. M. Claes, Laboratory of Marine Biology, Catholic University of Louvain, Place Croix du Sud, Kellner Building, B-1348 Louvain-la-Neuve, Belgium. E-mail: julien.m.claes@uclouvain.be

1. Bibliographical synthesis

1.1. Introduction

In the deep-sea, many organisms, from bacteria to fishes, possess structures to emit light. In this dark environment which offers no place to hide and only rare meals, bioluminescence assumes numerous functions including predation, defence against predators and reproduction [1,2,3]. While the majority of deep-sea Osteichthyes (circa 70 %) are luminous, only a small fraction of deep-sea Chondrichthyes (circa 6%) is endowed with this capability [1,4,5]. This led some authors to consider this amazing property like a burden in these fishes [6]. Even though it is absent in Holocephali (chimaeras), luminescence competence nevertheless appeared at least three times independently in cartilaginous fishes (figure 1), once in basal Batoidea (rays) and twice in Squalean sharks; thus, a total of at least 51 cartilaginous species are involved [5, 7, 8, 9].

Due to the relative inaccessibility of their environment, the logistical difficulties linked to their maintenance in experimental conditions and their rarity (some are only known from one of several specimens), luminescent sharks have been poorly investigated [6, 10]. This explains why only extremely limited and fragmented information is available in the literature on shark bioluminescence. This paper aims to provide the first synthesis on what is currently known on the phenomenon of visible light emission in these cartilaginous fishes.

1.2. Luminescent sharks

1.2.1. Symbiotic luminescence

In symbiotic luminescence the light production is only present through the association with another organism which is luminous. This kind of extrinsic luminescence is frequently observed in deep-sea squids and Osteichthyes harboring bacterial photophores [1,14,15]. Forms of symbiotic luminescence have once been suggested for some shark species: (i) the mouth of the megamouth shark, *Megaschasma pelagios*, was considered to be a bacteria-filled luminous lure allowing krill attraction [16], and (ii) ocular parasitic copepods of sleeper sharks (*Somniosus* species) were thought to attract prey items to the mouth of the shark [17]. Now, since any evidence to find luminescent tissues in the mouth of *M. pelagios* failed and since tested specimens of sleeper shark copepods were not luminous, symbiotic luminescence seems to be very unlikely in sharks [18, 19].

1.2.2. Intrinsic luminescence

Intrinsic bioluminescence appeared in two shark families: the Etmopteridae ("lantern sharks") and in the Dalatiidae ("dwarf mesopelagic

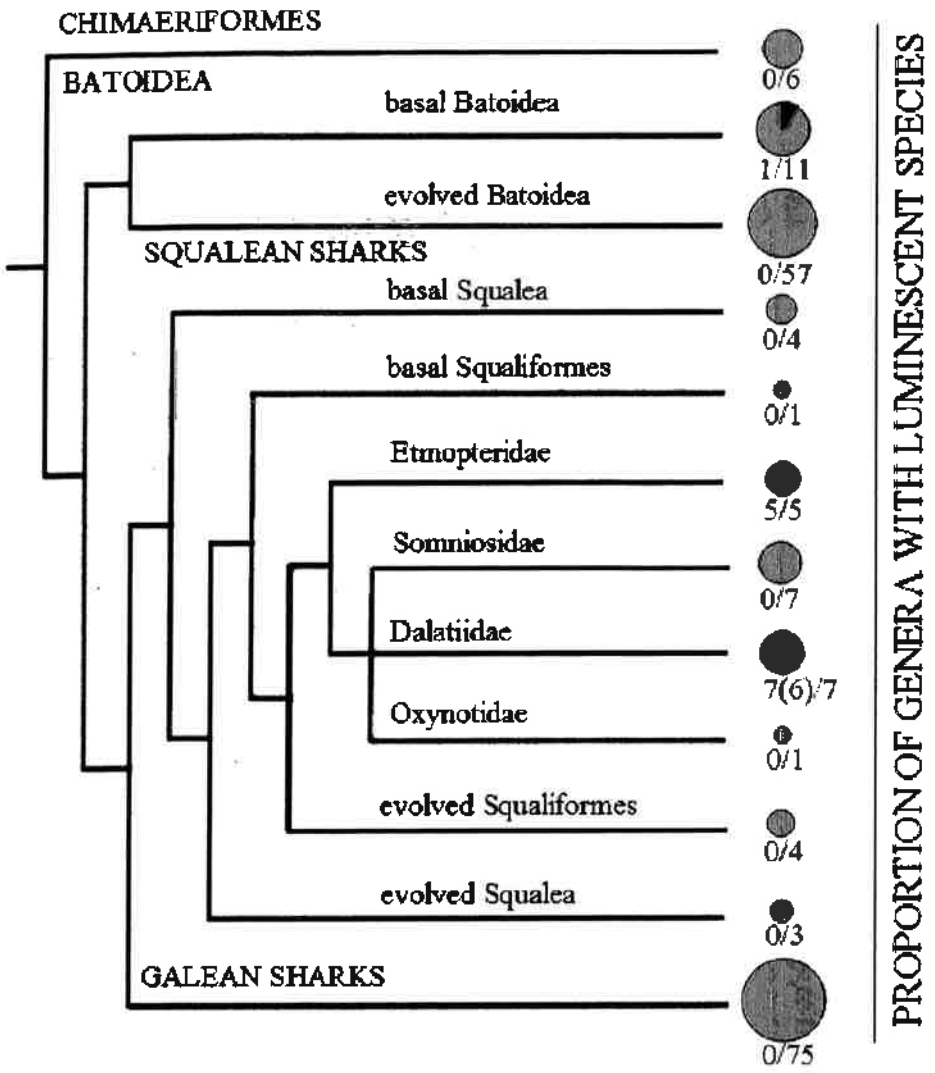


Figure 1. Cladogram showing the occurrence of bioluminescence in cartilaginous fishes [5,11,12,13]. Circles to the right indicate the proportion of luminous genera containing luminous species in a taxonomic group (black shade, luminous; grey shade, non-luminous). Circles are scaled to the number of genera in a given taxonomic group. The only luminous ray, *Benthobatis moresbyi* (dark blind ray), shows a characteristic row of minute photophores along the edges of its disk [7].

sharks"), which encompass small species which can be found everywhere in the water column, but generally at great (> 200 m) depths (only one dalatiid species, *Dalatias licha*, exceeds 1 m in total length; figure 2) [5,9,20].

The Etmopteridae radiated 90 millions years ago when they started to leave shallow water environments to occupy the empty deep water niches during the marine life turnover, which followed the mass extinction provoked by the global Cenomanian-Turonian anoxic event ("Bonarelli event") [21]. This shark family encompasses five different genera (*Aculeola*, *Centroscyllium*, *Etmopterus*, *Miroscyllium* and *Trigonognathus*), which are all luminous [5].

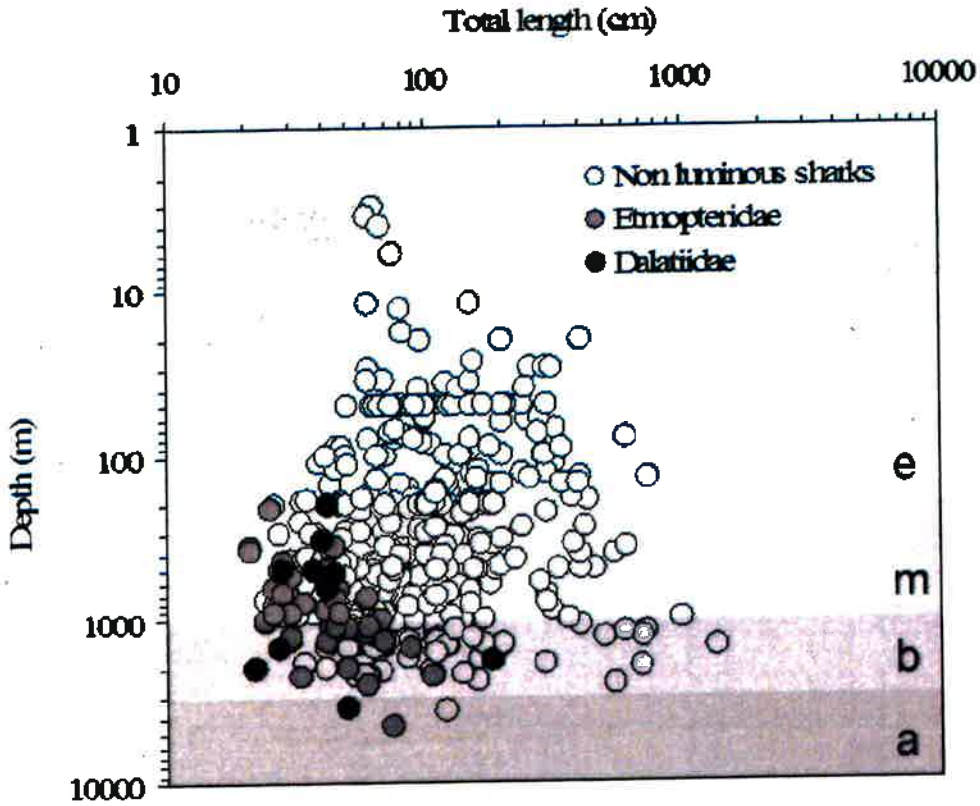


Figure 2. Maximum depth of occurrence for Etmopteridae, Dalatidae and non-luminous sharks plotted against their maximum recorded size [20,23]. Data set for maximum total length recorded from 455 species of sharks. Data are presented on a log-log scale. e, epipelagic zone; m, mesopelagic zone; b, bathypelagic zone; a, abyssopelagic zone.

The Dalatiidae, on the other hand, evolved later when they replaced extinct large fishes and marine reptiles in the epipelagic fauna of the Palaeocene after the Cretaceous/Palaeocene mass extinction (65 millions years ago) [21]. Seven genera are present in this family (*Dalatias*, *Euprotomicroides*, *Euprotomicrus*, *Heteroscymnoides*, *Isistius*, *Mollisquama* and *Squaliolus*), of which at least six are known to contain luminescent species (the genus *Mollisquama* contains the species *M. parinii*, which is only known from one individual whose luminescence needs to be confirmed) [5,12].

While Etmoperidae are demersal species, which generally live in groups, all Dalatiidae are essentially solitary pelagic (except *D. licha*, which is benthopelagic) predators, which undergo daily vertical migrations [20,22].

1.3. Photogenic structures

1.3.1. Luminous patterns

Etmopteridae and Dalatiidae show two different types of photophore organizations (= luminous patterns; figure 3) [6].

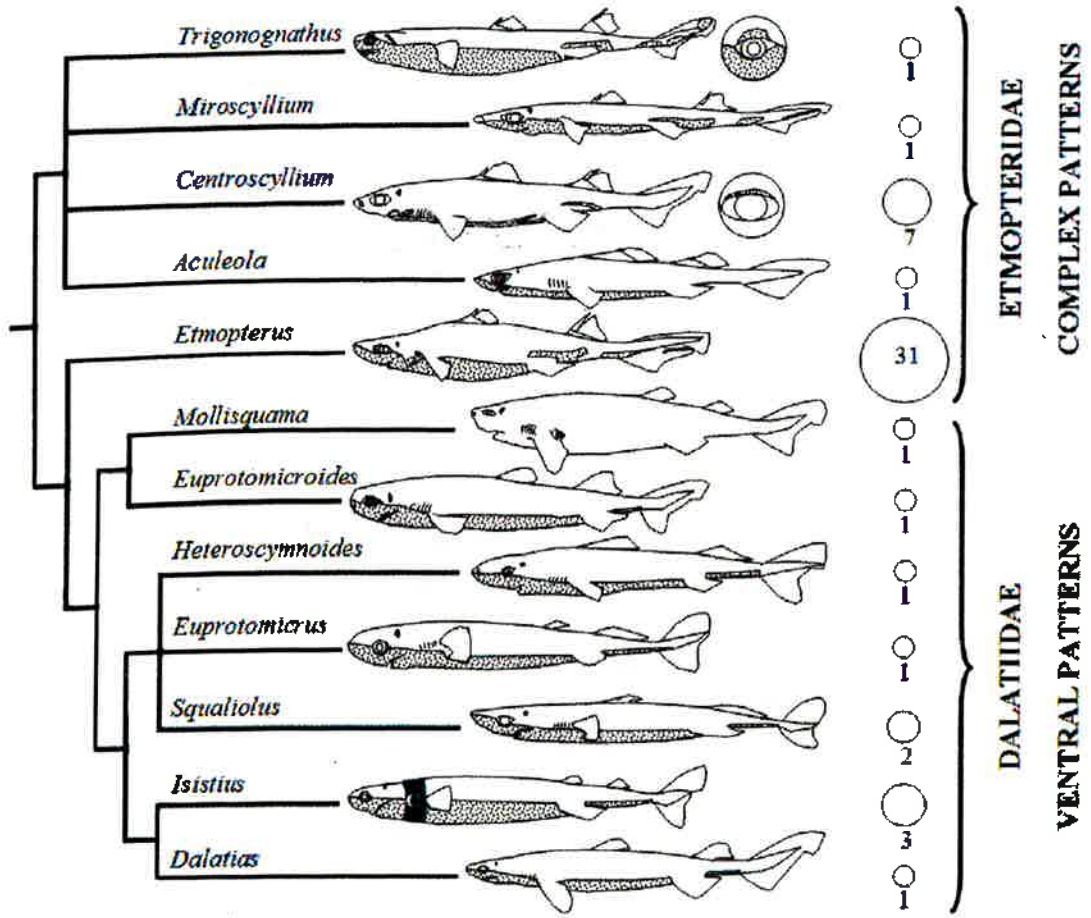


Figure 3. Cladogram of luminescent sharks based on dental characters and showing their different luminous patterns (dotted areas) [adapted from 5,6,12,21,30,31]. The genus *Heteroscymnoides* has been added into the same taxonomic grouping as *Euprotomicrus* and *Squaliolus*, based on morphological similarities. Circles to the right scale to the number of species in a given genus. Inserts for *Centroscyllium* and *Trigonognathus* represent their ocular photophores. c = dark collar of *Isistius* species.

In dalatiid species, photophores are arranged following a density gradient from dorsal to ventral region, where their number may attain 60 units per square millimeter [6,9,24]. Among the species possessing this pattern, one genus (*Isistius*) harbours a dark collar lacking photophores in front of the pectoral fins. Luminous patterns of the Dalatiid species are presented in figure 3. The luminous pattern of the genus *Mollisquama* is not represented, because it has never been described and as already mentioned, the luminous status of *M. parinii* remains uncertain.

Etmopterid sharks possess considerably more complex luminous patterns than the Dalatiidae, as they show aggregations of photophores on the ventral side, but also on the flanks, the tail and even (in *Centroscyllium* and *Trigonognatus*) on the upper eyelid (figure 3) [6,25,26]. In some species of

lantern sharks, the photophores can also be inconspicuous because they are not arranged in specific markings [5]. As the luminous pattern of lantern sharks is supposed to be species-specific and to stay constant between sexes and throughout the ontogeny, it has often been used for species determination [6, 27, 28, 29].

1.3.2. Photogenic organs

1.3.2.1. Photophores

Photophores of sharks are extremely small (100 μm and 150 μm on average, for the Dalatiidae and Etmopteridae respectively) and numerous (several thousands); so that sharks are probably the animals with the highest number of photophores present [1,9]. Compared with the photophores of the Osteichtyes, the structure of those of the sharks is simpler, being only composed of few elements [14,32]. Structural differences exist between the two families of luminescent sharks [9].

Dalatiid sharks harbour photophores that contain only a single photogenic cell (= photocyte) plus a pigmented sheath and a lens made up of a group of small cells overlying the apical surface of the photogenic cell (figure 4a). Photocytes contain granules supposed to be photogenic and a large nucleus

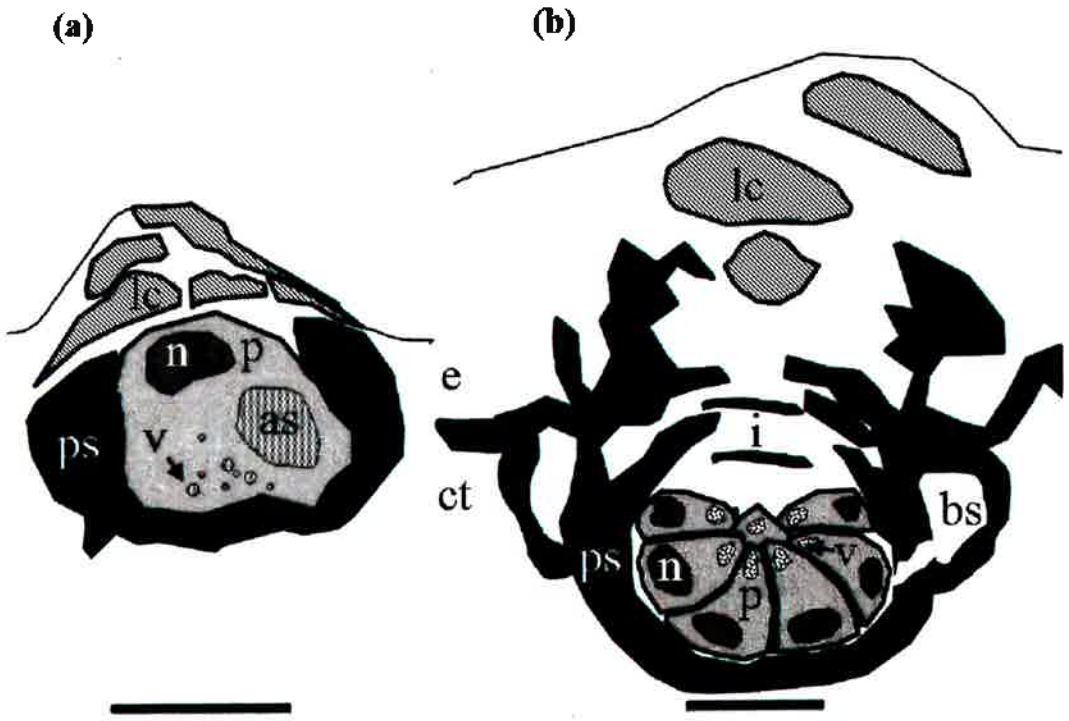


Figure 4. Photophores of luminescent sharks [adapted from 8,9,26,33,34]: (a) Dalatiidae (b) Etmopteridae. As, acidophilic secretion; bs, blood circulating sinus; ct, connective tissue; e, epidermis; i, iris; lc, lens cell; n, nucleus; p, photocyte; ps, pigmented sheath; v, vesicle presumed to be photogenic. Bars scale 50 µm.

generally placed at the apical side. The lens cells contain a homogeneous cytoplasm, presumably transparent in life. The photophores are present in the stratified squamous epidermis of the skin and lack reflectors or any kind of specialized structure [9, 33].

Photophores of Etmopteridae are more complex than those of Dalatiidae. They are composed of a black circular pigmented sheath encompassing a group of 6-13 photogenic cells in general, a lens formed by 2 to 3 lens cells, and an iris-like structure formed by chromatophores (sometimes divided into 3 groups: internal, horizontal, and external process following their distance to the photocytes; figure 4b). It has to be noted that etmopterid circular photophores sometimes fuse together to produce larger linear photophores (300 μm in length by 100 μm broad) with a higher number of lens cell. The photophores also lack reflectors in this group [8,9,26,34].

1.3.2.2. Secretory luminous tissues

In *Euproctomicroides zantedeschia* a putatively luminous tissue of another type has been found [35]. This tissue is a probably stratified epithelium, present into the abdominal pouch of this species, with a thickness of 50-75 µm (figure 5). It is composed of different cell types: (i) columnar cells possessing one huge yellow apical inclusion (30 x 25 µm) as well as a basal nucleus; (ii) flattened cells with basal nucleus extending from the basal lamina towards the free surface of the epithelium, separating the lateral cell membranes of tall columnar cells; (iii) superficial cells, with a nucleus close to the surface of the epithelium, forming a very thin cover over the dome-shaped distal surfaces of the columnar cells.

A tissue of this kind may also exist in the pectoral gland of *M. parinii* [5].

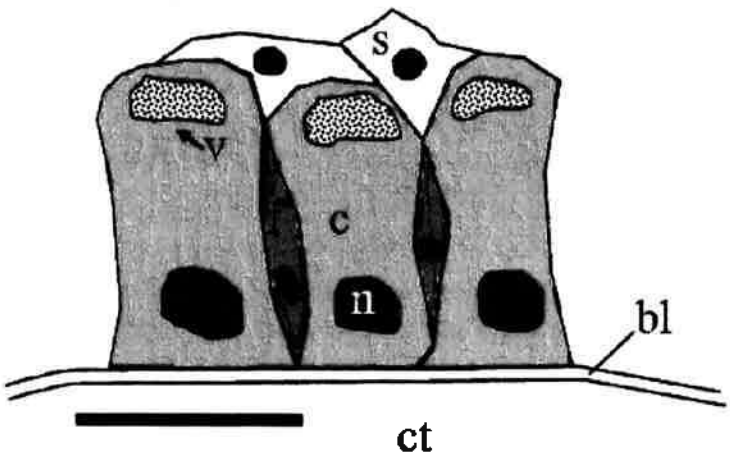


Figure 5. Secretory luminous tissue of *Euprotomicroides zantedeschia* [adapted from 35]. bl, basal lamina; c, columnar cell; ct, connective tissue; f, flattened cell; n, nucleus; s, superficial cell; v, vesicle presumed to be photogenic. Bar scales 50 µm.

1.3.3. Specialized squamation

Placoid scales of sharks evolved to assume many functions, including defence against parasites and predators, protection towards substrate abrasion, involvement in hydrodynamics and, finally, in connection with an accommodation of sensory and bioluminescent organs [6, 36]. Luminescent sharks had indeed to elaborate special squamation, which allows coexistence of epidermal photophores and placoid scales. Four major special squamation patterns are found in luminescent sharks and are presented in figure 6. The bioluminescent squamation patterns of luminescent sharks are so specific that they could be used as a tool to determine bioluminescence not just in current, but also in extinct sharks [6].

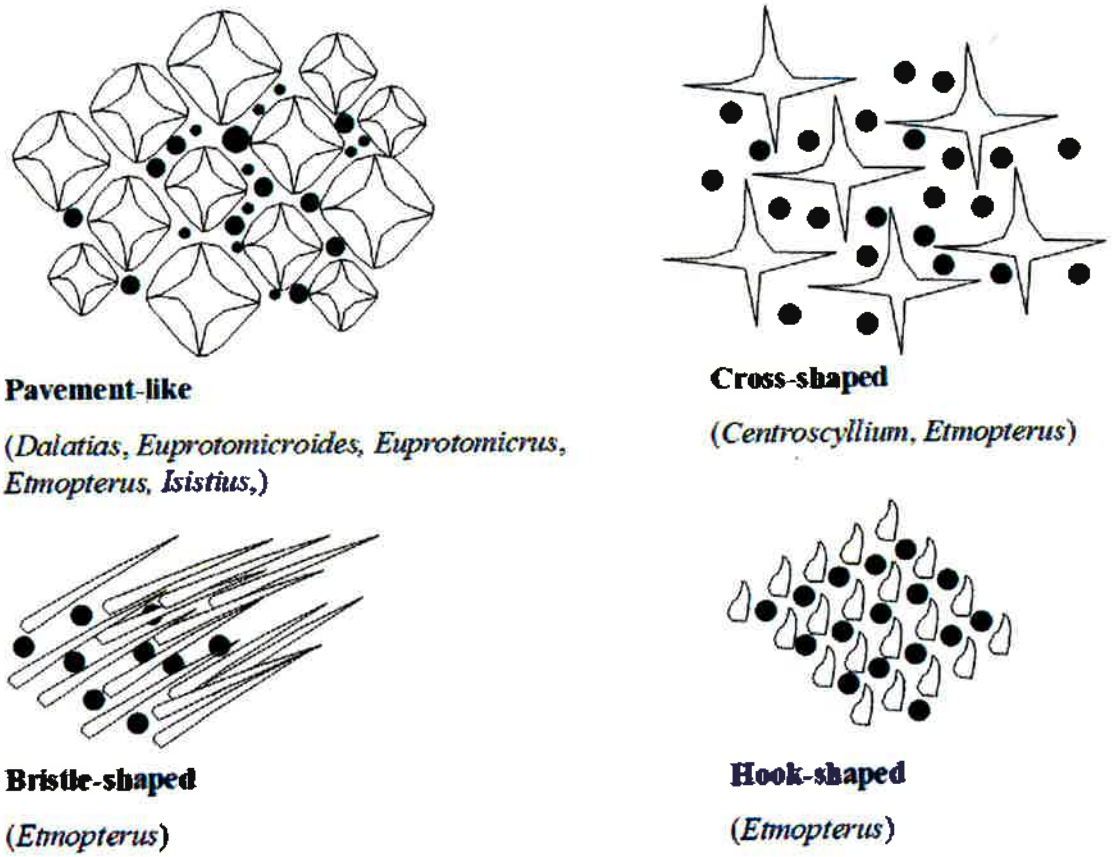


Figure 6. Squamation patterns of luminescent sharks [adapted from 6, 37]. Genus in which the specific pattern has been found are noted into brackets. Black dots = photophores.

1.4. Bioluminescence

1.4.1. Physical and chemical characteristics

Most of our knowledge of shark luminescence is almost exclusively based on simple visual observations, which are sometimes contradictory and

doubtful [31,34,38,39,40,41]. The only species for which spontaneous bioluminescence has been observed are *Euprotomicrus bispinatus*, *Isistius brasiliensis*, *Etmopterus spinax* and *Etmopterus pusillus*. Only the bioluminescence spectrum of *I. brasiliensis* has been precisely measured with a spectrophotometer [42]. This shark emits a bluish glow with a peak of emission at 455 nm and a half bandwidth of 73 nm (427-500 nm, figure 7a,b). *E. spinax* also shows a bluish glow with a peak of emission, which seems, however, to occur at higher wavelength, even though it has never been measured (figure 7c) [39, 40]. From the literature we know that *E. bispinatus* emits a greenish glow [31,41] while *E. pusillus* emits a "whitish" glow [34]. However, these assertions have to be considered with the highest care until spectral measurements are available.

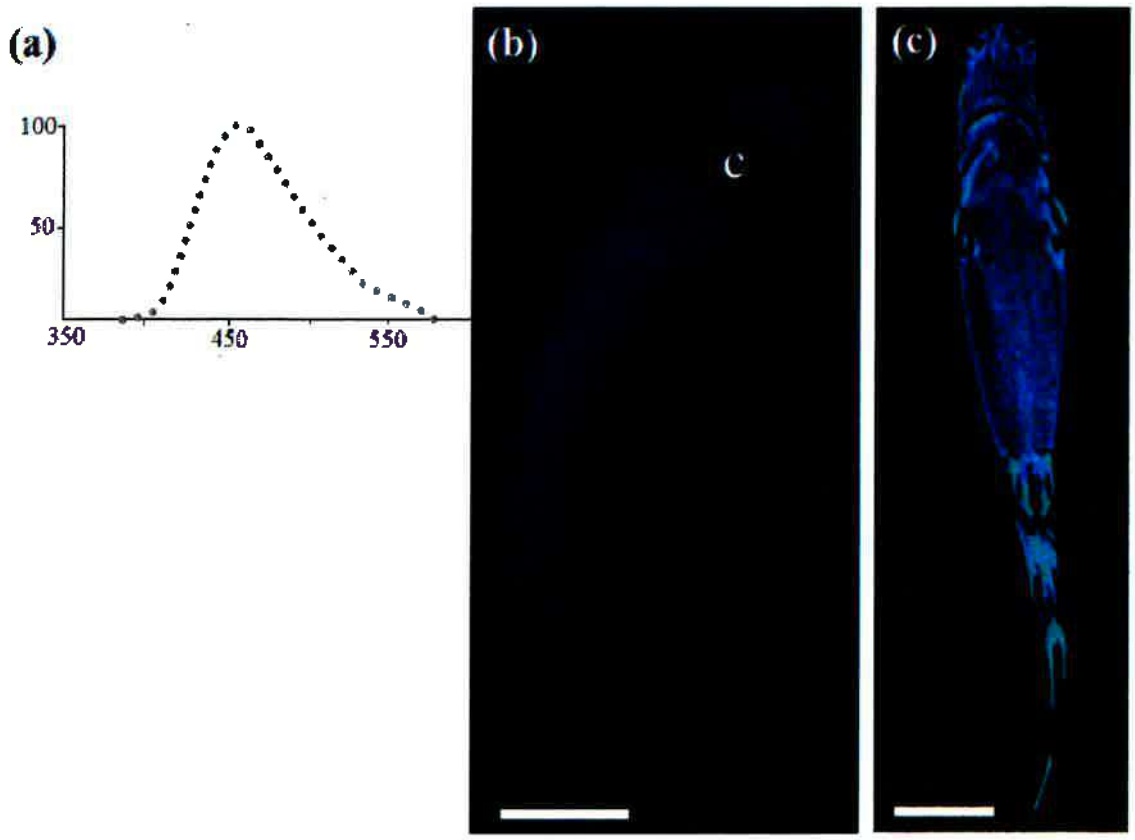


Figure 7. Bioluminescence of sharks. (a) Corrected emission spectra of *Isistius brasiliensis* [adapted from 42]. (b)Spontaneous luminescence of *Isistius brasiliensis* (© T. Frank - expedition Sonne 194). c, dark collar. Bars scale 5 cm (c) Spontaneous luminescence of *Etmopterus spinax* (© J. Mallefet).

1.4.2. Control

Physiological studies of luminous control mechanisms in sharks have never been systematically carried out [6,8]. The impossibility to find

innervations in photophores of the few species investigated, the slow onset of luminescence in *E. pusillus* and the absence of response to classical neurotransmitters such as adrenaline or acetylcholine in *I. brasiliensis* (only hydrogen peroxide stimulates light production in this species), led to the hypothesis that bioluminescence is in sharks hormonally controlled [1,8,34,39]. A control via the iris-like structure of the photophores through the action of chromatophores in Etmopteridae has sometimes been suggested, but never tested [1,9,26,34].

1.4.3. Function

Due to the absence of experimental data on bioluminescent behaviours, a biological role of bioluminescence in sharks has never been demonstrated as is often the case for other luminous deep-sea organisms as well [6]. Functions of light emission in sharks can only be deduced from life trait history such as global ecology, arrangement of photogenic organs and bioluminescence spectrum. The best current functional hypotheses are presented here.

Dalatiidae are small pelagic shark, which are particularly vulnerable to predation in the water column, assuming they are mainly found in the mesopelagic zone (figure 2). It is likely that their ventral patterns are used for camouflage by counter-illumination [6]. The ventral emission of *I. brasiliensis* produces however considerably shorter wavelength (455 nm) than what would be expected for a function of camouflage, as pelagic environments have peak of ambient light situated around 470-480 nm. Even though this could suggest that its photophores are not involved in camouflage, or at least not well adapted, one cannot exclude that this shark might also take advantage from the peculiar vision of deep-sea predators for which such differences in wavelength seems to have little effect [42]. The presence of a dark collar in *I. brasiliensis* has led authors to suggest a secondary predatory use of its bioluminescence. This collar could indeed allow this species to use its light emission as a lure for big pelagic animals on which this shark feeds by ectoparasitism. Indeed, if bioluminescence matches the luminous background, the dark ventral collar would be the only part of the shark visible from below (figure 8a). This collar would therefore looks like a smaller fish very attracting for upward looking pelagic predators which would then be attacked by the shark at close distance [43,44].

Luminous patterns of Etmopteridae are more complex, suggesting that either their bioluminescence is not involved in camouflage or it is used for more than one purpose [6]. Moreover, since they are benthopelagic species, counter-illumination would be less useful in these fishes. As their patterns appear to be species-specific the best current hypothesis is that they use their luminescence for species recognition and schooling (figure 8b) [6]. Function

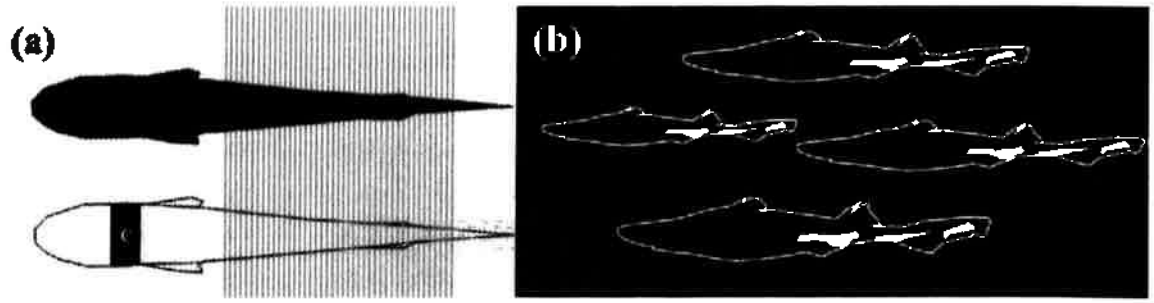


Figure 8. Putative luminescent behaviours in sharks. (a) Counter-illumination in *Isistius brasiliensis* [adapted from 44]. c, dark collar. When luminescent, the body of the shark (except the dark collar) matches well the background. (b) Schooling in *Etmopterus spinax*. Luminous lateral zones are presented in white colour. For clarity, the shark silhouettes are delimitated with white lines.

of ocular photophores of *Centroscyllium* and *Trigonognatus* is relatively mysterious but may be involved in prey attraction or vision improvement [25,26]. One cannot exclude these ocular photophores to play a reference function in countershading.

2. Recent work

Recently, experimental works on the bioluminescence of a deep-sea shark from the Etmopteridae family, *Etmopterus spinax* have been undertaken. This species, being commonly found in the East-Atlantic and surviving for more than one week in captivity, is indeed a perfect model species in order to investigate experimentally different aspects of shark bioluminescence.

2.1. Embryology

In *E. spinax*, the elaboration of luminous structure occurs before birth. The luminous pattern of ready to hatch specimens (and free-swimming sharks) is elaborated by 9 different luminous zones which appear sequentially during the embryogenesis of this shark (figure 9) [45].

Within the different luminous zones, the organogenesis of the photogenic organs follow the same steps: (i) apparition of pigmented cells between the epidermis and the connective tissue, (ii) elaboration of the pigmented sheath, the iris, and the lens; and (iii) apparition of fluorescent vesicles inside the photocytes which probably contain the luminous substrate [45]. Test with hydrogen peroxide (H_2O_2), a substance that is supposed to stimulate the maximum light emission, show that the capability of the photophores to emit light is linked to the presence of these fluorescent vesicles, as it is the case in the luminous teleost *Porichthys* [45, 46].

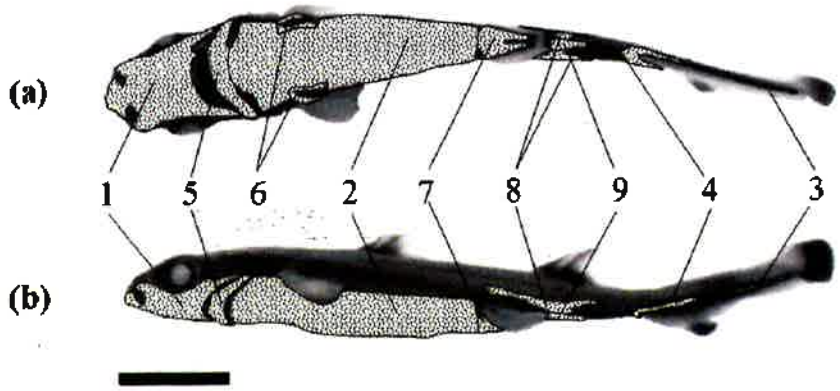


Figure 9. (a) Ventral and (b) lateral view of the luminous pattern of *E. spinax* presenting the different types of luminous zones (dotted areas). 1, Rostral; 2, Ventral; 3, Caudal; 4, Infra-caudal; 5, Mandibular; 6, Pectoral; 7, Pelvic; 8, Lateral; 9, Infra-pelvic. Numbers correspond to the order of their sequential appearance during embryogenesis. Scale bar = 2 cm [adapted from 45].

The luminous pattern of this shark is therefore fully operational before birth (figure 10a). It is interesting to note that adult specimens of *E. spinax*, as well as free-swimming specimens of *E. lucifer* (another species from the Etmopteridae family) also show fluorescent vesicles in their photocytes (figure 10b) [45, 47].

2.2. Ontogeny

The photophore density of the *E. spinax* luminous pattern is not homogenous but show differences between luminous zones as well as during

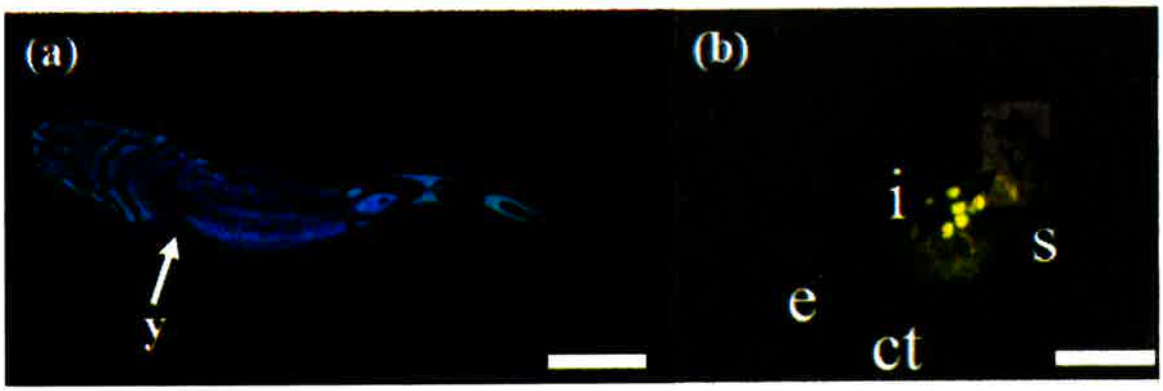


Figure 10. (a) Self-glowing embryo of *E. spinax* (© J. Mallefet). Arrow indicates yolk sac insertion. Scale bars = 2 cm. y, yolk sac insertion. (b) Photocyte's fluorescent vesicles present in the centre of a photophore from *E. lucifer* (epifluorescent microscopy; © J.M. Claes). e, epidermis; ct, connective tissue; I, iris; s, pigmented sheath; v, fluorescent vesicles present in photocytes. Scale bar = 50 μm [adapted from 48].

the growth of free-swimming specimens (figure 11). These differences in photophore density probably reflect a differential use of the luminous zones by the shark, whose luminescence intensity is directly dependant from their photophore density as it has been shown by peroxide stimulation and confirmed by direct observations of spontaneous luminescence (figure 7a, 11b).

The photophore diameter, on his side, does not show any ontogenetic variation in free-swimmings, even though it undergoes a high increase during embryogenesis [45].

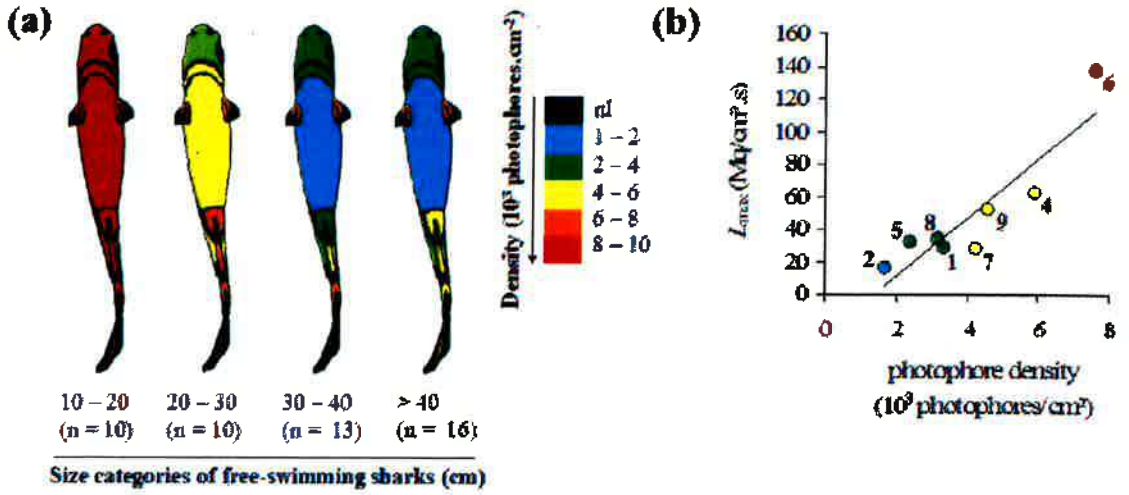


Figure 11. (a) Evolution of photophore density from the different luminous zones of *E. spinax* during ontogeny. nl, non-luminous area. (b) Relation between photophore density and maximum intensity of light emission recorded after hydrogen peroxide application for large (> 40 cm) sharks. Values are means (n = 16) and numbers correspond to the different zones tested (the caudal zone was not tested due to its small size). The colour code for photophore density is the same than the one used at figure 11a. [47].

Conclusion

This synthesis on shark bioluminescence is based on a careful review of all current available information in old and recent literature.

The two families of luminescent sharks, the Dalatiidae and the Etmopteridae, show differences in their luminous pattern, the organisation of their photogenic organs, and probably in the role of their bioluminescence. This support the hypothesis that bioluminescence appeared two times independently in sharks; firstly in Etmopteridae in response to the need of an efficient tool for species recognition in the deep-water environments, and secondly, in Dalatiidae, to provide an efficient camouflage in a pelagic environment with no place to hide.

Current knowledge of shark luminescence is rather limited and more researches are strongly needed to determinate the biochemistry, the physiology as well as the function(s) of their light emission. Works are in progress to fulfil this goal.

Acknowlegments

We thank T. Frank and H.-J. Wagner who provided us the picture of the glowing *Isistius brasiliensis* taken during the expedition Sonne 194. This work was supported by a grant from the F.N.R.S. to J. M. Claes. J. Mallefet is a research associate of F.R.S.-F.N.R.S. This is a contribution to the Biodiversity Research Centre (BDIV).

References

1. Herring, P. J. 1978, Bioluminescence in action, Academic Press, London.
2. Wilson, T., and Hastings, J. W. 1998, Annu. Rev. Cell. Biol., 14, 197-230.
3. Herring, P. J. 2007, J. Mar. Biol. Ass. U. K., 87, 829-842.
4. Bone, Q., and Marshall, N. B. 1982, Biology of Fishes, Blackie, Glasgow.
5. Compagno, L., Dando, M., and Fowler, S., 2004, Sharks of the world, HarperCollins, London.
6. Reif, W.-E. 1985, Acta Zool., 66, 111-118.
7. Alcock, A. 1902, A naturalist in Indian seas, London.
8. Harvey, E. N. 1952, Bioluminescence, Academic Press, New York.
9. Hubbs, C. L., Iwai, T., and Matsubara, K. 1967, Bull. Scripps. Instn. Oceanogr., 10, 1-64.
10. Wetherbee, B. M. 2000, Fish. Bull., 98, 189-198.
11. Shirai, S. 1996, Interrelationships of Fishes, Academic Press, New York.
12. Van Grevelynghe, G., Diringer, A., and Séret, B. 1999, Tous les requins du monde, Delachaux et Niestlé, Lausanne.
13. Naylor, G. J. P., Ryburn, J. A., Fedrigo, O., and López, A. 2005, Reproductive biology and phylogeny of Chondrichthyes, Science Publishers, Inc., Enfield.
14. Hansen, K., and Herring, P. J. 1977, J. Zool., Lond., 182, 103-124.
15. Nealson, K. H., and Hastings, J. W. 1979, Microbiol. Rev., 43, 496-518.
16. Diamond, J. M. 1985, Nature, 316, 679-680.
17. Berland, B. 1961, Nature, 191, 829-830.
18. Herring, P. J. 1985, Nature, 318, 238.
19. Nakaya, K., Yano, K., Takada, K., and Hiruda, H. 1967, Biology of the Megamouth Shark, Tokai University Press, Tokyo.
20. Carrier, J. C., Musick, J. A., and Heithaus, M. R. 2004, Biology of sharks and their relatives, CRC Press, Boca Raton.
21. Adnet, S., and Capetta, H. 2001, Lethaia, 34, 234-248.
22. Compagno, L. J. V. 1990, Env. Biol. Fish., 28, 33-75.
23. Priede, I. G., Froese, R., Bailey, D. M., Bergstad, O. A., Collins, M. A., Dyb, J. E., Henriques, C., Jones, E. G., and King, N. 2006, Proc. R. Soc. B., 273, 1435-1441.

24. Zidowitz, H., Fock, H. O., Pusch, C., and Von Westernhagen, V. H. 2004, J. Fish Biol., 64, 1430-1434.
25. Yano, K., Mochizuki, K., Tsukada, O., and Suzuki, K. 2003, Ichtyol. Res., 50, 251-258.
26. Iwai, T. 1960, Pacif. Sci., 14, 51-54.
27. Springer, S., and Burgess, G. H. 1985, Copeia, 3, 584-591.
28. Schofield, P. J., and Burgess, G. H. 1997, Bull. Mar. Sci., 60, 1060-1073.
29. Last, P. R., Burgess, G. H., and Séret, B. 2002, Cybium, 26, 203-223.
30. Burkhardt, R. 1900, Ann. Mag. Nat. Hist. Ser., 6, 558-568.
31. Parin, N. V. 1966, Trans. Inst. Oceanol. Acad. Sci. USSR., 73, 173-194.
32. Cavallaro, M., Mammola, C. L., and Verdigliones, R. 2004, J. Fish Biol., 64, 1552-1567.
33. Seigel, J. A. 1978, Copeia, 4, 602-614.
34. Oshima, H. 1911, J. Coll. Sci., Imp. Univ., Tokyo, 27, 1-25.
35. Munk, O., and Jorgensen, J. M. 1988, Acta Zool., 69, 247-251.
36. Raschi, W., and Tabit, C. 1992, Aust. J. Mar. Freshw. Res., 43, 123-147.
37. Stehman, M., and Krefft, G. 1988, Arch. Fisch. Wiss., 39, 1-30.
38. Bennet, F. B. 1840, Narrative of a whaling voyage around the world from the year 1833 to 1836, London.
39. Johann, L. 1899, Z. wiss. Zool., 66, 136-160.
40. Hickling, C. F. 1928, Nature, Lond., 121, 280-281.
41. Dickens, D. A. G., and Marshal, N. B. 1956, Mar. Obs., 26, 73-74.
42. Herring, P. J. 1983, Proc. R. Soc. Lond. B, 220, 183-217.
43. Jones, E. C. 1971, U. S. Fish. Bull., 69, 791-798.
44. Widder, E. 1998, Env. Biol. Fish., 53, 267-273.
45. Claes, J. M., and Mallefet, J. 2008, J. Fish. Biol., 73, 1337-350.
46. Anctil, M. 1977, J. Morph., 151, 363-396.
47. Claes, J. M., and Mallefet, J., in prep.
48. Claes, J. M., and Mallefet, J. 2009, 15th International Symposium on Bioluminescence and Chemiluminescence proceedings, Shanghai-China, 5, 15-19.

Research signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 67-83
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

5 Echinoderm bioluminescence: Where, how and why do so many ophiuroids glow?

J. Mallefet
Laboratory of Marine Biology, Catholic University of Louvain, 3 Place Croix du Sud, Kellner Building, B-1348 Louvain-la-Neuve, Belgium

Abstract

Luminescence occurs in four of the five Echinoderms classes: Crinoidea, Holothuroidea, Asteroidea and Ophiuroidea. Until recently, in many cases information on echinoderm luminescence was entirely descriptive and limited to morphological and ecological observations with few additional remarks on some features of the bioluminescence. One major reason for this poor documentation of the phenomenon in echinoderms is related to the limited accessibility of species and individuals that can be studied. During the last five years, a series of field trips and participation at one deep-sea cruise allowed

Correspondence/Reprint request: Dr. J. Mallefet. Laboratory of Marine Biology, Catholic University of Louvain
3 Place Croix du Sud, Kellner Building, B-1348 Louvain-la-Neuve, Belgium
E-mail: jerome.mallefet@uclouvain.be

this author to discover and describe the luminous capabilities of numerous ophiuroids. A multidisciplinary approach allowed him to obtain physiological, morphological, ethological and finally biochemical data for certain light-producing ophiuroid species mainly because they belonged to species present from the intertidal zone down into deeper waters. This chapter attempts to summarize recent information on ophiuroid bioluminescence.

1. Introduction

"The only luminous stars the man can reach are the luminous seastars"
Glowly yours - J.Mallefet

The emission of visible light by living organisms is a fascinating phenomenon that has attracted the attention of naturalists for centuries, but it was only in 1952, that Harvey [1] attempted to produce a first list of luminous organisms, among these echinoderm species appeared. Since then, Herring's revisions in 1978 and 1987 enable us to have a better view of the phylogenetic distribution of bioluminescence [2,3]: it can be found from bacteria to fish (see numerous examples from various phyla in this book). Although quite rare considering all known species (only 0.2% of all the known genera possess luminous species), the vast majority of luminous organisms are found in the sea. At least 13 marine phyla contain luminous representatives. These include bacteria, unicellular algae, cnidarians, ctenophores, nemerteans, molluscs, annelids, arthropods, bryozoans, echinoderms, hemichordates and finally vertebrates (of which only some fishes are luminous). Natural luminescence is unknown in higher plants and in vertebrates above pisces; the complete absence of bioluminescence in several invertebrate taxa remains unclear and no doubt numerous luminous species are still undescribed [2-7].

Despite the reasonable number of luminous species, little is known about echinoderm luminescence. A literature survey of the last twenty five years provided us with limited information: fact is that luminous echinoderms exist [2,3,8] and that for some species we have information regarding the morphological structures involved in light production [3,10-12] and a certain amount of ecological data [5,13-21]. Yet, except for one or two species, the functions luminescence plays in these organisms is still a matter of discussion [8,22-28] and until recently, nearly no information about the physiological control mechanisms of the light emission had appeared in the literature [9, 29-45]. It must be pointed out that, when available, echinoderm luminescence studies have been mostly carried out on ophiuroids, largely because some species are easily observable *in situ* and can be collected and kept in captivity; thus allowing experimental work.

2. Distribution

The first luminous ophiuroid description mentioned in the scientific literature was that of Viviani in 1805 [46], who published a figure of *Asterias noctiluca* further identified as *Amphiura squamata*. In echinoderms, four of the five classes contain luminous representatives (Table 1) and a total number of 121 luminous species have been reported to date [8,47-50]. This number represents less than 2% of the total number of echinoderm species [51] and corresponds to approximately 4% of the genera. Within the echinoderm phylum bioluminescence is not uniformly distributed in each of the classes: with 66 and 31 species respectively, ophiuroids and holothurians represent 81 % of the echinoderm luminous species while asteroids and crinoids contribute 16 and 3%, respectively. The total absence of luminescent echinoid species remains enigmatic (Fig. 1).

It must be pointed out that the high number of known luminous ophiuroids compared with luminescent representatives of the other classes of echinoderms is mainly due to the recent research activity in this class since

Table 1. Total number of species and known luminous species within each of the five echinoderm classes.

Classes	Species number Total	Luminous
Echinoidea	838	0
Ophiuroidea	2278	66
Holothuroidea	1430	31
Asteroidea	1745	20
Crinoidea	576	4

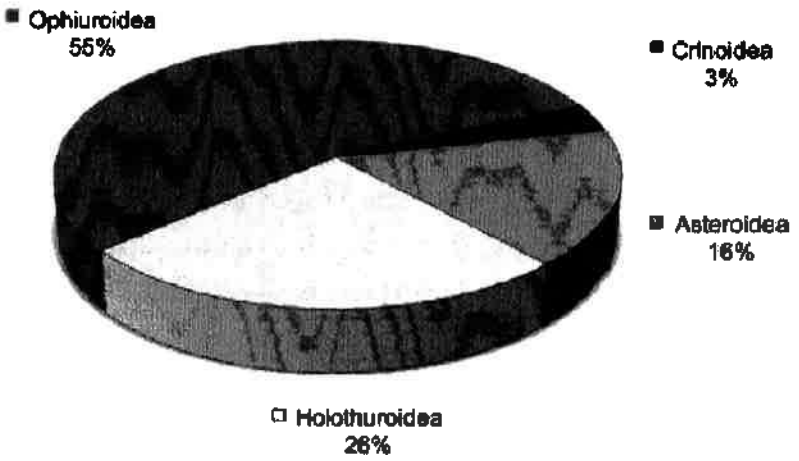


Figure 1. Relative abundance of species within four classes of echinoderms known to contain luminous representatives.

the number of known luminous ophiuroid species increased from 34 in 1995 to 38 in 2005 [49] to reach 66 in 2009. Over the same period, only two other new luminous echinoderm species were found, one crinoid and one holothuroid [50].

Luminous ophiuroids can be found in all kinds of biotope (soft and hard sediment) from intertidal zone down to deep-sea, in temperate or sub-tropical waters and even in tropical reefs [50,52]. The light emitted is mostly green [30,53] and in only two species was reported as blue, namely *Amphiura filiformis* and *Ophiomusium lymani* [9,13]. During a deep-sea cruise in 2005 (organised by CSIRO – RV Southern Surveyor), two new blue emitters were discovered and identified as *Amphilimna transacta* and *Ophioleuces seminudum*. When feasible, still and digital pictures of luminescence were taken to document the luminescence of ophiuroids and some examples are illustrated in Fig 2. *Ophionereis schayeri* is a green emitter commonly found under rocks from temperate shallow waters of Southern Australia; *A. filiformis* is a blue emitter found buried in the soft sediments of north-eastern European waters. *Ophiacantha alternata, O. lymani, O. seminudum* were collected on the continental slope (200 to 1000m depth) of Western Australia and finally *Amphipholis squamata* is a cosmopolitan species found from the intertidal zone down to 1300 m depth. It must be pointed out that *O. lymani* luminescence is present on the arms of the animal and not restricted to ovaries as stated earlier by Herring [9]. All ophiuroids species collected during the field trips and the deep-sea cruise in Australia are registered in the Victoria Museum collections for further identification by Dr. T. O'Hara of the "Invertebrates Department". Work is in progress and the analysis of the data will provide hints to understand the links between biotopes, phylogeny and light capabilities of the ophiuroids. It must be pointed out, however, that some of the deep-sea luminous species are still undescribed; [54].

3. Morphology

In ophiuroids, no specialised luminous organ (photophore) has been found so far and morphological studies indicated that photocytes are mainly present in the arm, associated directly or not with the nervous system [1,9,11,12,53]. The description of the luminous area as well as the fine structure of the photocyte was successful in *A. squamata,* and the presence of intracellular vesicles (μ-sources) undergoing ultrastructural changes during photogenesis was also documented [11,12]. Pictures of the luminous areas of *O. schayeri* are given in Fig. 3 as an example of repartition of photocytes along the arm of one ophiuroid. Taking advantage of the fact that most luminous substance fluoresce under UV or blue light excitation, one can use this property to trace

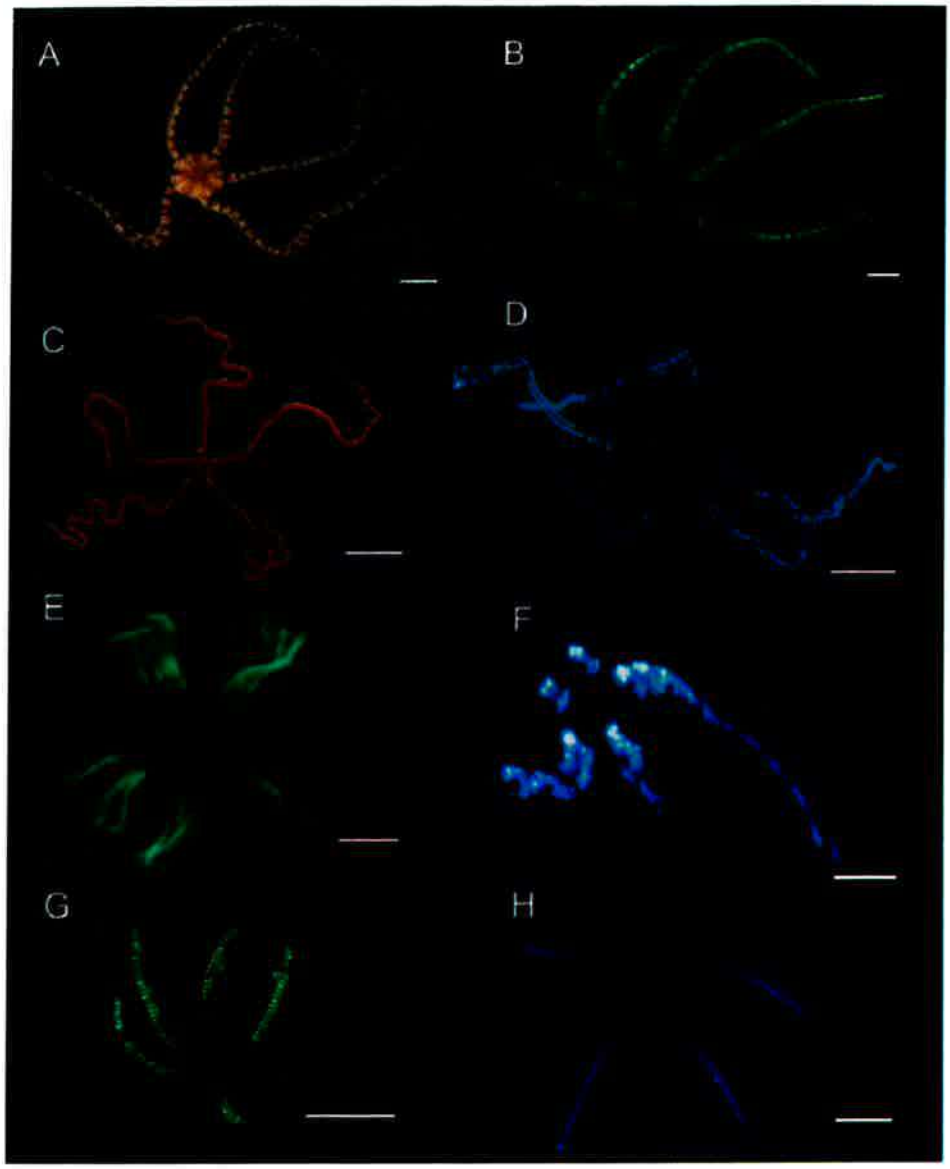


Figure 2. Examples of brittle star luminescence. A & B: *Ophionereis schayeri* natural light and luminescence; C & D: *Amphiura filiformis*, natural light and luminescence; E: *Ophiacantha brachygnatha*; F: *Ophioleuce seminudum*; G: *Amphipholis squamata*; H: *Ophiomusium lymani*. scale bar = 1cm (©photo J.Mallefet).

photocytes in the whole arm or just a section. However, fluorescence is not always present and, for example in *A. filiformis,* fluorescence has never been observed, either due to a complete lack of fluorescence after the luminous response or because it is too weak and too rapidly fading away to be noticed.

Arms and sections of *O. schayeri* arms were observed under blue light excitation in order to visualise photocyte positions, revealed by epi-fluorescence (arrows in Fig. 3). Photocytes are mainly located just under the oral arm plates (A-C), but also under aboral and lateral plates (B-C). Numerous

photocytes have been observed in some spines (D-E). Cross section examinations showed that the radial nerve cord (rnc) does not contain fluorescent sites (F-G). A similar situation has been observed in *Ophionereis fasciata*, another closely related ophiuroid, but no photographic documents were obtained due to the rapid fading of the fluorescent signal. In some species, disc luminescence has been recorded, but it must be pointed out that disc light intensity represents only a few percent of that of the arm [48,55].

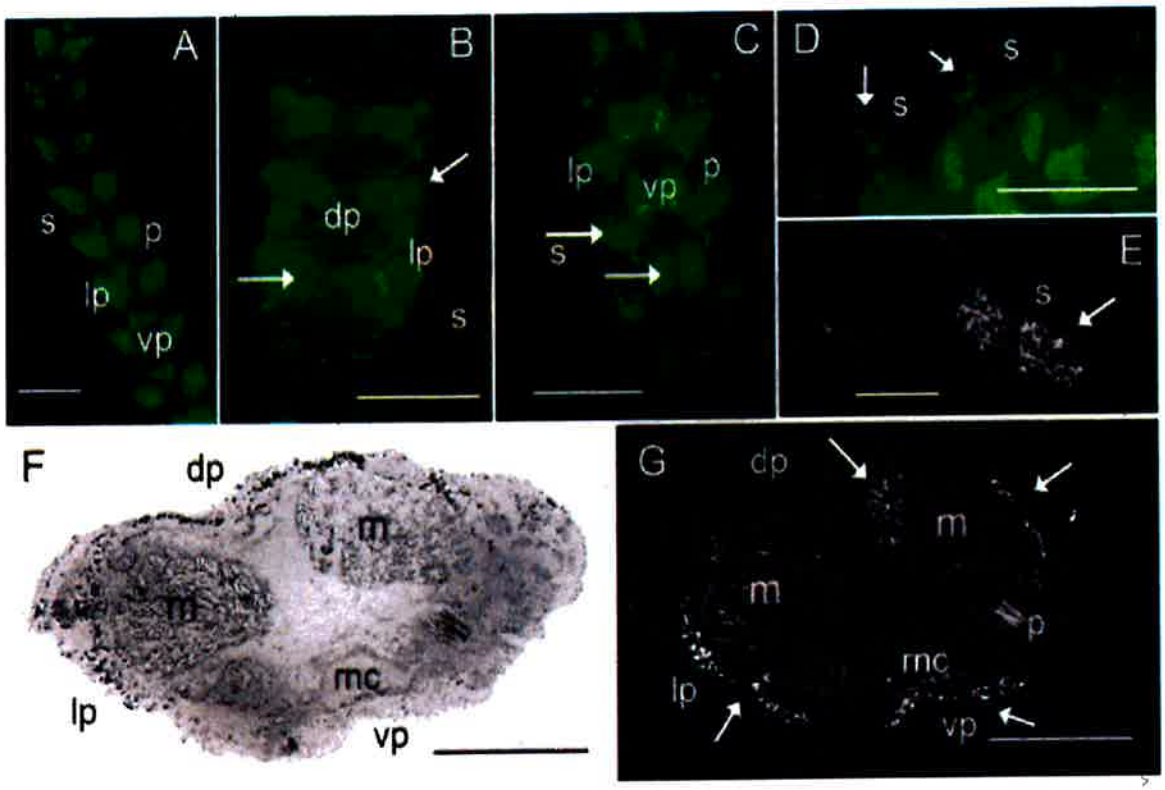


Figure 3. Epi-fluorescent images from *O. schayeri* arms A; B= aboral view of arm segments, C= oral view; D= spines E=cross section of a spine; F=cross section of the arm, light microscopy; G= cross section in epifluorescence. Abbreviations: dp: dorsal plate; lp: lateral plate; m: muscle, p: podia s: spine, rnc: radial nerve cord. Scale bars = 3mm (A-D); = 0.5mm (E); = 1 mm (F-G).

4. Pharmacology

4.1 Extrinsic control mechanisms: Nature of neurotransmitters and modulators

Initially focusing on *Amphipholis squamata*, a small cosmopolitan luminous ophiuroid, the study of luminescence control mechanisms has now been extended to other ophiuroid species. Using isolated arms, it was suggested that luminescence was under nervous control. Pharmacological studies permitted the description of the nature of the nervous control, and in the

studied species light emission is now known to be under cholinergic, trace aminergic, and Gaba-ergic control. The characterisation of specific receptors on the photocyte membranes, using specific agonists and antagonists, has been successful in *A. squamata, A. filiformis, A. arcystata, O. schayeri* and *O. fasciata,* while in other species such characterisation remains uncertain (Table 2). The presence of neuromodulatory mechanisms has also been observed.

Table 2. Extrinsic control mechanisms in ophiuroids species, nature of neurotransmitters and neuromodulators. Ach. : acetylcholine, GABA : Gamma butyric acid ; Tryp. Tryptamine Octo. : Octopamine ; Tau. : Taurine ; Cat : Catecholamine. + : weak luminescence ; ++ : strong luminescence; 0: no effect. mR : muscarinic receptor ; nR : nicotinic receptor, GABAB : Gaba B receptor. Neuromudolatory effects are represented in bracket [+] : potentiation ; [-] : inhibition ; nt : not tested.

Species	Ach	GABA	Tryp	Octo	Tau	Cat	References
Amphipholis squamata	++ mR	(-)	(+)	nt	nt	[- adr]	[31-33, 40-42,56]
Amphipholis sp	++	nt	nt	nt	nt	0	[55]
Amphiura filiformis	++ nR&mR	0	+	+	+	[+dopa]	[36,44]
Amphiura arcystata	++ mR>nR	nt	nt	nt	nt	nt	[55,57,58]
Ophiopsila californica	+	0	++	0	+	[+dopa]	[36,44]
Ophiopsila aranea	0	0	0	0	+	0	[36,44,59]
Ophionereis fasciata	++ nR	[+]	nt	nt	0	0	[60,61]
Ophionereis schayeri	+ mR	++ GABAB	nt	nt	0	0	[62,63]

4.2. Intrinsic control mechanisms: Second messengers

Adaptation and development of an enzymatically based dissociation method for photocytes [32] allowed work at the photocyte level and the study of the second messengers' role during photogenesis. Pharmacological studies

were developed using classical permeable analogs (dibutyryl-c-AMP and dibutyryl-cGMP), and drugs altering the major second messenger pathways (such as forskolin, an adenylate cyclase activator, MDL, an adenylate cyclase inhibitor, U-73122, a phospholipase C inhibitor) in order to analyse intracellular control mechanisms. Major findings are summarized in Table 3. The implication of AMPc and IP3 pathways is suggested in *A. squamata* luminescence, while GMPc seems to be involved in one inhibitory control in *A. filiformis*. It has been suggested that calcium could represent a major pathway involved, since incubation of dissociated photocytes in Ca-free seawater either lowered or totally inhibited luminescence in all ophiuroid species studied to date [37,64,65].

Table 3. Implication of second messengers in ophiuroid luminescence. (√= positive, 0 = no effect, - = inhibition, nt = not tested).

Species	cAMP	cGMP	IP3	Ca2+	References
A. squamata	√	0	+	√	[66]
A. filiformis	0	-	0	√	[37,44]
O. aranea	0	0	0	√	[37,44]
O. californica	0	0	0	√	[37,44]
A. arcystata	nt	nt	nt	√	[58]
O schayeri	nt	nt	nt	√	[48]
O. fasciata	nt	nt	nt	√	[48]
Amphipholis sp.	nt	nt	nt	√	[55]

5. Ethology

In the late eighties, research conducted on two ophiuroid species, *Ophiopsila riseii* and *Ophiopsila californica,* led to the hypothesis of an aposematic use of bioluminescence [22-24]. In this case, light emission would warn the predator that the prey possesses a bad taste (i.e., is unpalatable) and should not be attacked. Despite some strong opposition [67,68], it has generally been accepted that the role of luminescence in ophiuroids must be associated with antipredatory mechanisms [8]. Although commonly admitted, the sacrificial lure hypothesis, i.e., losing one luminescent arm portion by autotomy in order to distract predator attention in order to achieve escape, has rarely been properly documented (Table 4).

Here I report first experimental evidence for the sacrificial lure role as well as a deterrent effect of ophiuroid luminescence. Analyses of interactions

Table 4. Major functions of luminescence documented (D) or suggested (S) for some ophiuroid species.

Species	Function	References
Amphipholis squamata	Sacrificial lure (D), burglar alarm (S)	[26,27]
Amphiura filiformis	Sacrificial lure (S)	[8]
Ophiopsila californica	Aposematism (D/S)	[22-25]
Ophiopsila aranea	Aposematism (D)	[69,70,71]
Ophionereis schayeri	Sacrificial lure (S)	[48].

between predators and ophiuroids using intensified camera footage revealed various types of behaviours. Some examples are illustrated in the following figures.

Light emission of the five arms of *Amphipholis* sp. is induced by mechanical contact with forceps as illustrated in Fig. 4 A. But it was observed that contact with predators induced a brief bright emission of light (Fig. 4 B) and when the predator held the arm of the ophiuroid, the distal part of the arm exhibited a wriggling motion, flashing at the same time (Fig. 4 C).

In another series of experiments, in which *A. squamata* was placed in a close chamber, decorated with a stone in order to enable the ophiuroid to seek protection from the predator, it was observed that prolonged interaction with a crustacean (*Galathea squamifera*) induces luminescence followed by arm autotomy of the luminous part, which remained bright in the predator's oral cavity (Fig. 5). In this case the possibility of burglar alarm use of luminescence has been suggested since the crustacean, i.e., the predator, becomes clearly visible to a secondary predator like a fish or octopus, perhaps.

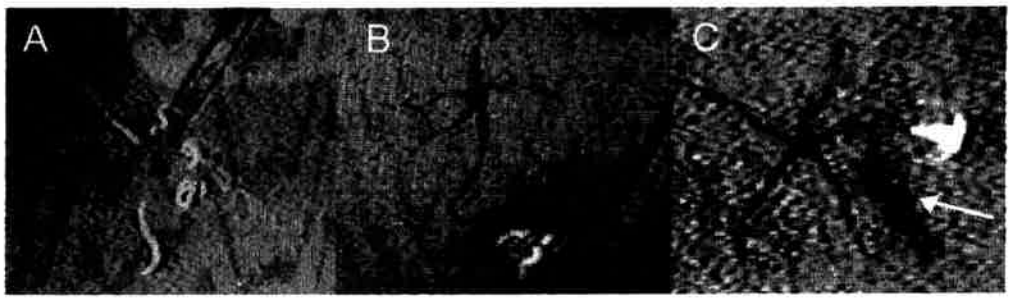


Figure 4. Light emissions from *Amphipholis* sp. in response to mechanical stimulation with forceps (A) and contact with predators *Pugettia producta* (B) and *Alpheus bellinanus* (arrow, C).

Figure 5. Interaction between *A. squamata* and one crustacean, *Galathea squamifera*.

Studying interactions between *Ophiopsila aranea* and *Carcinus maenas* during multiple serial trials, it was shown that predation events were most frequently observed during first encounters, the sacrificial lure response being observed readily with the predator becoming visible and the ophiuroid itself getting away. The effect of *Ophiopsila aranea* luminescence as a deterrent mechanism was observed after the second and third feeding trial, suggesting that the crab had learned that the luminescence signal acted as a warning signal i.e., pointing towards a role of the luminescence in aposematism [69,70,71].

6. Biochemistry

Despite the reasonable number of luminous echinoderm species, very little is known about the echinoderm luminescence system. The chemical reactions responsible for the emissions of visible light by organisms, generally, are usually based on an oxidation of a substrate, termed luciferin, by molecular oxygen under the catalytic activity of an enzyme, termed luciferase. Many chemically different luciferins and luciferases have been isolated from luminous organisms; but while some luciferins are common to phylogenetically different organisms, luciferases are always specific. The term photoprotein was introduced by Shimomura and Johnson in 1966 to characterize a luminous reaction that do not require oxygen and where the light emitted is proportional to the amount of reacting protein [72]. It is now accepted that the photoprotein corresponds to a stable enzyme-substrate intermediate, whose light emission is triggered by the presence of a cofactor.

First information about the luminous system in ophiuroids was available in Herring's work of 1974 [9], in which it is mentioned that some luciferine luciferase reactions were obtained from deep sea ophiuroid crude extracts. In 1985 the first photoprotein, named Ophiopsilin, was characterized by Shimomura [73]. A biochemical characterization aiming to isolate the luminous system of *A. squamata* suggested the presence of another photoprotein, but even if purification failed, results indicated that calcium did

not seem to be the cofactor of the luminous system (Mallefet & Shimomura, unpublished). More recently, a new biochemical study revealed that the luminous system of *A. filiformis* is based on a luciferine/luciferase reaction. Crude extracts of the enzyme were tested. Results indicated that coelenterazine is the substrate of the reaction; luciferase activity is relatively stable (60% of the activity remaining after 4 days at –80°C). Temperature does not affect activity in the range encountered by the animals *in vivo* and the light emitted is stable from 6 - 20°C; optimal pH-activity has been estimated to occur at 7.4. Effects of NaCl, KCl, $MgCl_2$ and $CaCl_2$ concentrations have been studied and light emissions increase linearly with salt concentrations. Purification of this luciferase was initiated using two successive column chromatographies: ion exchange followed by hydrophobic interactions. The following table is giving an overview of the main results from these purification attempts [74].

The fraction showing most of the activity after these purification steps was analysed by mass spectrometry with no success. Further work is needed to isolate and purify this new luciferase; a comparative study might reveal evolutionary links of the luminous systems in ophiuroids to other animals.

Table 5. Purification of *A.filiformis* luciferase.

	Vol. (ml)	Protein (mg)	Lase activity (10^8 . RLU . s^{-1})	Lase specific activity (10^8 . RLU.s^{-1}.mg^{-1})	Recovery rate (%)	Purification factor
Extraction	6,2	6,42	9,63	1,5	100	1x
Centrifugation + dilution	33	5,47	9,08	1,66	94	1,1x
Ion exchange chromatography	15	1,51	7,31	4,85	76	3,2x
Hydrophobic exchange chromatography	1	0,18	1,92	10,9	20	7,3x

7. Ophiuroid luminescence applications

7.1. Luminous capabilities to monitor pollution

In one study, it has been suggested that luminescence of the cosmopolitan ophiuroid *A. squamata* could be useful in order to monitor water quality [75]. Although working with living ophiuroids could be advantagous since they would embody toxic effects at cellular, tissue and organism level, problems are likely to arise in terms of distinguishing the different effects and the levels affected by them, rendering the interpretation of the results very difficult. Using isolated ophiuroid photocytes might offer an

alternative, but the dissociation protocol would render this method way more difficult than well established bacterial kits that still represent the fastest and easiest way to monitor pollution [76]. Further work will be necessary to establish in which way and how ophiuroid luminescence can be used.

7.2. Luminous capabilities and functional recovery during regeneration in ophiuroids

Ophiuroids are noted for their great capacity of regeneration, frequently losing arms at all levels, with subsequent and usually quite rapid regeneration. That the nerve [77] and cell divisions (i.e., proliferations) are involved in this phenomenon is well known [78]. Numerous morphological studies have focused on the ecological [79,80,81] and cellular aspects of the regeneration process in ophiuroids [82]. Knowing that many ophiuroids are luminescent and this luminescence is under nervous control [47], it is possible to monitor regeneration progress and to assess the effectiveness and extent of functional recovery using luminescence as a natural marker [83-85]. In a study performed on a model ophiuroid, *A. filiformis*, it was shown that regeneration rate is rapid, since only 10 days after amputation 1.32 ± 0.08 mm (n=20) of the newly formed arm is present. After 303 days, more than 5 centimetres of the new arm can be seen. Knowing that regeneration took place in an aquarium supplied with natural sea water whose temperature fluctuated naturally, expression of the size of the regenerate as a function of time multiplied by the average temperature for the appropriate period allows a curve to be obtained that fits a sigmoidal equation (Fig. 6). A plateau is observed after 1500 tdeg (day*°C), corresponding to a period of 130 days at

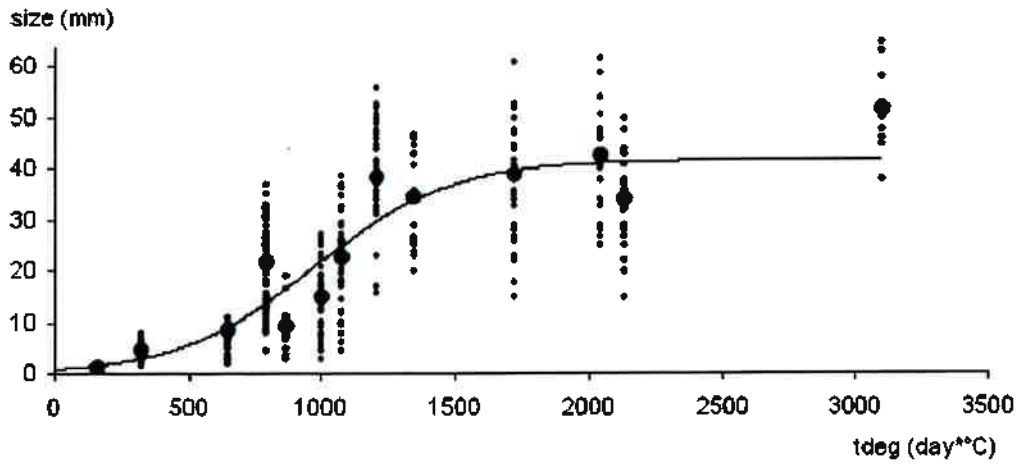


Figure 6. *Amphiura filiformis* size of newly formed arms (size in mm) as a function of time multiplied by mean water temperature for the corresponding period (tdeg in day*°C). Curve fitting using non linear model (●= mean value ; $p<0.01$).

12°C. Regeneration rates are not constant and statistical analyses reveal two phases of regeneration: a first of approximately 130 days, with an increasing regeneration rate followed by a second phase that shows a decrease in the regeneration rate.

This result showed that the length of the regenerated arm varies for a similar period of time as a function of seawater temperature, i.e., the season. The shortest regeneration sizes were found in winter when the lowest temperatures are recorded. The effect of temperature has been integrated in the analysis by expressing regeneration and light recovery as a function of time multiplied by temperature. When taking into account time and temperature in this way, regrowth size shows a sigmoidal relationship reaching a plateau after 130 days. This kind of regrowth is typical for *A. filiformis*, since a similar pattern for natural growth was described for this ophiuroid species [86]. Regrowth rate showed two phases, the positive slope indicating that most energy is placed into length increase, while the negative slope could indicate that most growth energy is allocated to an increase in *arm width.*

Bioluminescence recovery, expressed as a percentage of the control value, i.e., the light emitted by the regenerated arm divided by the light emitted by the section of arm originally amputated, shows large variations over a period of 300 days: a minimal value of 2.67 ± 0.67% being calculated after 125 days, while a maximal value of 66.19 ± 7.79% was measured after 57 days (Fig. 7).

Bioluminescence recovery rates are not constant over a period of 300 days. This large variability is not due to temperature, since this parameter was integrated in the analyses. Moreover, it has been shown that the light emission reaction is not affected by temperature within the normal natural temperature range encountered by the animal [87] and taking into account that

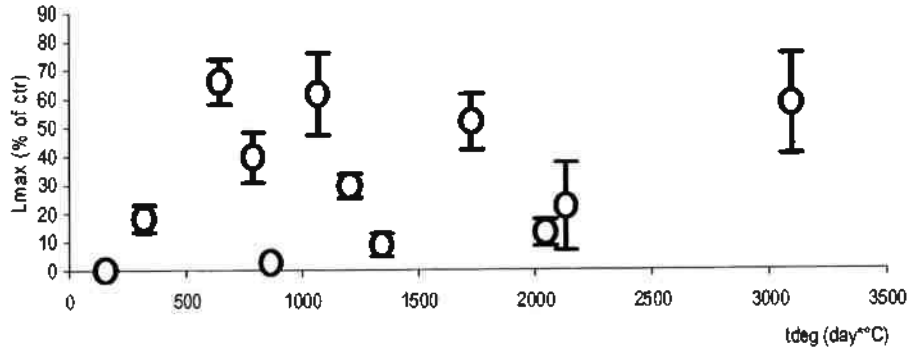


Figure 7. Luminescence recovery as a function of days*temperature. Luminescence maximal intensity (Lmax) of the regenerated part is expressed as percentage of the control value (Mq/s.mm) induced by potassium chloride depolarisation 200mM.

it was recently described that regeneration rate is proportional to the length of the missing part of the arm [81], one can argue that the recovery of light emission should be expressed in terms of body mass instead of the percentage of the control maximal light intensity normalised by arm length. Further and additional research should be designed to examine whether light recovery had better be expressed as a function of arm volume instead of arm length.

The recent discovery of the nature of the luminous system in *A. filiformis* allows us to monitor the amount of luminous compounds in the arm [88]; future work will be devoted to measure the luminous compounds in the regenerated arm in order to better understand the functional recovery of ophiuroid luminescence.

8. Final conclusion

There can be no doubt that luminescence in Ophiuroids is more widespread than initially thought. Much additional field and laboratory work will be necessary to understand why so many Ophiuroidea glow in the dark and participation at benthic deep sea cruises will provide access to living echinoderm specimens, allowing comparisons of luminous capabilities within ophiuroids and an extension of this research program to species of other echinoderm classes.

Acknowledgements

I am very grateful to Dr. T. O'Hara (Museum Victoria-AU), Prof. M. Jangoux (ULB-BE); M.C. Thorndyke (Kristineberg-SE) ; J. Case (UCSB-USA); M. Barker (Otago-NZ); M. Byrne (SYDU-AU); A. Williams & crew of R.V. Southern Surveyor (CSIRO-AU) who have made this research program possible. I want to thank all the field station directors that provided me with facilities to collect the animals. I must not forget my research team members (PhD and Master students): their invaluable contributions to this work were essential. This research program was supported by various grants from the Fonds Léopold III, Fondation Agathon de Potter, F.N.R.S., EU–LSF & TARI; the Australian Museums. This chapter is a contribution to the Biodiversity Research Centre (BDIV) and «Centre interuniversitaire de biologie marine » (CIBIM) and was solicited by Prof. Dr. V.B. Meyer-Rochow (Jacobs University, Bremen, Germany), whose attention to the text I gladly acknowledge. J. Mallefet is a research associate of F.R.S.-F.N.R.S.

References

1. Harvey, E. N. 1952, Bioluminescence, Academic Press, New York.
2. Herring, P. J. 1978, Bioluminescence in action, Academic Press, London.

3. Herring, P. J. 1987, J. Biolum. Chemilum., 1, 147-163.
4. Campbell, A.K. 1989, Essays in Biochem., 24, 41-80.
5. Hastings, J.W. 1983, J. Mol. Evol., 19, 309-321.
6. Hastings, J.W. 1995, Bioluminescence. Cell Physiology, Academic Press, N Y, 665-681.
7. Hastings, J.W., and Morin, J.G. 1991, Bioluminescence. In: Neural and integrative animal physiology (Prosser, C.L., ed.), Wiley-Liss Inc, 131-171.
8. Herring, P. J. 1995, Proc. Int. Echinoderm Conf. 9-17.
9. Herring, P.J. 1974, J. Zool., Lond., 172, 401-418.
10. Brehm, P., and Morin, J.G. 1977, Biol. Bull., 152, 12-25.
11. Deheyn, D., Alva, V., and Jangoux, M. 1996, Zoomorphol., 116, 195-204.
12. Deheyn, D., Mallefet, J., and Jangoux, M. 2000, Cell Tissue Res., 299, 115-128.
13. Emson, R.H., and Herring, P.J. 1985, Proc Int. Echinoderm Conf., 5, 656.
14. Emson, R.H., and Wilkie, I.C. 1982, Proc. Int. Echinoderm Conf., 11-18.
15. Emson, R.H., Jones M.B., and Whitfield, P.J. 1989, In: Reproduction, genetics and distributions of marine organisms, Ryland J.S. and Tyler. P.A. eds., Fredensborg, Olsen & Olsen, 75-81.
16. Robison, B.H. 1992, J. Mar. Biol. Ass. U.K., 72, 463-472.
17. Alva, V. 1996, PhD Thesis, Université Libre de Bruxelles
18. Deheyn, D., Mallefet, J., and Jangoux, M. 1997, J. Mar. Biol. Assoc. UK., 77, 1213-1222.
19. Dupont, S. 1998, Master Thesis, Université Catholique de Louvain, Belgium.
20. Dupont, S., and Mallefet, J. 2000, Hydrobiologia 440, 137-144.
21. Dupont, S., Mallefet, J., and Dewael, Y. 2001, Belg. J. Zool., 131, 89-94.
22. Basch, L.V. 1988, Proc. Int. Echinoderm Conf., 503-515.
23. Grober, M.S. 1988a, J. Exp. Mar. Biol. Ecol., 115, 157-168.
24. Grober, M.S. 1988b, Anim. Behav., 36, 493-501.
25. Grober, M.S. 1990, Am. Soc. Zoologists 77-87.
26. Mallefet, J., Dupont, S., Vandemeulebroeck, G., Baguet, F., and Deheyn, D. 1998, Eos, Trans. Am. Geophysic. Union 79, 175
27. Deheyn, D., Mallefet, J., and Jangoux M. 2000, J. Mar. Brit Assoc. UK., 80, 179-180.
28. Vandemeulebroeck, G. 1998, B.Sc. Thesis, Université Catholique de Louvain, Belgium.
29. Brehm, P. 1977, J. Exp. Biol., 71, 213-227.
30. Widder, E.A., Latz M.I., and Case, J.F. 1983, Biol. Bull., 165, 791-810.
31. Mallefet, J., Baguet, F., and Jangoux, M. 1989, Arch. int. Physiol. Biochim., 97, 37.
32. Mallefet, J., Germain, G., and Baguet, F. 1991, Belg. J. Zool., 121, 29.
33. Mallefet, J., Vanhoutte, P., and Baguet, F. 1992, Proc. Int. Echinoderm Conf., 125-130.
34. Mallefet, J., Ajuzie, C.C., and Baguet, F. 1994, Proc. Int. Echinoderm Conf., 455-460.
35. Dewael, Y., and Mallefet, J. 2001 Proc. Int. Echinoderm Conf., 249-251.
36. Dewael, Y., and Mallefet, J. 2002, J. Exp. Biol., 205, 799-806
37. Dewael, Y., and Mallefet, J. 2002, Comp. Biochem. Physiol., 131C, 153-160.
38. De Bremaeker, N., Mallefet, J., and Baguet, F. 1993, Arch. Int. Physiol. Biochim., 101, 30.

39. De Bremaeker, N., Mallefet, J., and Baguet, F. 1993, Bioluminescence Symp., 40, 73.
40. De Bremaeker, N., Mallefet, J., and Baguet, F. 1996, Comp. Biochem. Physiol., 115C, 75-82.
41. De Bremaeker, N., Deheyn, D., Thorndyke, M.C., Baguet, F., and Mallefet, J. 1997, Proc. R. Soc. Lond., B 264, 667-674.
42. De Bremaeker, N. 1999, PhD thesis. Université catholique de Louvain, Belgium.
43. Vanderlinden, C., Dewael, Y., and Mallefet, J. 2003, J. Exp. Biol., 206, 3007-3014.
44. Vanderlinden, C., and Mallefet, J. 2004, J. Exp. Biol., 207, 3749-3756.
45. Vanderlinden, C., Vanhemelen, M., Nilius, B., Gailly, P., and Mallefet, J. 2004, Biolum. Chemilum., 39-42.
46. Viviani, D. 1805, Quattuordecim lucescentium animaculorum novis speciebus illustrata. Genova. 17 pp.
47. Mallefet, J. 1999, Proc. Int. Echinoderm Conf., 93-102.
48. Mallefet, J., Barker, M., Byrne, M., and O'Hara, T. 2004, Proc. Int. Echinoderm Conf., 299-304.
49. Mallefet, J. 2005, Proc. 13th Int. Symp. Biolum., 19-22.
50. Mallefet, J., and O'Hara, T. 2009, Proc. Int. Echinoderm Conf., 45.
51. Uthicke, S., Schaffelke B., and Byrne, M. 2009, Ecol. Monogr., 79, 3-24.
52. Mallefet, J., and O'Hara, T. 2006, Proc. Int. Echinoderm Conf., in press.
53. Brehm, P., and Morin, J.G. 1977, Biol. Bull., 152, 12-25.
54. Mallefet, J., and O'Hara, T. 2009, in prep.
55. Mallefet, J., Case, J., McDougall, C., and Hendler, G.2004, Proc. Int. Echinoderm Conf., 305-310.
56. Dupont, S., Mallefet, J., and Vanderlinden, C. 2004, Comp. Biochem. Physiol., 138C, 59-66.
57. Quevy, F. 2007. B.Sc. Thesis, Université Catholique de Louvain, Belgium.
58. Mallefet, J. 2009, Proc. Int. Echinoderm Conf., 72.
59. Mallefet, J., and Dubuisson, M. 1995, Belg. J. Zool., 125, 167-173.
60. Vanhemelen, M. 2005. Master Thesis, Université Catholique de Louvain, Belgium.
61. Vanhemelen, M., Mallefet, J., and Vanderlinden, C. 2005, 12th BCZ, 75.
62. Renard, J. 2007, B.Sc. Thesis, Université Catholique de Louvain, Belgium.
63. Mallefet, J., Renard J., and O'Hara, T. 2008, Luminescence 23, 84.
64. Mallefet, J., Ajuzie C.C., and Baguet, F. 1994, Proc. Int. Echinoderm Conf., 455-460.
65. Mallefet, J., Chabot, B., De Bremaeker, N., and Baguet, F. 1998, Proc. Int. Echinoderm Conf., 387-392.
66. De Bremaeker, N., Dewael, Y., Baguet, F., and Mallefet, J. 2000, Luminescence 15, 159- 163.
67. Guilford, T., and Cuthill, I. 1989, Anim. Behav., 37, 339-341.
68. Grober, M.S. 1989, Anim. Behav., 37, 341-343.
69. Hurbin, O. 2007, B.Sc. Thesis, Université Catholique de Louvain, Belgium.
70. Jones, A. 2009, Master Thesis, Université Catholique de Louvain, Belgium.
71. Mallefet, J., Hurbin, O., and Jones, A. 2009, Proc. Int. Echinoderm Conf., 44.

72. Shimomura, O., and Johnson, F.H. 1966, In Bioluminescence in Progress , F.H. Johnson and Y. Haneda, eds, 495-521, University Press, Princeton, NJ.
73. Shimomura, O. 1986, Photochem. Phobiol., 44, 671-674.
74. Mallefet, J., Parmentier B., Mulliez X., Shimomura O., and Morsomme, P. 2009, Proc. Int. Echinoderm Conf., 74.
75. Deheyn, D., Jangoux, M., and Warnaux, M. 2000, Sci Tot. Environ., 247,41- 49.
76. Deheyn, D., R Bencheikh-Latmani R., and Latz, M. 2004, Environ Toxicol., 19, 161-178.
77. Morgulis, S. 1909, Proc. Am. Acad. Arts Sci., 44, 655-659.
78. Dobson, W.E. 1988, Ph.D. thesis, University of South Carolina, USA.
79. Stancyk, S.E., Golde, H.M., Pape Lindstrom, P.A., and Dobson, W.E. 1994, Mar. Biol., 118, 451-462.
80. Sköld, M., and Rosenberg, R. 1996, J. Sea Res., 35, 353-362.
81. Dupont, S., and Thorndyke, M. 2006, , J. Exp. Biol., 209, 3873-3881.
82. Thorndyke, M., Patruno, M., Dewael, Y., Dupont, S., and Mallefet, J. 2003, Proc. Int. Echinoderm Conf., 193-199.
83. Mallefet, J., Dupont, S., Dewael, Y., Patruno, M., and Thorndyke, M. 2001, Proc. Int. Echinoderm Conf., 301-305.
84. Dupont, S., Vangeluwe, D., and Mallefet, J. 2001, Proc. Int. Echinoderm Conf., 255-260.
85. Thorndyke, M.C., and Candia Carnevali, M.D. 2001, Can. J. Zool. 79, 1171-1208.
86. Sköld, M., Josefson, A.B., and Loo, L-O. 2001, Mar. Biol., 139, 519-526.
87. Bruggeman, O., Dupont, S., Mallefet, J., Bannister, R., and Thorndyke, M.C. 2003, Proc. Int. Echinoderm Conf., 177-180.
88. Delval, S., and Mallefet, J. 2006, Proc. Int. Echinoderm Conf., 13.

Research signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 85-104
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

6

Hitherto unreported aspects of the ecology and anatomy of a unique gastropod: The bioluminescent freshwater pulmonate *Latia neritoides*

V.B. Meyer-Rochow[1] and S. Moore[2]
[1]Faculty of Engineering and Science, Jacobs University, D-28759 Bremen Germany; Department of Biology, University of Oulu, SF-90014 Oulu Finland; [2]Landcare Research, Tamaki Campus, University of Auckland Private Bag 92170, Auckland, New Zealand

Abstract

Latia neritoides is a basommatopheran pulmonate, rarely exceeding 10 mm in length, that inhabits lotic habitats of New Zealand's North Island and which upon disturbance releases a mucus of greenish luminescence of 510 nm wavelength. Most likely the luminescent mucus, which leaves the snail via the

Correspondence/Reprint request: Prof. Dr. V.B. Meyer-Rochow, Faculty of Engineering and Science, Jacobs University, D-28759 Bremen, Germany. E-mail: b.meyer-rochow@jacobs-university.de

pneumostomal opening, detracts and confuses the predator (which could be fish, crayfish, or larvae of aquatic insects). In the aquarium Latia *displayed no homing behaviour or feeding rhythmicity. Eggs were laid throughout the year and observed to develop best at a water temperature of 18-22°C. Hatching occurred as early as 21 and as late as 45 days later. Approximately 10% of the eggs failed to develop normally. Individuals are likely to attain a length of 4.5 mm in the first year and may live 2-3 years. Information on internal and external anatomy is provided, the radula is described and pharyngeal bulb together with oesophagus, salivary glands, the locations of male and female gonopores, the pneumostomal lappet, the intestinal tract and the reproductive apparatus are identified and illustrated.*

1. Introduction

Gastropod phylogeny has recently attracted renewed interest and the examination of cell lineage data from 30 gastropod taxa has led to a re-interpretation of older phyletic trees [1]. However, the position of *Latia neritoides*, first described by Gray [2] and the only freshwater gastropod known to be luminescent, as a member of basommatopheran pulmonates remains unchallenged (Fig. 1). Formerly placed within the Ancylidae, Hubendick [3] decided that together with the family Acroloxidae, *Latia neritoides* was derived from a South American chilinid ancestor. *Chilina* is the sole genus in the family Chilinidae and shares with *Latia neritoides* the lotic habitat, a non-contractile pneumostome, pneumostomal lappet, non-invaginable tentacles, and separate male and female gonopores [4]. Phenotypic plasticity in gastropods, generally, and freshwater limpets in particular [5] is, of course, always a problem, but with regard to the organization of the reproductive system, eggs, and egg capsule morphology, *Latia* most closely resembles *Acroloxus* [6]. All three genera *Chilina*, *Latia*, and *Acroloxus* have the same chromosome number (n = 18 [3]), which further supports the view of a close relationship between the three respective families.

Naturally *Latia neritoides* (Fig. 2), because of its ability to produce a greenish and brightly luminescent slime (Fig. 3), has been the subject of a variety of studies. Detailed observations on its bioluminescence were first published by Bowden [7], and later supplemented by examinations of the biochemical reaction behind the light production [8, 9]. Information on the general biology and biogeography of *L. neritoides* was published by Meyer-Rochow and Moore [10] and some details of its reproductive behaviour were provided by Moore and Meyer-Rochow [11]. A comparison of the respective eye anatomies and retinal ultrastructures in equally-sized individuals of *L. neritoides* and non-luminescent *Ancylus fluviatilis* by Meyer-Rochow

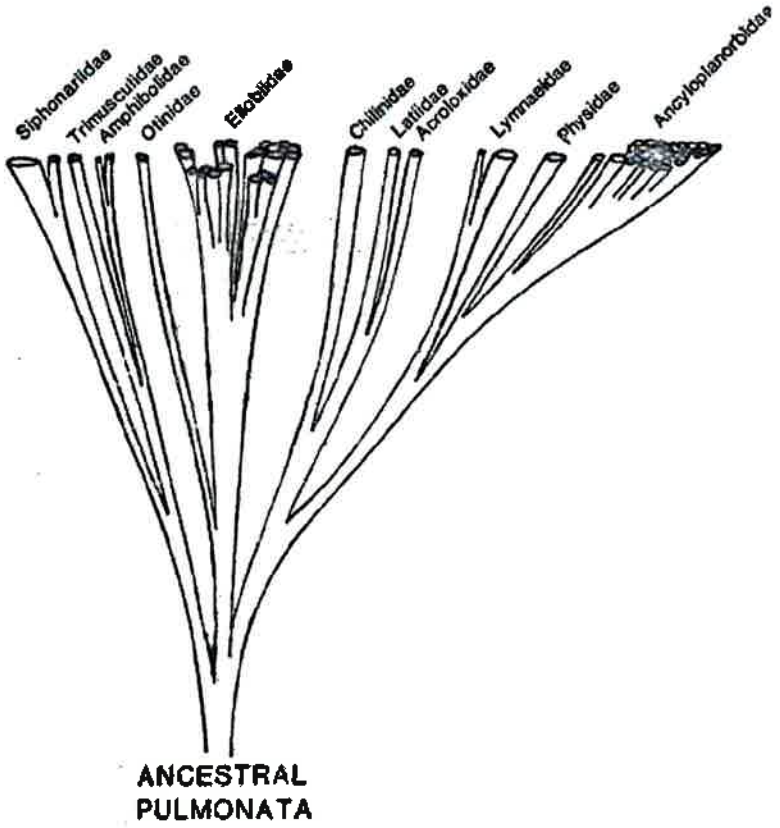


Figure 1. Likely phylogeny of the pulmonate gastropods with the position of the Latiidae [3].

Figure 2. *Latia neritoides* adult, with shell size of ca. 7 mm in length.

and Bobkova [12] demonstrated that the eyes of the luminescent species had only a slightly larger lens, but a very much thicker and extensive retinal layer than those of the non-luminescent *Ancylus*. However, behavioural observations in the field and tests in the aquarium failed to provide any evidence

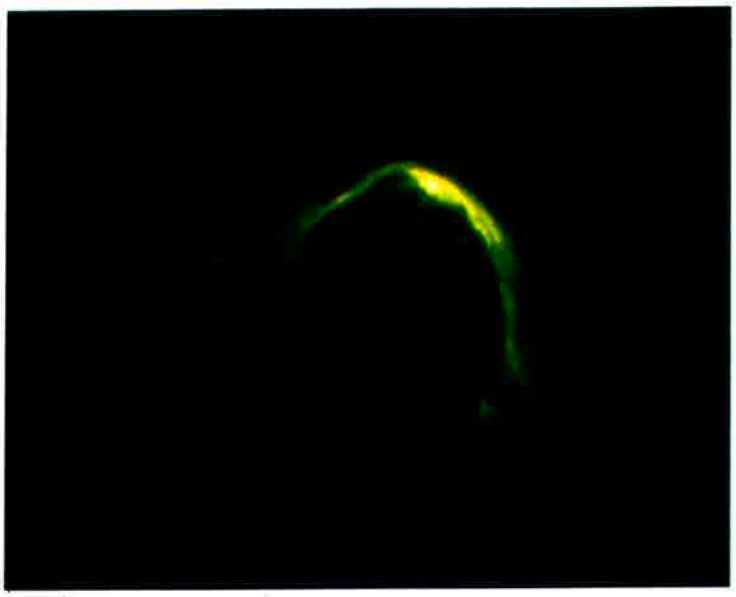


Figure 3. *Latia neritoides* adult, with luminescing muscus, photographed by its own light, cf. [27].

for a communicative role of the luminescence in *L. neritoides* and it seems as if the luminescent slime, carried downstream by the current, functions as a decoy to lure predators like eels, other fish and perhaps some invertebrate predators like crayfish away from the dark brown or black limpets that remain firmly attached to a rock or otherwise hard substrate [10].

Larvae of the large predatory dobsonfly *Archichauliodes diversus* were unable to harm attached adult *Latia* and neither was the freshwater shrimp *Paratya curvirostris* capable of preying upon attached *Latia*. However, although the common bully (*Gobiomorphus cotidianus*) was not seen to dislodge any adult individuals or egg capsule, this fish did attack falling and inverted *Latia*. The freshwater crayfish *Paranephrops planifrons* readily ate inverted *Latia* and, given an opportunity, removed egg capsules, but showed no interest in attached *Latia*. Short-finned eels clearly removed *Latia* from stones and, thereby, just like the larger predatory invertebrates, induced luminescence, but convincing evidence of an unpalatability of *Latia* is lacking and to a human, *Latia*'s luminescent slime is taste- and odourless. Since dragonfly larvae and crayfish were seen to require hours to remove the apparently sticky and seemingly unpleasant slime from their mouthparts, the slime's luminescence may lead long-lived aquatic predators to associate the greenish light with the unpleasant experience of having to spend a considerable amount of time freeing their mouthparts of the sticky mucus. Nevertheless, *Latia* shells were usually found in the stomachs of three species of fishes, which shared the habitat with *Latia* (e.g., *G. cotidianus*, *A. dieffenbachi*, and *Salmo trutta*) and whose stomach contents were analysed.

From these few introductory remarks it is obvious that *L. neritoides*, known only from some freshwater streams in the North Island of New Zealand, is a zoologically (and bio-geographically) extremely exciting animal. Convergence in shell forms is a widespread phenomenon in gastropods and anatomical studies of the alimentary canal and reproductive system can be of taxonomic and phylogenetic importance [13]. *Latia*'s internal anatomy has not previously been described in any great detail [14] and some aspects of its behaviour and ecology have also remained unreported until today. We therefore decided to use this chapter and present information on those aspects of *Latia* biology that had not sufficiently well been covered earlier; supplementing existing publications [2-4, 7-12, 14-16] on this fascinating bioluminescent member of the mollusc phylum.

2. The Ngutunui Stream: Figures on abundance, density, and size of *L. neritoides*

In the following account we shall limit ourselves to *L. neritoides* from the Ngutunui Stream, because we noticed morphological differences in shell shapes and sizes of populations of *Latia* from the Waitara and Waikato Rivers, Waikuku Stream and Lake McLaren. In the Ngutunui Stream densities of up to 350 *L. neritoides* per square metre can be encountered, but considerable differences between sites in the same stream were noticeable. Distribution tends to be clumped and stones measuring 20 – 40 cm in diameter could harbour 40 – 80 individuals, usually in the vicinity of egg capsule clusters (Fig. 4), mostly on the upstream or downstream sides of the

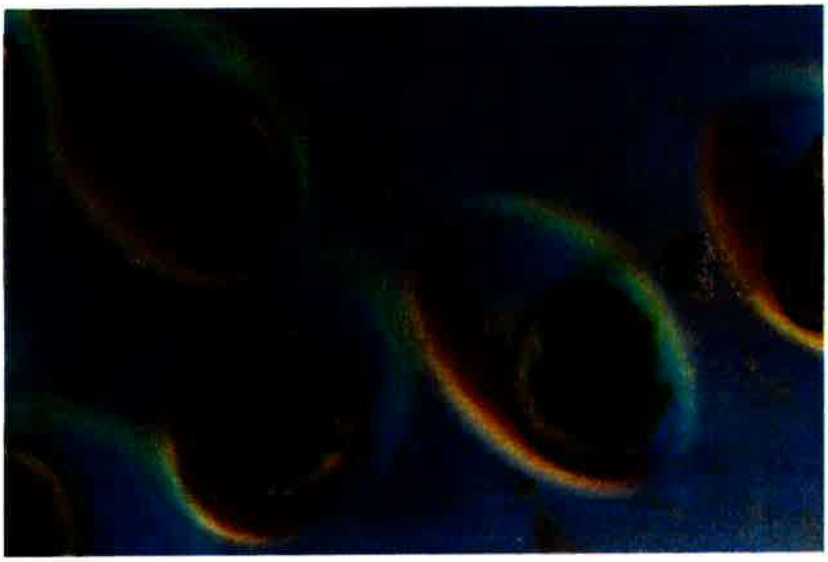


Figure 4. Individual eggs (from a cluster of 36), each ca. 0.3 mm long, with embryos in them.

stones, but rarely on the stones' uppermost surfaces (although occasionally snails might be present there, but less commonly occurring in clusters). Pieces of hard wood were acceptable to *Latia* in the same way and small specimens were found on beds of the introduced oxygen weed *Elodea canadensis* in Lake McLaren.

Egg capsules, not overlapping, could be as dense as 4 per square cm on clear, solid surfaces and together with the adults were most common in sections of the stream of around 40 cm depth or less. An analysis of the size clusters on individual stones suggested that most of the stones' resident snails were of the same age, pointing to very poor mobility of *L. neritoides*. Egg capsules were least common in the winter months and most abundant in spring, summer, and autumn. However, it is possible that in the colder winter months large boulders in the deeper sections of the stream are preferred as substrates for egg-laying. This aspect of possible seasonal distributional changes in site preferences requires further investigation. It is also possible that the emission peak of the luminescent mucus, reported earlier as 535 nm [15] and measured by us as 510 nm [10], varies seasonally or in individuals of different populations. This, too, would require additional research.

Across the projection of the internal shelf, shells of *L. neritoides* individuals from Ngutunui Stream display distinct trenches (Fig. 5), which increase in number with age. Individuals with shell lengths of up to 2 mm exhibit no trenches, while those with 5 - 7 mm large shells possess on average

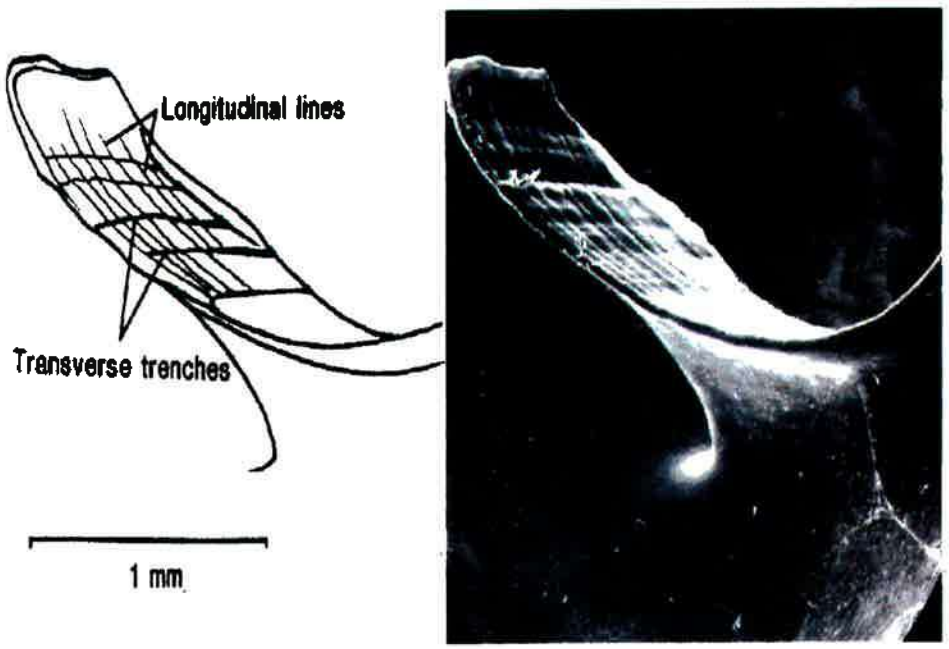


Figure 5. Schematic drawing (left) and scanning electron micrograph (right) of the inner projection of the internal shelf with transverse trenches and longitudinal age lines.

4 trenches and individuals with shells 9 mm or longer have 6-7 trenches. Although there is a clear and virtually linear relationship between shell length and number of trenches, we cannot conclude that the trenches and/or shell growth are indicative of age alone as nutritional supply, food composition and other environmental conditions like temperature, $CaCO_3$ content of the water, etc. could influence the speed of growth and longevity of *Latia* individuals. Moreover, there could be genetic differences between populations of different localities, for considerable variations in shell sizes and shapes (e.g., length to width ratio) could be observed. Specimens from the Waikuku Stream exhibited shell lengths of up to 12 mm, while those of other habitats, including the Ngutunui Stream, rarely exceeded 8 mm.

3. Behavioural observations of *Latia neritoides* in the aquarium

In captivity *Latia* preferred the surfaces of stream stones to the smooth, clean sides of glass aquaria, although they will commonly inhabit aquarium glass that has a visible algal cover. Loose sand or gravel was even less attractive than glass. When leaf litter was the only substrate other than glass, no individuals were ever seen on leaf surfaces. *Latia* would, however, attach to any solid wooden surfaces and would readily move over glass and stone surfaces that were covered by fine algal mats. None of the 20 marked individuals that were followed for several consecutive days showed a tendency to return to the same site at any time of day or night. Therefore, at least under aquarium conditions, *Latia* showed no homing behaviour, but did exhibit favourite places. For example, relatively high numbers of individuals were found close to the rising columns of bubbles from air stones and near the surface of the water. An accumulation of *Latia* towards the brighter, illuminated end of an aquarium where most of the algae were located, was recorded in the first two days following the random introduction of *Latia* to the tank. After 9 days, however, as the supply of edible algae was depleted in the illuminated half of the aquarium, a general movement back towards the darker half brought the numbers in the two halves to equal figures. Over the next 7 days approximately 60% of all of the specimens were located in the darker half of the tank. *Latia* did not tend to follow older feeding trails or the same path of movement.

Latia fed at any time of day and individuals were usually observed to make at least occasional feeding movements when moving over bare glass. The mouth was seen to open as the radular apparatus pulled its rows of teeth forward to produce a half-second long scraping motion. Following this action, the mouth closed, completing one cycle of movement, which from

beginning to end took about two seconds before the next scrape could commence. Usually 10 to 15 scrapes were completed in one minute.

As the individual made on average 7 or 8 scrapes in one direction, leaving an unbroken trail, the head was swept sideways by turning the whole body up to a quarter turn. This sweep was reversed as the animal moved slightly forwards, resulting in a zig-zag trail of curved sweeps. A 6-7 mm adult would leave two wedge-shaped scrape marks per one mm, with sweeps averaging 3 mm long and trails about 1.5 cm in length. Typical feeding trails were left in fine mats of green algae and diatoms on the glass. Microscopic inspection revealed that the radula cleared almost all algae in the scraped areas. No feeding trails were found in any patch of Cyanophyte (= Cyanobacteria) or *Cladophora* (a filamentous green alga) growth.

A comparison of different living conditions in captivity with 20 individuals at the outset, thus making it a starting density of approximately one *Latia* per 200 square cms, resulted in the following survival lengths:

- 15 days in an aquarium filled with sand and leaf litter, kept in the dark and a water temperature of 15°C;
- 21 days in an aquarium with Cyanophyte (=Cyanobacteria) growth and 18-22°C warm water under normal daily dark/light cycle;
- 50 days in an aquarium with water of 18-22°C under normal daily dark/light cycle, but filamentous green algal bloom from approximately day 35;
- 100 days in an aquarium with water of 18-22°C under normal daily dark/ light cycle, but without any algal bloom.

Latia can be found within 800 m of the mouth of the Waitara River, where only 400 m downstream the water is brackish at high tide, and experiments in the laboratory showed that *Latia* could tolerate concentrations of up to 60% sea water, but only for a few days. Yet, even in only 20% sea water, feeding movements were rare and rapid lethality began to commence when water reached levels of 30-40% sea water (through a gradual rise by 5 % per day). *Latia* survived well, but grew more slowly, in aquaria lacking any $CaCO_3$ source. The longest surviving specimen in any aquarium during our observations spent its whole 9 months without a $CaCO_3$ supply. The shell of this individual grew in length by 0.05 mm per month over most of this period, and the newly formed shell was translucent and thin. Specimens kept in aquaria with abundant shell fragments in the gravel grew in length by approximately 0.1 mm per month. Such individuals had thicker and darker shells, were usually more active, and were commonly observed to be feeding.

Four *Latia*, which survived for 30 days in a long tank (120 x 20 x 30 cm) without water circulation, were moving around freely and feeding normally without showing signs of stress such as dormancy or withdrawal. The depth of the water had been 15 cm and the water temperature a nearly constant 17°C. One individual per 700 sq cm was the highest density tolerated in an aquarium with stagnant water. When in an aquarium *with* water circulation containing one individual per 100 sq cm, the circulation was cut off, one third of the population died within the next three days. As to the optimal temperature, it was noted that *Latia* survived in water of 10°C as well as in water of room temperature, but at 10°C the growth rate of edible algae was too low to support many individuals for any length of time. As the temperature increased in summer to over 22°C, an increase in the death rate followed, with larger individuals tending to die first. An indirect effect of the warmer summer temperatures was the rapid spread of Cyanophyta (= Cyanobacteria), accelerating the death rate.

4. Luminescence, development, and growth

4.1. Light production in *L. neritoides*

Since Gray [2] described the species, luminescence in *Latia* has been the subject of several publications [8-11, 15, 16,] and is known to involve a greenish-glowing mucus extruded from the limpet into the environment via the pneumostome. The green light spreads around the oval-shaped space between the foot of the animal and the rim of its shell (Fig. 3). Luminescence was produced at all temperature conditions, in which *Latia* individuals were kept (from 10 to 22° C). It was noticed, however, that in warmer water the luminescent mucus was less viscous and tended to streak out from the specimen with the current. In cooler water the mucus broke away from the specimen in more discrete globular units.

Only one other basommatophoran genus, the marine *Planaxis*, is known to contain species that luminesce in response to disturbance. Unlike *Latia*, however, *Planaxis* produces intracellular light. The light of the terrestrial gastropod *Dyakia striata* (also known as *Quantula striata* [18]) is also intracellular, but it does not respond to disturbance. In *Dyakia* two types of faint light, a continuous one emitted from cells scattered over foot and mantle, and flashes, yellow-green in colour, produced by a discrete photogenic organ behind the mouth, are produced. Young *Dyakia* snails flash more than old ones, but both can lose the ability to luminesce due to starvation [19].

In the field luminescence was visible around the shell of *Latia* for up to a minute, but only in relatively slow flowing streams. Most luminescence

accumulated to the right side of the animal. In fast currents, little luminescence could accumulate around the shell or remain on stone surfaces, but most was rapidly washed downstream almost as soon as the mucus was released from the animal (Fig. 6). Some specks of light were seen swirling around in the eddies behind and between stones. Some specks simply hopped to a different stone surface, where they stuck and remained until the light eventually faded. If the mucus was broken into many specks, a combination of some or all of the patterns could be observed.

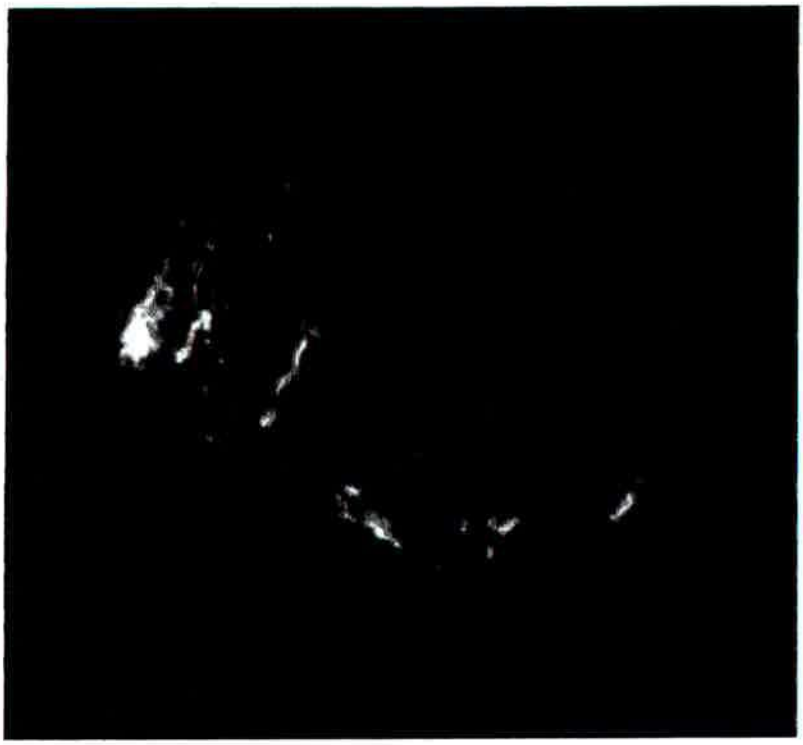


Figure 6. Greenish luminescent slime, produced by *Latia*, is taken by the current downstream. The producer of the luminescent mucus remains hidden. Original photo in colour from [10].

4.2. Growth and development in *L. neritoides*

Details on reproduction, embryology and development, including first appearances of luminescence, have been published earlier and shown that luminescence can occur in the egg at least 10 days prior to hatching [11]. About 10% of the developing eggs produced deformed or otherwise abnormal specimens. When after an average of 30 days the small snails hatched, they usually took only seconds to eat through the egg capsule, but many individuals stayed on the capsule for one or two days feeding on the growth that had meanwhile occurred on the egg capsule (mainly diatoms of the genus *Cocconeis*, some filamentous green algae and associated ciliates).

Whether a certain epibiontic growth aids the juveniles to escape from their eggs (as reported for some reptilian eggs [20]) is an intriguing, but untested idea for *Latia*. Torn or otherwise damaged egg capsules or those containing unfertilized eggs were rapidly colonized by fungi and bacteria, but undamaged egg capsules containing normally developing eggs did not share that fate.

Thicker algal mats and long filamentous algae, however, often trapped young snails and killed them, which is perhaps why some newly hatched *Latia* moved up to 8 cm out of the water to remain in the spray-zone, feeding there on the scant growth of algae present. Although not further investigated, the presence of rotifers, which managed to pass behind the head of the juveniles underneath the mantle, seemed to be correlated with the premature death of *Latia*, but oligochaete worms appeared to be regular commensals on and around the shells of juvenile *Latia*. Average heartbeat frequency of a newly hatched individual at 18°C was ca. 1 Hz and increased to 1.2 Hz in juvenile *Latia*, but owing to the increase in shell pigmentation heartbeat measurements could not be continued beyond10 days post-hatching. Based on shell length, growth at 18-22°C was approximately linear for the first 120 days with increases from 0.5 mm to 2.0 mm. Shell lengths were estimated to reach a length of ca. 4.5 mm in the first year of growth.

Some 'milestones' in the development of young *Latia* occurred on days 6 and 7 of development, when the until then spherical embryo developed three approximately even sized protrusions, which by day 10 allowed a distinction of the head/foot area, inner organs, and shell. By day 17 the internal organs could be broadly identified, the radular apparatus was clearly visible and the gut could be traced. The eyes became recognizable on day 19 due to their dark screening pigment as two dark spots near the middle of each semicircular lobe of the velum. A respiratory current could be seen to the right side of the shell by day 21; at the same time a heartbeat of 1 per second was noticeable. Around day 25 an increase in shell pigmentation was apparent and hatching could commence as early as day 21 and as late as day 45. Newly hatched individuals possessed shell lengths ranging from 0.35 to 0.48 mm. The shells grew faster on the right and anterior margins changing the appearance of juvenile *Latia* from snail to limpet-shape. A thin and smooth periostracum layer covered the outside of the shell and was responsible for giving the shell of *Latia* its dark colouration.

5. Morphology and anatomy

Appearances of adult *L. neritoides* from above, below and the side are shown in figures 7, 8, and 9. These figures will facilitate an understanding of

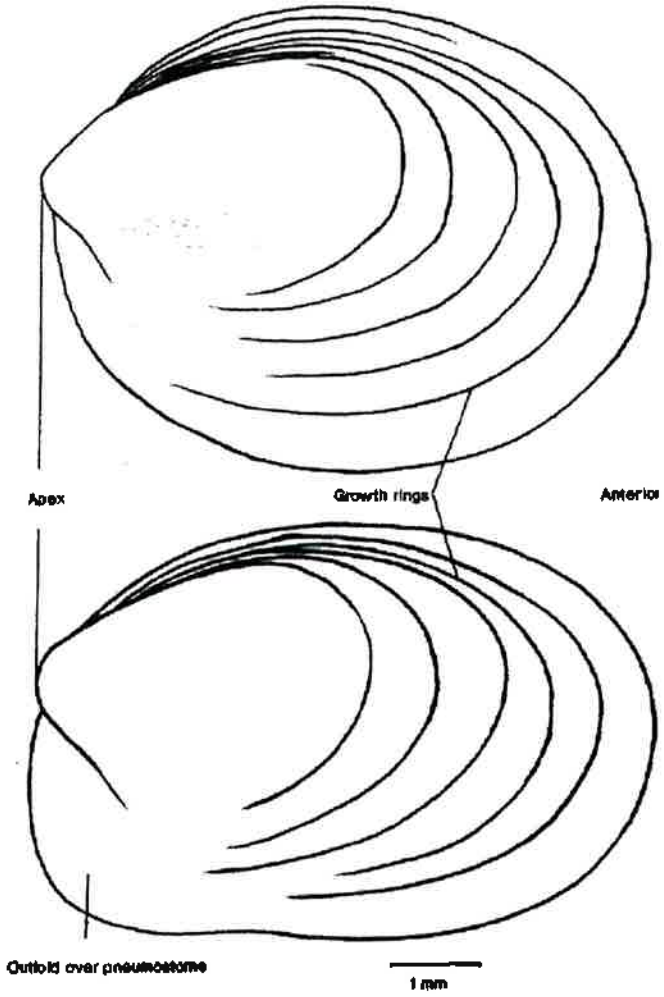


Figure 7. Dorsal view of the shells of adult *Latia neritoides* from (top) Ngutunui Stream and (bottom) Waikato River.

the anatomical and functional elaborations given further below. *Latia* has a large flexible foot, cream-coloured on its base, but grey on its sides. Glands in the foot secrete abundant mucus and the foot's ciliated undersurface (as with other snails [21]) ensures strong adhesion to the substrate. The cushion-like oral lappets in front of the foot have the same colouring, but are divided from the anterior of the foot by a distinct furrow. Situated between the oral lappets is the mouth, which is commonly 'T'-shaped when closed (Fig. 10). The pulmonary sac (i.e., modified mantle cavity) of *Latia* is revealed by removing the dorsal mantle wall (Fig. 11). A thick spongy tissue covers part of the inside of the roof of the mantle cavity. The mantle is fused to the neck region anteriorly, forming an enclosed chamber (apart from the one opening at the pneumostome).

Beneath the anterior half of the pulmonary sac lies the pharyngeal bulb (buccal mass), which houses the radular apparatus. When the covering of this

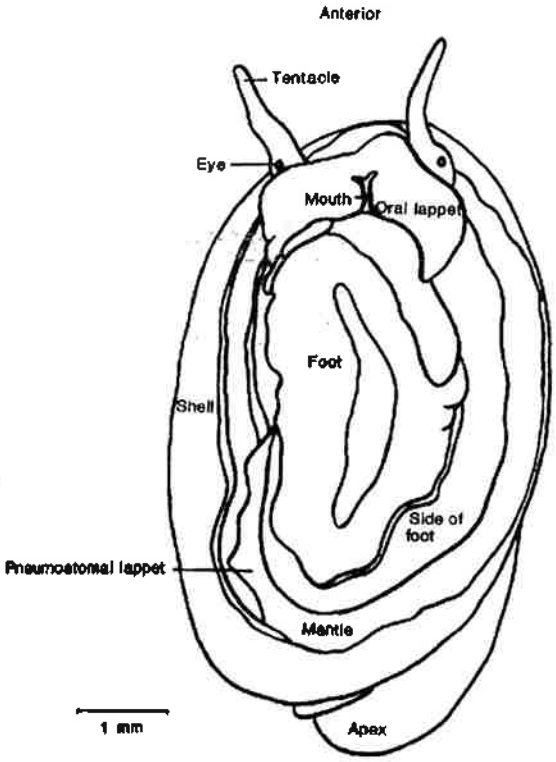


Figure 8. Light microscopic view of the ventral surface of an adult *Latia neritoides* from Ngutunui Stream.

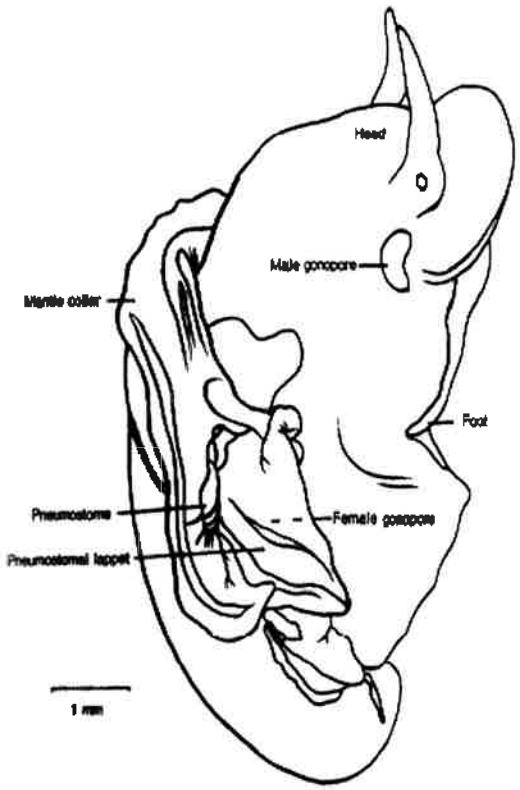


Figure 9. Light microscopic view of the right side of an adult *Latia neritoides.*

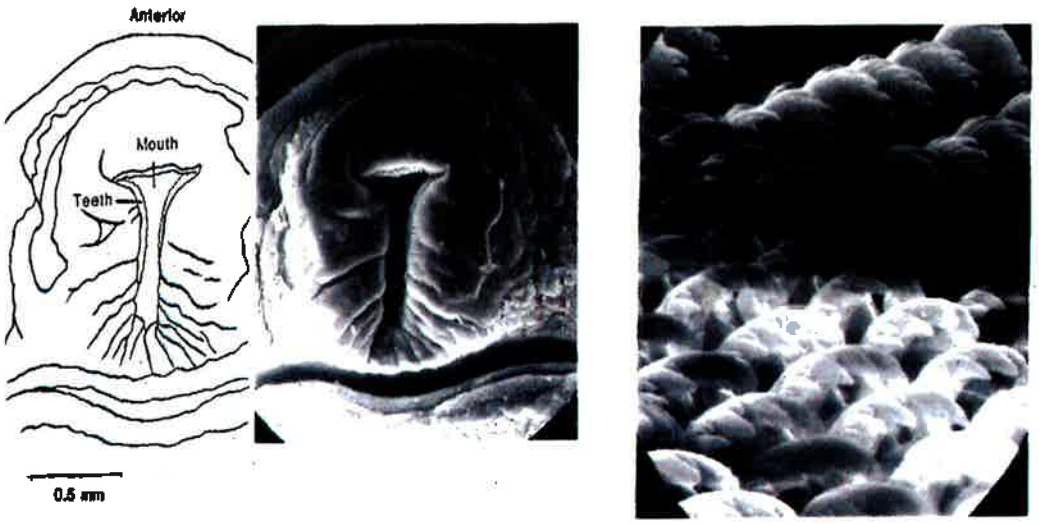


Figure 10. Oral region: left semi-schematic; centre scanning electron micrograph; right, individual teeth with same scale bar length as on the left, but representing 0.01 mm.

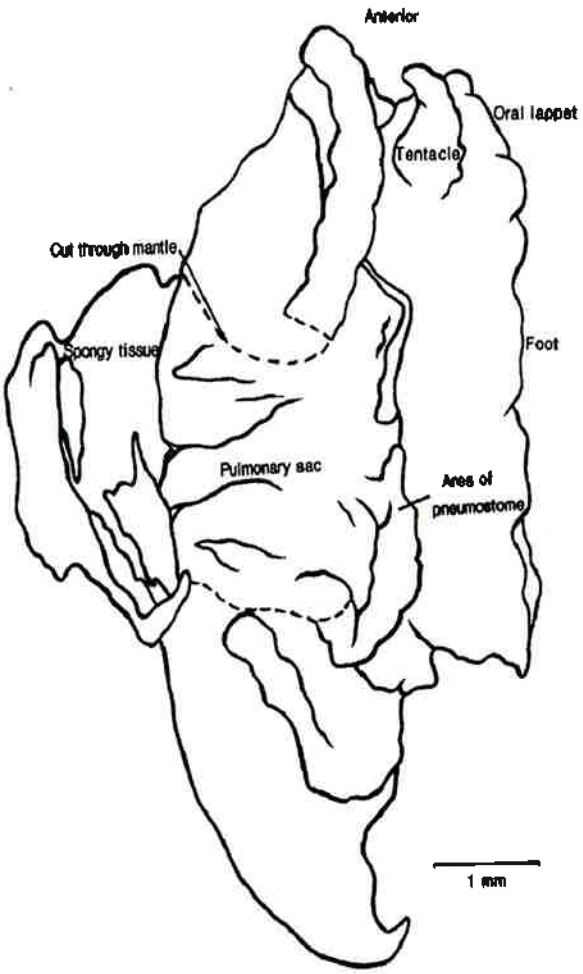


Figure 11. Lateral view of the right side of an adult *L. neritoides* after removal of the shell and lifting the roof of the pulmonary sac.

apparatus is removed, the musculature on the dorsal surface of the apparatus and the anterior part of some of the rows of its teeth are revealed (Fig. 10). The posterior end of the radular apparatus can be lifted out from under the anterior part of the main gut region to show the full extent of this feeding organ. An estimated 20-25% of the body is occupied by the radular apparatus. By pulling the whole organ away from the head area, the underlying site of attachment, the mouth and ventral surface of the apparatus, but no 'jaws', are visible.

In an individual of 7 mm shell length the opening of the mouth was 0.54 mm long and 0.42 mm wide at its widest part. The radula extended over 1.5 mm and possessed a width of 0.39 mm. The lateral teeth were curved discs that bore large cusps around their margin. Each tooth was about 13 μm long and maximally 7 μm thick. There were 5-6 large, rounded cusps on each side of the tooth, but smaller cusps were located between them. The larger cusps were approximately 3 μm long, while the smaller ones were close to half that length (Fig. 10).

Posterior to the mantle cavity, a thin membrane covers the visceral mass. Figures 12, 13, and 14 show the appearance of the digestive and reproductive

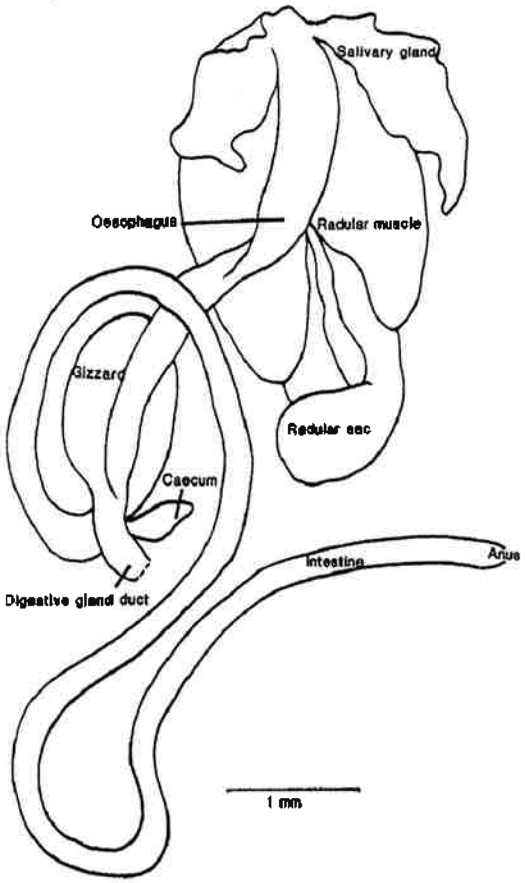


Figure 12. The alimentary canal of an adult *L. neritoides* with the large digestive gland removed.

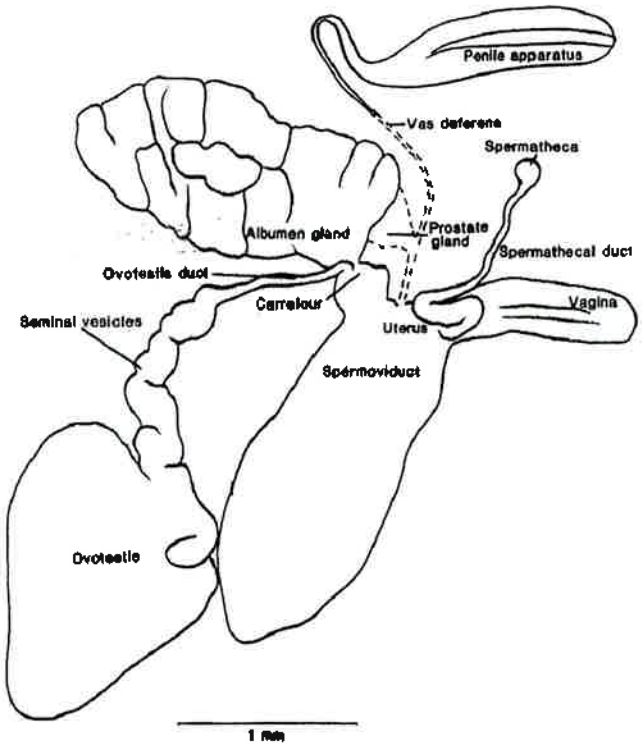


Figure 13. The reproductive system of an adult *L. neritoides*. The broken line indicates part of the system not identified in the dissection. A comparable dissection of *Lymnaea* sp. can be found in [28].

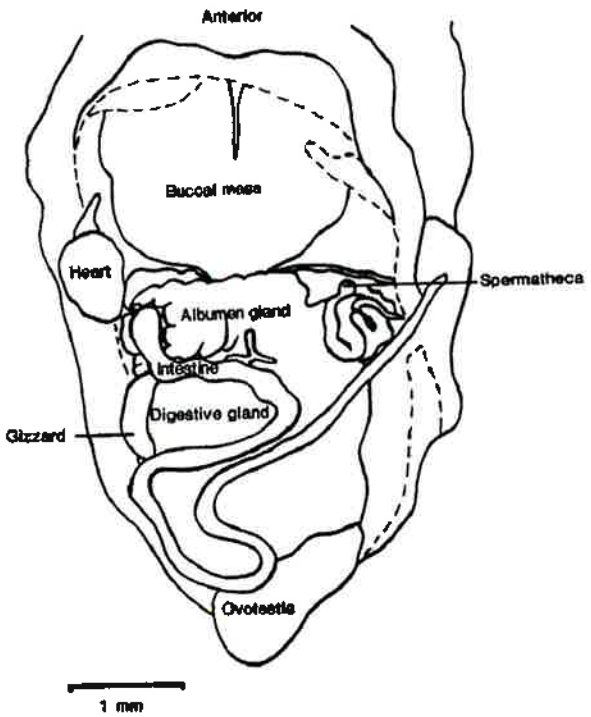


Figure 14. Dorsal view of the visceral mass of an adult *L. neritoides* with shell, mantle and body wall removed.

systems when this membrane is removed. The intestine lies dorsally over all other organs in the posterior half of the body. When the digestive system is pulled away from the neighbouring reproductive system, individual organs like the heart, gizzard, digestive gland, spermoviduct, ovotestis (Fig. 13) etc. can be identified (Figs. 13, 14). The large green digestive gland of *Latia* is attached by one duct to the stomach and fills much of the posterior half of the body. The digestive gland lies beneath the intestine and covers most of the other organs in this area of the body. This gland has a complex outline being branched into many ducts (Fig. 15).

The stomach of *Latia* is located on the left side of the animals and a shiny-surfaced gizzard is present in the anterior part of the stomach. An inconspicuous crop separates the gizzard from the long, white oesophagus. The oesophagus originates from the dorsal surface of the anterior end of the pharyngeal bulb and has inputs from the salivary glands (shown in Fig. 1 of Moore and Meyer-Rochow [11]). On the ventral side of the stomach region an egg-shaped caecum enters beside the duct of the digestive gland just before the beginning of the intestine (Fig. 12). The intestine is not particularly long and follows a relatively simple path to the ciliated rectum and anus in the posterior wall of the pneumostomal opening.

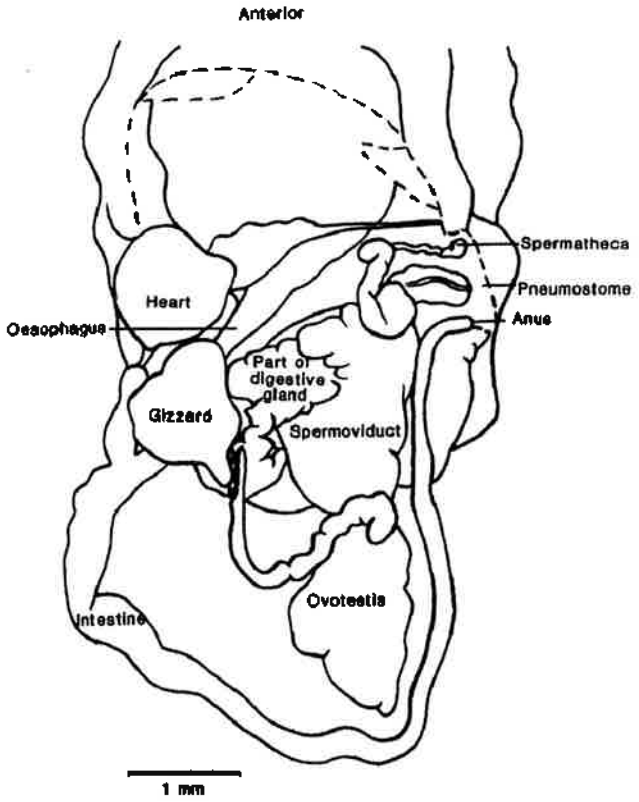


Figure 15. Dorsal view of the partly separated visceral mass of an adult *L. neritoides* with shell, mantle, body wall, and part of the digestive gland removed.

In the muscular gizzard of captive individuals the diatom genus *Cocconeis* was most commonly encountered, followed by the diatom genera *Achnanthes, Epithemia* and *Stauroneis*. Specimens captured in the field, however, had ingested a much greater variety of food types and much unidentifiable fine particulate matter seems to have been mixed with the diatoms taken from the surfaces of the stones in the stream. In these 'field individuals' most food seen in the alimentary canal was well broken down.

In close association with the posterior end of the digestive gland is the yellow-coloured ovotestis. From this organ, the thick white vesicles lead to the long narrow ovotestis duct, which curves towards the right side of the body. An overview of the reproductive system is depicted in Fig. 2 of the publication by Moore and Meyer-Rochow [11]. The ovotestis duct links up at the carrefour, the site of junction of the two other large organs of the reproductive system. Dorsally is the white albumen gland and ventrally is the large oval-shaped spermoviduct. The uterus is located towards the right extremity of the spermoviduct. A spermathecal duct joins up at the uterus region and further still to the right, opening to the right side of the body beneath the pneumostomal lappet, was the location of the female gonopore. Developing sperm was only seen in specimens from the field, but not in any of the aquarium specimens. One part of the system not identified during this study was the prostate gland and the vas deferens (which should lead to the prostate gland as indicated by Pelseneer [14]). The large penile apparatus was easily located, opening at the male gonopore behind the right tentacle (Figs. 9, 13).

The eye of *Latia* and its retinal ultrastructure have already been described in detail [12]. Comparisons with the photoreceptors of the equally large *Ancylus fluviatilis*, a gastropod with similar habitat preferences to *Latia*, but unable to produce luminescent slime, had shown that *Latia* possessed a very significantly more extensive retina, although the lens of its eye was only marginally enlarged over that of *Ancylus*. On the basis of estimations of the resolving power of the eye of *Latia* in comparison with those of other pulmonates, it was concluded that image formation was not possible [22], which can hardly speak for a role of the eye in *Latia* in the context of intra-specific visual communication. More likely the eye can merely perceive the approach of shadows and large shapes and is reasonably sensitive to changes in brightness, but no evidence was found in support of the view that individuals might warn each other by light signals of impending danger. However, that a chemical message, contained in the luminescent mucus droplets extruded by a distressed *Latia*, could alert other as yet unattacked individuals to be on guard, is still a possibility.

6. Conclusion

Latia neritoides is a basommatophoran pulmonate of lotic freshwater habitats in the North Island of New Zealand, which does not need access to the surface of the water. It has a permanently open pneumostome through which water enters and leaves the pulmonary sac and from which the greenish luminescent mucus is extruded. *Latia* has a wide temperature tolerance, from well below 10°C to just over 22°C, and can survive brackish water for a couple of days, but not longer, and is not harmed by direct sunlight. It would seem that the life span of *Latia* can be at least 3 years, but that differences with regard to maximum size, shell shape and length, and luminescence emission peaks exist between different stream populations. The luminescent mucus appears to be primarily a detraction for predators, a decoy that the latter may follow while *Latia* itself remains hidden in the dark, protected by its brown-black shell.

Unlike most pulmonates, *Latia* has no jaw, the ability to luminesce, a particularly large pharyngeal bulb with the oesaphagus and salivary glands originating from the anterior end, and a relatively short intestine. In common with other pulmonates *Latia* has an eye located at the base of each of the two non-retractable tentacles, the typical locations of male and female gonopores, a pneumostomal lappet, a spongy tissue in the roof of the pulmonary sac, typical lateral teeth, a long oesaphagus, a short crop, a gizzard and a caecum as well as a ciliated foot. Similarities between *Latia, Chilina*, and *Acroloxus* with regard to their reproductive systems [3, 4, 23], suggest a closer phylogenetic relationship between these three genera.

What remains an enigma, however, is the zoogeography of *Latia neritoides*. Gastropod families other than the Latiidae like, for instance the Bulimulidae, support the view that in the Mesozoic, according to Skipworth [24] New Caledonia, New Zealand, the New Hebrides and Tasmania had been in contact with each other and have had affinities to South America (Cherel-Mora 1983, cited in [25], but no *Latia* relatives have ever been reported from any of the aforementioned regions or Australia, except for the New Zealand North Island. No reports of *Latia* from the South Island of New Zealand exist, even though stream habitats are very similar throughout the North and South Islands of New Zealand and numerous aquatic insects are shared between them [26]. Some freshwater gastropods may have become extinct in the South Island during the glacial advances of the last two million years [26] and it is at least conceivable that afterwards colonization or re-colonization by *Latia* could not occur, because of *Latia*'s intolerance to water of more than 60% seawater salt content. On the other hand, Russel-Hunter [5] noted examples of the stream limpet *Ancylus* being found alive, in flight, on

the elytra of water beetles in Europe and North America. But, as we said earlier, *Latia*'s zoogeography for the time being remains an enigma.

References

1. Lindberg, D.R., and Guralnick, R.P. 2003, Evol. Dev., 5, 494-507.
2. Gray, J. E. 1850, Proc. Zool. Soc. Lond., 17, 164-169.
3. Hubendick, B. 1978, Pulmonates: Systematics, Evolution, and Ecology, V.Fretter and J. Peake (Eds.), Academic Press, New York, 1-48.
4. Hyman, L.H. 1967, The Invertebrates: Mollusca, McGraw Hill, New York.
5. Russel-Hunter, W. 1978, Pulmonates: Systematics, Evolution, and Ecology, V.Fretter and J. Peake (Eds.), Academic Press, New York, 135-384.
6. Bondesen, P. 1950, Natura Jutlandica (Aarhus) 3, 1-208.
7. Bowden, B.J. 1950, Biol. Bull., 99, 373-380.
8. Shimomura, O., Johnson, F., and Haneda, Y. 1966, Bioluminescence in Progress, F.H. Johnson and Y. Haneda (Eds.), University Press, Princeton, 391.
9. Cormier, M., Wampler, J.E., and Hori, K. 1973, Fortschr. Chem. Org. Naturst., 30, 1-60.
10. Meyer-Rochow, V.B., and Moore, S. 1988, Int. Rev. ges. Hydrobiol. 73, 21-42.
11. Moore, S., and Meyer-Rochow, V.B. 1988, Verh. Int. Ver. Limnol., 23, 2189-2192.
12. Meyer-Rochow, V.B., and Bobkova, M.V. 2001, N. Zld. J. Mar. Freshwater Res., 35, 739-750.
13. Strong, E.E., and Glaubrecht, M. 2008, Acta Zool. (Stockholm), 89, 289-310.
14. Pelseneer, P. 1901, Mém. Acad. Sci. Roy. Belgique, 54, 1-76.
15. Shimomura, O., and Johnson, F.H. 1968, Biochemistry, 7, 1734-1738.
16. Shimomura, O., Johnson, F.H., and Kohama, Y. 1972, Proc. Nat. Acad. Sci. USA., 69, 2086-2089.
17. Buck, J.B. 1978, Bioluminescence in Action, P.J. Herring (Ed.), Academic Press, New York, 419-460.
18. Haneda, Y. 1981, Bioluminescence and Chemiluminescence, M.A. DeLuca and W.D. McElroy (Eds.), Academic Press, New York, 257-265.
19. Counsilman, J.J., Loh, D., Chan, S.Y., Tan, W.H., Copeland, J., and Manera, M. 1987, The Veliger, 29, 394-399.
20. Ferguson, M.W. 1981, Science, 214, 1135-1137.
21. Aboul-Magd, L.A., and Sabry, S.A. 1985, Malacologia, 26, 201-211.
22. Zieger, M.V., and Meyer-Rochow, V.B. 2008, Am. Malacol. Bull., 26, 47-66.
23. Duncan, C.J. 1975, Pulmonates: Systematics, Evolution, and Ecology, V.Fretter and J. Peake (Eds.), Academic Press, New York, 309-365.
24. Skipworth, J.P. 1974, N. Zld. J. Geography, 57, 1-13.
25. Brescia, F.M., Pöllabauer, C.M., Potter, M.A., and Robertson, A. 2008, Molluscan Res., 28, 111-122.
26. Winterbourn, M.J. 1973, Tuatara, 20, 141-159.
27. Meyer-Rochow, V.B. 2001, The Biologist, 48, 163-167.
28. Morton, J.E. 1969, Practical Invertebrate Zoology: A Laboratory Manual, R. Phillips Dales (Ed.), Sidgwick & Jackson, London, 147-210.

Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 105-138
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

7 Lights on the ground: A historical survey of light production in the Oligochaeta

Emilia Rota
Department of Environmental Sciences, University of Siena
I-53100 Siena, Italy

Abstract

Earthworms and potworms are usually not sampled or examined in conditions that makes their capacity to produce light noticeable. Furthermore, fewer records of luminosity can nowadays be expected from common observers, due to urban development, light pollution and people less likely to take night-time nature walks in the dark. Nevertheless, the description in 1990 of luminous potworms from the forests of east Siberia has revived interest in the subject. This chapter offers a retrospect of the discovery

Correspondence/Reprint request: Dr. Emilia Rota, Department of Environmental Sciences, University of Siena I-53100 Siena, Italy. E-mail: rota@unisi.it

of bioluminescence in the Oligochaeta, from the first reports in 1600-1700 to our modern understanding of the chemical components involved in light production. The various luminous species from around the world are presented, whenever possible, in the fascinating words of the original sources. Although such reports began relatively late and were initially seen with suspicion, the increasing spread of the exotic Microscolex phosphoreus *across Europe and other parts of the world, helped promoting awareness and raising scientific curiosity about the phenomenon. Early students tried to understand which environmental conditions and what type and amount of stimulation might be triggering the light production. The chemistry of earthworm bioluminescence was first approached around the middle of 1800, with J.-H. Fabre carrying out perhaps the first laboratory experiments and establishing in vivo a dependence on oxygen. Accurate histological studies in the first half of 1900 unequivocally identified the granular cells suspended in the coelomic fluid as the seat of light production in megadriles, definitely ruling out a role for epidermis, or infection by luminous microorganisms. The disruption of these coelomocytes appeared essential for luminescence to take place. A luciferin-luciferase reaction could not be demonstrated until 1938, but cross-reactions with extracts of other bioluminescent organisms were always unsuccessful. The situation changed dramatically in the late 1960s when studies on the North American* Diplocardia longa *documented the stimulatory effect of hydrogen peroxide on earthworm luminescence. In the following two decades,* Diplocardia *luciferin (the aldehyde N-isovaleryl-3-aminopropanal) and luciferase (a Cu-containing, 300 kDa protein) were purified and identified, and a peroxide adduct of the former was found to be the true substrate of this type of bioluminescence. Whilst luciferin resulted to be chemically identical in all luminous megadriles in the superfamily Megascolecoidea investigated, luciferases appeared more specific and responsible for the position of the emission spectra (λ_{max} range from 500 to >570 nm). Cross reactions with luminous members of Lumbricidae have not yet been attempted. The bioluminescence of potworms (Fam. Enchytraeidae) is known since 1838, but in lesser detail than that of megadriles. Possibly confined to the sole genera* Henlea *and* Fridericia, *it was recently found, surprisingly, to involve completely different systems in the two genera with regards to photogenic organs, methods of lighting and biochemistry. In* Henlea, *luciferin is an unstable compound, and luciferase is a 72 kDa dimer protein; the system requires oxygen and calcium ion. Light (λ_{max} 464 nm) appears as a weak internal luminescence and, on stronger stimulation, as bursts of light associated with emission of coelomic fluid. In* Fridericia, *luciferin is a stable compound of 0.5-0.7 kDa, and luciferase is a 60 kDa dimer protein; the system requires oxygen, ATP and magnesium ion. Light*

(λ_{max} 478 nm) appears as a continuous glow of the body wall with a segmental pattern similar to that of the epidermal glands but not associated with secretion of luminous mucus. The ATP-dependence and alkaline pH are reminiscent of terrestrial arthropods with intracellular luminescence systems. Enchytraeid luciferins and luciferases do not cross react with each other and with Diplocardia *reaction components. The morphology, evolution and behavioural aspects of bioluminescence in the various oligochaete taxa are discussed.*

1. The earliest records re-examined

In 1683 Herman Nicolas Grimm (1641-1711), a Swedish-born physician and botanist who spent a long time in the East Indies, reported on some "rare luminous worms" inhabiting the woods of Coromandel: "In a dark night I noticed something luminous, the sight of which left me astonished and amused. As I approached closer, I perceived some motion, thus I left it undisturbed. Then, when day was breaking, I looked again carefully and found that the light was produced by worms, which appeared coiled together into a silky scarlet ball where neither eyes nor wings nor feet were observable. I took home with me several of those specimens and some of the soil on which they were laying, to later enjoy their luminosity. Placed in a glass cup, they kept glowing for a whole month, and so brightly that I could have read and written with their aid. After that time, however, they lost their life as well as their light" [1].[1] Although the described phenomenon and the accompanying drawing leave some uncertainty regarding the type of worms involved, at some stage Grimm entered the literature as the earliest reporter of luminescence in earthworms [e.g. 2-5].

The next two records, after nearly a lapse of a century, were more detailed and unequivocal, although taxonomically undefined (at the time all earthworms were still named collectively *intestina terrae* or confounded as varieties under the name *Lumbricus terrestris* L., 1758). They both occurred in southern France, in nearby areas on the right bank of the Rhone River, but the two narratives differed from each other in many respects. The first record [6] was by Honoré Flaugergues near Viviers in October 1771. The earthworm, measuring 8 by 0.6 cm, recalled a piece of rotten wood and gave out a bluish light which was most intense around the clitellum and disappeared within one or two days from capture. Similar sightings occurred again in October of the following years, making Flaugergues suspect some relationship between luminosity and the breeding season. The second record

[1] Often miscited as 1670. This is my translation from the original Latin text; French translations appeared in 1755 and 1767.

was due to Jean Guillaume Bruguière, who saw plenty of earthworms shining among hedges near Rochemaure [7]. They resembled *L. terrestris*, except in having broader heads and tapering tails. Their luminosity, much brighter than that of fireflies, came mostly from the hind body parts and was still visible after ten days in captivity.

The involvement of Bruguière, one of the greatest authorities of his time in zoology, made the discovery of earthworm luminescence pass directly into the French synopses of natural history and attracted the interest of colleagues. Bosc in his *Histoire naturelle des vers* spent only few words on the phenomenon, but had evidently gathered further evidence: "Earthworms are sometimes phosphorescent; but we ignore the cause of this condition, which is not due to mating, because it can be seen in summer, autumn and spring time" [8: p. 214]. Also Cloquet in the *Encyclopédie Méthodique* made a positive statement: "Earthworms are phosphorescent under certain circumstances, as I had the chance to see for myself more than once" [9: p. 555]. Vallot [10] too had witnessed the phenomenon and in fact suggested that the so-called "luminous urine" [11, 12] might be easily explained by the presence of luminous earthworms or centipedes on the spot where the urine was discharged [see also 13: p. 128].

Outside France, however, there was strong skepticism about the veracity of the above accounts. Macartney [14] stated that it was "next to impossible that the common earthworm should be endowed with so remarkable a property without every person having observed it". Morren [15: p. 24] appeared less conditioned by preconceptions, but was not ready to see earthworm luminescence as an inborn capacity.[2]

2. Specific records in megadriles

2.1. *Microscolex phosphoreus*

It was Anton Dugès [16], one of the pioneers of earthworm taxonomy, who first accorded specific rank to a luminous worm. Found in abundance in the hothouse of the Jardin des Plantes in Montpellier, his *Lumbricus phosphoreus* [= *Microscolex phosphoreus*] (1-3.5 by 0.1-0.2 cm, semi-transparent, red-blooded, with eight rows of chaetae and clitellum in XIII-XVI), emitted luminous fluid from the body surface, "a fluid undoubtedly similar to that released through the dorsal pores by many other earthworms".

[2] "Quum certis quibusdam circumstantiis submittuntur Lumbrici, phosphorescentes evadunt. Quum mihi talia videndi occasio non data fuit, huius proprietatis investigationi operam navare non potui. Quum extra tempus coitus locum habeat, non mihi videtur esse tribuenda cuidam actioni a natura generata".

The species was initially classified in *Lumbricus*, even though it differed from all known "lombrics" precisely by the lack of dorsal pores and by the anterior position of the clitellum. Between Dugès' description and the end of the XIX century, a number of new records identified as, or likely to be referable to, *L. phosphoreus*, occurred at this or that locality in France (from Toulouse and Pont-Saint-Esprit to Lille and Pas de Calais), always in association with artificial or disturbed habitats (greenhouses, private gardens, degraded lands) [13, 17-20].

At that time even Jean-Henri Fabre had *M. phosphoreus* in his courtyard in Avignon [21]. In his *Recherche de la phosphorescence de l'agaric de l'olivier*, Fabre [22] described the luminous material of this earthworm as a greasy fluid, which adhered to the fingers and left traces on everything it came in touch with. During physical and chemical laboratory experiments (the first ever perhaps on an earthworm), he saw the luminescence extinguish in the vacuum as well as in "unbreathable gases"(i.e. hydrogen, carbon dioxide, etc.), while it maintained unchanged brightness whether in aerated water, in ambient air or in pure oxygen. A letter to Léon Dufour dated 1857, cited in [22], documents that Fabre had ascertained that luminescence appears at birth and saw in it a process of oxidation, a sort of respiration, especially active in certain tissues.

In Germany and the British Isles references to this species were made much later. *Lumbricus phosphoreus* was first listed in a *Catalogue of the British Worms* [23] on the ground of vague reports from boglands of the south and west of Ireland [24] and from Liverpool [25]. But a second record from Liverpool [26], which I have never seen cited, may be confidently assigned to this species. It was communicated by the botanist W. Harrison to the President of the local Literary and Philosophical Society, the entomologist H.H. Higgins, who confirmed that an earthworm was involved, and not a centipede. That account appears today so insightful and suggestive of new perspectives, that it is worth being quoted at length: "On a gravel walk in a garden, at Walton, I frequently at night remarked the appearance of luminous particles, moving in various directions, and so numerous and bright as to remind me of the stars in the sky. It was soon apparent that the lights were produced by a very small worm, which varies from a quarter to three quarters of an inch in length;[3] being nothing more, so far as I could discern, than the young of the common earthworm. It was not the worm itself that was luminous, but something which appeared to exude from various parts of its body. On lifting up some of the worms with a pin, portions of the luminous matter occasionally fell to the ground, and broke into smaller pieces. Each

[3] i.e. 0.6-1.9 cm.

light lasted about ten minutes. It was interesting to observe the shining globules apparently running about the walk. On throwing the light of a lamp on any one of these moving pieces, I found that it was being conveyed away by a beetle, several species of which were thus engaged, and every one fought hard before it would allow me to rob it of its load. The thought struck me that the worms required to be wounded before they emitted light; accordingly, by the aid of a lamp, I found seven worms that were not luminous; and taking them one by one, I pricked them with a pin; the result was that each of them shone beautifully. The beetles then were feeding on the worms, and everyone they attacked produced light. The specimens I took continued to give out light after they were immersed in spirits. The gravel on the walk had been the ballast of a ship" [26].

The first German reference is by Friedrich von Stein [27] who, in a village near Potsdam, one night in September, observed that the damp, gravelly ground surrounding the fountain in a garden, was sprinkled with luminous spots. By scraping the gravel, the spots increased in number and then many small earthworms of "an undetermined species of *Lumbricus*" emerged. The slime secreted by the worms not only caused their whole body surface appearing luminous, but also produced luminous trails that glowed for a long time. Ten years later Matzdorff [28] reported on *L. phosphoreus* having established a dense population in a private garden in Berlin, possibly owing to a year-round reproduction and good spreading capacity. Luminescence, however, appeared to be spontaneous only in summer and autumn. The garden had been supplied with plants arrived from harbours in northern Germany. Indeed, *M. phosphoreus* (a member of Acanthodrilidae) was the first non-lumbricid megadrile described to science and would be soon recognized as an exotic species [18, 29], South American in origin [30, 31]. During his stay in Argentina, the mycologist Carlo Spegazzini collected it "among the roots of grass in all meadows" and described it as very conspicuous at night, "like a rubbed wax-match" [31, cited as *M. modestus*].[4]

Today *M. phosphoreus* is known as one of the most brightly luminous earthworms and one easily triggered to 'switch on' by the slightest irritation [33]. Its introduction in Europe by human agency (plant trade, ship ballast, etc.) during the last centuries sounds *a posteriori* as a logical explanation for the lack of published records in earlier times. However, since its accidental introduction, the species has shown in Europe an extraordinary invasive capacity (see distribution in [34]) and adaptability, as illustrated by these two

[4] For nomenclatural reasons, the genus name *Microscolex* Rosa has priority over *Photodrilus* Giard and the species name *Microscolex phosphoreus* (Dugès) has priority over *Microscolex modestus* Rosa [32].

references: (i) Skowron [35] so described his visit to one of the coal-mines situated near Cracow: "I observed in one passage not used for over two years, about 230 m below the surface, great quantities of *Microscolex* which in these special conditions of constant temperature and moisture had propagated very rapidly. Walking in darkness, hundreds of luminous points were seen, glowing brilliantly after every step. This species seems to need a higher temperature and that explains why it can live in mines, where it was occasionally introduced. Contrary to the individuals I have examined in Naples and others which were recently sent to me from Naples, the coal-mine forms do not show the day-night rhythm in luminescence, a fact probably connected with the constant darkness in which they are living". (ii) In central Hungary the species has been able to survive outdoor in the sandy soil of a garden down to winter temperatures of -20°C [36].

The question arises: were then all European cases in records referable to one and the same species, introduced from abroad and endowed with an exceptional ecological adaptability and spreading capacity? And what about those luminous earthworms described as having body sizes and pigmentations comparable to *L. terrestris* or other lumbricids (e.g. Flaugergues and Bruguière's records)?

2.2. Records in Lumbricidae

2.2.1. *Eisenia lucens*

In 1854 a group of naturalists from Warsaw led by zoologist Antoni Waga, conducted the first physiographic research of the Cracow-Częstochowa Jurassic Upland. They published their results in Polish (=*A Report From a Journey of Naturalists to Ojców in 1854*, Biblioteka Warszawska, 1855-1857), strongly emphasizing the distinctive character of the Jurassic Upland and its unique wildlife (nowaday, the Ojcow area alone is known to harbour 12% of the total animal species recorded from Poland [37]). Among Waga's discoveries was a new earthworm species, *Lumbricus lucens* [= *Eisenia lucens*], which lived in rotting logs and under the bark of standing dead firs, up to two ells[5] above the ground (Fig. 1). Waga [38] described the circumstances as follows: "One fine day in August, I stayed in the forest above the rocks until after dark, when I perceived that the earthworms which I was collecting in abundance from a stump were shining in my hand, and after being thrown in alcohol, lit the whole flask. Their light was not green, like that emitted in similar cases by electric centipedes and which also is transferred to the fingers, but it was white in colour, like that of wet decaying wood which sometimes becomes luminous and shines steadily,

[5] i.e. 115 cm.

but does not leave trails on contact. Earthworms held in a close hand shone brightly when I opened it, and the alcohol in the flask where I had thrown one or two of them lighted up wholly and maintained its shining for several seconds. Watching the phenomenon in daylight, I noticed that upon irritation the earthworms issued a milky liquid from the body, which at first whitened the alcohol, but afterwards coagulated and fell in clumps to the bottom of the vial, loosing the property of shining. [...] The size at maturity of the luminous earthworms is similar to that of the species named by Hoffmeister *Lumbricus agricola* [= *L. terrestris*] [...] Figure 5 in Hoffmeister [*Lumbricus olidus* = *Eisenia fetida*] gives an idea of its colour pattern".[6] The species, claimed Waga, was surely new, not only because of the peculiar habitat and the capacity to luminesce, but also because of its beautiful pigmentation pattern, i.e. an alternation of dark purple segmental stripes with colourless intersegmental areas; pigment was also lacking ventro-laterally below the dorsal chaetal lines.

Unfortunately, his Polish account was totally overlooked for more than a century and at least three different authors redescribed the same species as

Figure 1. The native European luminous earthworm, *Eisenia lucens*, as it appears in daylight in its natural habitat. (Photo courtesy J. Novák; source: www.biolib.cz).

[6] My translation from Polish.

new.[7] None of those authors, however, noticed the taxon's bioluminescence, which was rediscovered by Komárek [39] in Czech specimens (identified as *Eisenia submontana*; see Section 4). Komárek described the light production as a latent, somehow pathological, property, only elicited in response to a strong (mechanical or chemical) stimulation, which apparently contrasted with Waga's [38] observation in the field.[8] An explanation of the lesser readiness to luminesce in the laboratory can be found in Plisko's [42] words: "I found that specimens of this species always exert the capacity of shining if they do not stay too long in culture. After staying very long in culture, maintained on earth compost and leaf cuttings of lowland forest, specimens give off a fluid with considerably lower capacity of shining, and in some cases (over six months in culture) shining is not observed even in alcohol 75%".

Nowadays this quite large-sized worm (10-18 by 0.5-0.6 cm) is known to occur throughout central Europe, from Austria to Ukraine and from Poland to the Balkan Peninsula, and also in the Pyrenées, but is mostly confined to natural forests, where it plays an important role as decomposer of organic matter [43].

2.2.2. Problematic records: *Eisenia fetida* and other Lumbricidae

Historical records of bioluminescence involving lumbricid earthworms associated with agricultural/rural environments and particularly with manure heaps remain difficult to interpret. Phipson [13] recalled that in his childhood in England, whilst digging at night in a large dunghill for fish-baits, he and his schoolfellows had "turned up many hundred lumbrics in a highly luminous condition". Cohn [44] reported the observations by an apothecary in Kruszwica (central Poland) of many small luminous earthworms found amid soil and potato debris in a cellar where potatoes had been stored for a few months. Their light was bluish white, brighter than phosphoric light and continuous, not sparkling. It appeared over one third of the body length, but only upon irritation: i.e. any time the potatoes were shovelled, the soil was rubbed, or the animals were rolled between two glass slides. The light emitted *in vivo* lasted two hours, whereas the secreted slime shone for only a minute; the light ceased with the animal's death. Cohn had received only fragments of the worms, 2.5-5 cm long, and attempted in vain a spectral

[7] From the Krknose mountains, northeastern Czech Republic, as *Lumbricus submontanus* Vejdovský, 1875 [=*Eisenia submontana*]. From the Herculane Spa, by Mehadia, southwestern Romania, as *Allolobophora tigrina* Rosa, 1893. From down to 300 m depth in St. Canzian cave (= Škocjanske jame), Slovenia, as *Helodrilus (Allolobophora) latens* Cognetti, 1902.

[8] In later studies Komárek admitted that light could appear as soon as the coelomic fluid was exposed to air or pure water [40, 41].

analysis; through the authoritative assistance of Adolph Eduard Grube, he identified the material as probably belonging to *Lumbricus olidus* [=*Eisenia fetida*] or *L. tetragonus* [=*Eiseniella tetraedra*].

Franz Vejdovský [4] had his own experience in Prague: while searching through a manure-heap at night, he noticed several luminous spots of a soft bluish white colour, which changed their position, now disappearing, now re-appearing in more places. He therefore removed part of the manure from the spot in question and the light appeared again and brighter and shone for a longer time, whereas in other places it had disappeared. Using a lantern, he found many specimens of *Allolobophora foetida* [=*Eisenia fetida*] which he stored in a vial for further study. To his surprise, his fingers too were shining in the dark and especially the parts which had been in contact with the worms. Vejdovský erroneously concluded that it was the slime secreted by the epidermal glands of the worm that caused the luminescence.

In 1919 Friend [45] listed *Octolasium* among the luminous earthworms of the British Isles and commented: "I believe that the yellow extremities of *Octolasium* serve the purpose of dazzling underground foes by emitting light". This remains a rather mysterious record, because no reference was given and the taxon was not mentioned in the chapter on luminosity of the author's book [46] on British Annelids. In that book, however, Friend quoted from one of his correspondents: "I have seen earthworms luminous on many occasions between Woolston Moor and Sampford Brett, in West Somerset. The soil there is red marl, and in the district are red sandstone quarries. I used to search for these worms, as they were particularly wanted for fishing, and used to find them under big stones or slates in damp places. The worms were not so big as the ordinary garden worm, but were of a much redder colour. The luminosity was not so bright as that shown by the luminous centipede, but it had the same property of being seen in the ground after the worm had left it. Sometimes I have found four or five under one stone, and then the combined light was very apparent. I have never seen them in any other locality".

Very few earthworms in the world are physiologically equipped to exploit a dunghill environment, and among lumbricids in Europe *Eisenia fetida* and *E. andrei* are the species most likely involved in such circumstances, but the question remains whether the observed luminescence was self-luminosity or due to contact or infection with luminous bacteria or fungi. The first explanation is problematic (e.g. no instance of bioluminescence has ever been reported by the many modern vermicomposters using these species), but the possibility exists that the biochemical endowment of coelomocytes could vary from population to population (see Section 7).

2.3. Records in the Southern Hemisphere and Asia and first histological studies

2.3.1. New Zealand: *Octochaetus* (Fam. Octochaetidae)

European oligochaetologists of the XIX century had limited themselves to describe the phenomenology of luminescence without investigating the precise seat of light production. This had left space for uncertainty and alternative explanations as to the cause and origin of the light [e.g. 32].

Soon after his arrival in New Zealand, William B. Benham caught the opportunity to study microscopically the unusual properties of a local earthworm [9] and quickly published his preliminary observations: "Our large white earthworm (*Octochaetus multiporus*) has a milk-coloured coelomic fluid of very great tenacity; it can be drawn out into strands, and soon hardens on exposure to air. In the dark, when the worm is handled [...] the fluid is brilliantly phosphorescent when freshly discharged, and the fluid sticks to one's fingers very persistently; but it soon looses its phosphorescence. The fluid contains numbers of elaeocytes [... that] are colourless, not yellow, [...and cells] containing a threadlike structure [...] I am now endeavouring to locate the phosphorescence – that is, to ascertain which of these two cells is the seat of the phenomenon" [48].

Two years later Benham [49] added new details of the species' morphology and behaviour: "The worm is pale, almost white, owing to the absence of pigment in the body wall, which allows the opaque white fluid contents of the coelom to show through. The worm is curiously sluggish and inert; if one be taken in the hand it makes no attempt to wriggle out of it, but, by contraction of the longitudinal muscles the worm shortens itself, and at the same time the circular muscles are contracted, so that it becomes quite tense and firm to the touch. [...] When handled roughly a small amount of coelomic fluid issues from the dorsal pores [...] when placed in alcohol or vapour of acetic acid the discharge is abundant and a similar fluid is copiously discharged from the mouth. This fluid is opaque-white, resembling cream in appearance, with a consistency recalling gum-mucilage or clotted cream. It does not flow over the body surface but spreads slowly over it. [...]*Octochaetus multiporus* and *O. antarcticus* are highly photogenic or phosphorescent, and when handled in the dark it is at once seen that this light has its seat in the coelomic fluid as it issues from the dorsal pores and slowly spreads over the surface of the worm. The effect is much more brilliant if the worm be stimulated by a little vapour of acetic acid; then the abundantly

[9] *Octochaetus multiporus* measures up to 30 by 1 cm. According to Miller [47], small luminous earthworms are also known to the Maori communities, under the names 'piritaua' or 'titiwai'.

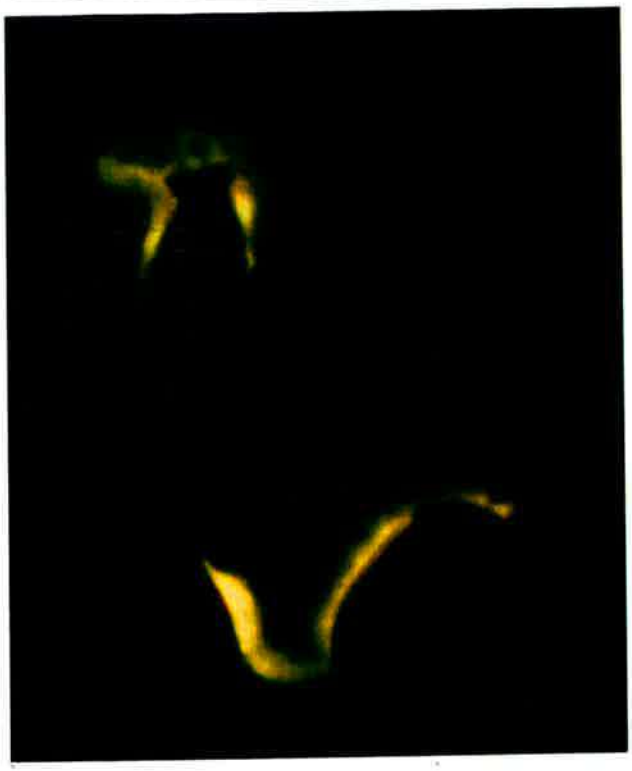


Figure 2. The large New Zealand earthworm, *Octochaetus multiporus*, giving out glowing coelomic fluid in the dark. (Photograph taken with the worm's own light. Courtesy V.B. Meyer-Rochow [118]).

discharged fluid gleams with considerable brilliance. [...] The eleocytes are large rounded cells crowded with colourless, highly refringent oily globules. [...] In the eleocytes of the fluid, it seems to me, we have just the very conditions for the emission of light [i.e. "metabolism and rapid oxidation of fat"], and we need not summon bacteria to their aid. As a matter of fact, I have seen no bacteria in this photogenic fluid". Further observations and experiments on this species and its spontaneous level of emission (Fig. 2), very bright and easily seen by the non-dark-adapted eye, would be conducted by [50, 51] (see Section 5).

2.3.2. South Africa: *Parachilota* (Fam. Acanthodrilidae)

The luminescence of South African earthworms was investigated for the first time by John D.F. Gilchrist [52]. Several sightings had been reported to him in the Cape Peninsula about luminous earthworms crawling up on damp nights and leaving trails or patches of luminous material. Gilchrist examined live specimens of "*Chilota* sp." (probably *Parachilota bergvleitanus*; [53]) collected on the slopes of Table Mountain. In the field, when first dug up, this worm usually assumed a rigid (death-feigning?) attitude, but after a while it began to move very lively and, by a series of strong flexures, scattered

masses of luminous substances to some distance in all directions. By placing the animals in contact with photographic plates in the dark, Gilchrist documented how the species, devoid of dorsal pores like *M. phosphoreus*, was able to give off its luminous coelomic fluid from the mouth and anus and spread it from there over the body. Amazingly, in *Parachilota* the fluid was sometimes ejected with considerable force, also on the clothes of the observer.

In daylight, if shaded by the hand, the fluid appeared of a greenish colour and luminous. Under the microscope, among the various types of cells floating in the fluid, the largest and most numerous ones were laden with liquid greenish inclusions. These cells were interpreted by Gilchrist as chloragogen cells containing fatty inclusions, suitable for undergoing oxidation with light production. Examined microscopically in a dark room by the aid of its own light, the fluid glowed uniformly without any differentiation, because the light was produced during the process of the breaking up of the cells and the scattering of their contents. However, by placing water in contact with some dried-up fluid on a slide, it was possible to establish a correspondence between the luminous particles and some of the discharged granules of the cells. Thus Benham's hypothesis [48, 49] of certain coelomocytes to be photogenic in some megadriles was confirmed. Gilchrist [52] sought evidence of a luciferin-luciferase system but (probably due to impurity of the preparations) obtained negative results.

2.3.3. Myanmar, India and far East: *Eutyphoeus* and *Ramiella* (Fam. Octochaetidae), *Lampito* and *Pontodrilus* (Fam. Megascolecidae)

Gates [54] organized a round-up of Rangoon earthworms, gathering a total of seventeen species to test for luminescence. Four species [three belonging to the genus *Eutyphoeus* and one to *Lampito*] gave positive results but to varying degree, and only upon violent mechanical or chemical stimulation (a dilute ammonia solution). The light was not produced immediately on the discharge of the fluid but appeared only after the lapse of a short interval. The most powerful light was exhibited by *Eutyphoeus peguanus*, a species with unpigmented, transparent body wall, as is the case in *M. phosphoreus* and *O. multiporus*. Later investigations by Gates [55] in Allahabad confirmed the capacity to luminesce (upon chemical stimulation) to be widespread in *Eutyphoeus* and also to characterize *Ramiella nainiana*. The luminescence of the marine littoral genus *Pontodrilus* was first recorded near Yokohama, Japan (*Pontodrilus matsushimensis*; [56]), but found later to be widespread in the genus in tropical countries of Asia [57]. Microscopic examination did not reveal any luminous bacteria. On blotting paper the luminosity of the mucus faded out after a few minutes while the paper was

drying, but dropping water on the blotting paper revived the luminosity. The colour of the light of luminous mucus on the blotting paper was pale blue, which changed to yellowish when the paper was rubbed. The intensity of luminosity was also increased by rubbing [57].

The luminescence of Australian earthworms belonging to four genera (*Diplotrema*, Fam. Acanthodrilidae; *Digaster*, *Spenceriella* and *Fletcherodrilus*, Fam. Megascolecidae) was later demonstrated and investigated by Barrie G.M. Jamieson and John E. Wampler [51, 58, 59] (see Section 5).

3. Early records in Enchytraeidae

Enchytraeidae, also called 'potworms' or 'white worms' are small oligochaetes (microdriles) found in terrestrial, freshwater and marine habitats in all regions of the world. Their bodies can measure between 1-170 mm in length and between 0.1-2.9 mm in diameter [60], but mainly range between 5-25 by 0.2-2 mm. Because of their transparent teguments and the gut contents matching in colour the background substrate, they are generally difficult to see in the field, even in daylight. The earliest case of bioluminescence assignable to this family dates back to 1838, when Eduard A. Eversmann [61], professor of zoology and botany at Kazan University in Russia, was informed by his rector, Nikolai Lobachevsky,[10] that many luminous worms were crawling about in his *Agapanthus* flowerpot. Having removed the plant from the pot, they saw that "the worms were so numerous that [in the dark] the soil appeared to be mingled with fire. [...] Their whole bodies shone with a light as strong as that of a firefly. Light emission occurred both in the dry and when the worms were plunged into water".

Eversmann [61] described the animals as 8.5-17 by 0.5-0.7 mm large (Fig. 3a), comprising 50-60 segments, each bearing laterally a short 'spine-like' and thin 'hair-like' chaetae; he named the species *Lumbricus noctilucus*.[11] Owsiannikow [63] also observed luminous worms in Kazan, and believed to recognise in them and in Eversmann's description the species *Enchytraeus albidus* Henle, 1837 (one of the very few terrestrial enchytraeids named at the time). He wrote: "Its light is very weak. It is not associated with a specific organ but flickers here and there, now coming from the head, now from the tail, now from the whole body. Sometimes a faint blue glow remains on one's fingers upon handling the worm". In 1887 Harker [64] reported on "a remarkable phenomenon of luminosity exhibited on a large scale on a peaty

[10] One of the father of non-Euclidean geometry.

[11] A taxon placed by Michaelsen [62] among the *Genera dubia et species dubiae Enchytraeidarum*.

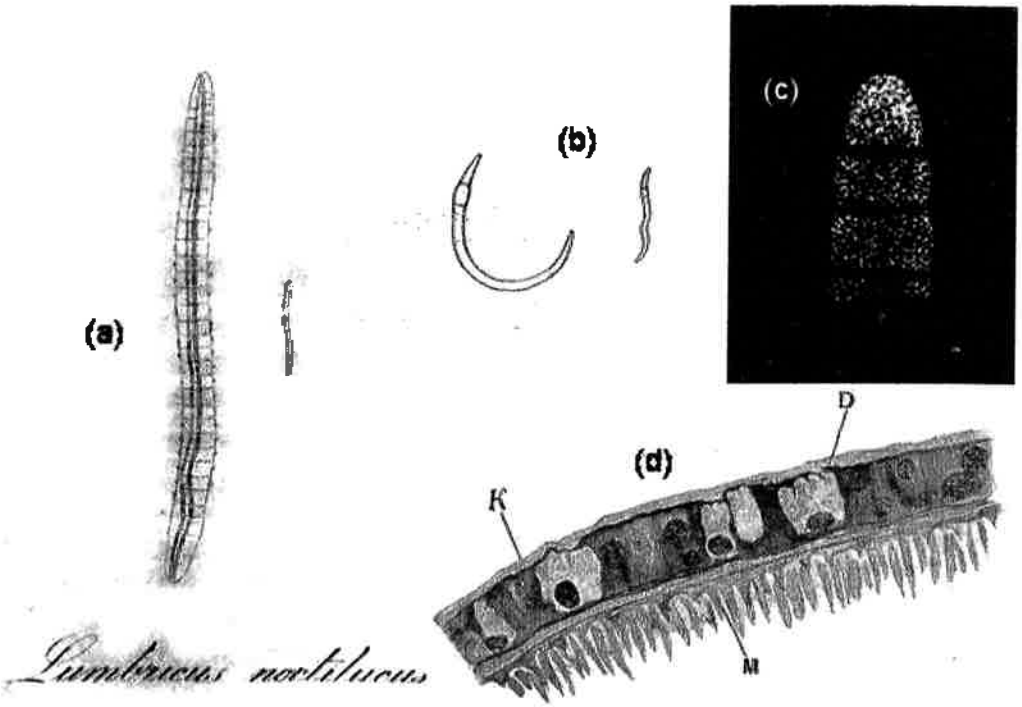


Figure 3. Old illustrations of luminous enchytraeids: (a) *Lumbricus noctilucus*, original drawing by Eversmann [61]. (b)-(d) The putative *Henlea ventriculosa* studied by Walter [66]. (b) Body size and shape (right) relative to *Microscolex phosphoreus* (left). (c) Anterior body end, self-illuminated. (d) Cross section of the body wall.

moor in Northumberland [England] at an elevation of 600 feet. The imprint of recent footmarks on the peaty ground shone with a brilliance recalling similar effects on sea-shores [...], while the feet of the horses of a riding party galloping across the wet peaty soil threw off the luminous mud in what appeared to be showers of white glowing fire. [...] Innumerable small worms [...] were proved to be the producers of the luminosity. In a darkened room a single worm on being gently rubbed glowed like a fine streak of phosphorus. The worm is a small *Enchytraeus*". By 1905, *E. albidus* was considered "a typical luminescent oligochaete" and seen as an example of extracellular light coming from the secretion of a weakly luminescent slime [65], even though the organ producing the slime was not specified.

The luminescence of *Henlea ventriculosa* (specimens identified by Wilhelm Michaelsen) was noticed simultaneously in two different localities of European Russia, i.e. Kaluga district and the city of Perm [66]. In Kaluga the worms lived under leaf litter in an old garden by a linden alley; they were not immediately visible, but by scratching lines on the ground with a stick, one could see many luminous stripes appear, each formed by separate lights (the worms were not on the soil surface). For its intensity and blue-green colour, the light resembled that of *Lampyris* [=*Phausis*] *splendidula*. Light

production by each worm was subject to fluctuations and always appeared more intense at the body ends. Under the microscope, the light was seen to come from a host of luminous dots scattered all over the body surface, but shining more intensely on head and tail (Fig. 3c). The worms showed little light when at rest, but if disturbed mechanically, thermally or chemically (water, salt solutions, weak acids, ammonium compounds), they had a brilliant response accompanied by discharge of luminous slime. The exuded slime, which Walter [66] speculated might be secreted by epidermal gland cells (Fig. 3d), glowed uniformly and steadily for tens of seconds. Issatschenko [67], who had demonstrated infection of luminous midges by *Photobacterium*, examined Walter's worms, but was unable to isolate or grow luminous bacteria from them, "although to judge by all characteristics the light of *Henlea ventriculosa* likewise proceeded from microorganisms". According to Walter [66], Issatschenko had seen that light emission occurred in dead worms after nine days in a sterile broth.

Walter [66] raised the question of the luminescence of *E. albidus*. He had himself been unable to observe any light in this taxon and correctly remarked that *Enchytraeus albidus* was "one of the synonyms of *H. ventriculosa*", intending that many terrestrial enchytraeids, including *Enchytraeus* [=*Henlea*] *ventriculosus*, were for a long time confused under the name *E. albidus* (see [62]).[12] *Enchytraeus ventriculosus* was described as a separate species in 1854 but some confusion continued till the end of the century (e.g. [68]). Thus the properties stated by Owsiannikow [63] and held as a typical case of extracellular luminescence by Pütter [65], could indeed refer to enchytraeids misclassified as *E. albidus*.

Henlea ventriculosa is a common, widespread species and Walter [66] justified the fact that nobody had noticed before its luminescence with its dwelling below the soil surface; however, there have been no further instances of luminescence in the species since his report. Friend [45] referred of *Henlea nasuta*, [13] and not of *H. ventriculosa*, as being luminous. As with the lumbricid genus *Octolasium* (see Section 2.2.2 above), it is not clear whether this was a lapsus or an emended identification. After these early reports, no further study of luminous Enchytraeidae was made, until the description of *Fridericia heliota* [69] (see Section 6).

4. From bacterial to self-luminosity

In Italy Paolo Panceri, who had investigated the luminescence of many species of polychaetes [70, 71], dismissed earthworm luminescence as

[12] And indeed sometimes still are.
[13] Also called *Michaelseniella nasuta*.

accidental and caused by feeding or some other factors. According to Beddard and other workers [32, 72-75] the production of light in earthworms could indeed, at least in some cases (e.g. *Eisenia fetida*), be attributed to infection by luminous microorganisms: photogenic bacteria entangled in the slime upon the skin or fungi occasionally met with in humid soils and ingested. However, "the regularity, and the mode of excitation, of the luminosity seemed to show that *Microscolex* is phosphorescent in its own right" [32].[14]

Dubois [76] and Linsbauer [77] were unable to show in the luminous slime of earthworms any luminous microorganisms. The same had done Issatschenko [67] for enchytraeids, and Gilchrist [52] had investigated very carefully the phenomenon in his South African earthworms and pointed to the self-luminosity. Nevertheless Umberto Pierantoni [78-80] saw in the phenomenon the possibility of further evidence for his "hereditary symbiosis theory", being convinced that *M. phosphoreus* owed its luminescence to intracellular symbiotic bacteria. These would correspond to the granules and rods visible in the coelomic cells, which Pierantoni believed to be capable of passing through the connective and muscular layers of the body wall into the lumen of the epidermal gland cells, to be then secreted with the epidermal mucus. Growth of the 'bacteroids' in an artificial medium, however, was not successful.

It was against this background that Stanislaw Skowron [35, 81] conducted his scrupulous investigations on *Microscolex phosphoreus*, which partly confirmed and partly improved the insightful results obtained by Gilchrist [52] on *Parachilota.*[15] All the *M. phosphoreus* specimens examined, both from Italy and Poland, were luminescent and with characteristics, which did not agree with the properties of bacterial light. The luminous slime, laden with granular cells, was seen to originate in the coelomic cavity, pass into the rectal and buccal regions through preformed openings at the gut extremities, and then exit from the anus and mouth; the epidermal glands and the nephridia did not take part in this secretion. The luminous material consisted of small, greenish, highly refractile granules which usually began to glow after the slime had been discharged and the granules were liberated from the cells. If the animals were dying, however, they became luminous within the body cavity,[16] perhaps because the cells break up inside the body cavity. The

[14] Luminous bacteria glow continuously, the luminescence being quite independent of stimulation.

[15] The two genera are phylogenetically close.

[16] Pierantoni had postulated both an "external" and an "internal" luminescence of *M. phosphoreus*.

disruption of the cells was essential to obtain the luminescence. The fact that the granules had to dissolve to become luminous disproved entirely their identity as infecting luminous bacteria.

Skowron [81] also posed the question of the regulation of luminescence: "The slime continues to glow for about half an hour if kept moist, while on the contrary the light of the extract [from ground worms] dies quickly, and on addition of water, immediately"; this fact was seen as if some enzymes liberated from the cells by grinding had an inhibiting effect upon the luminous material (cf. Section 5). In 1928 he [35] suggested the hypothesis that the different system components may be segregated by membranes: "I am not quite certain what prevents the luminescence of the granules inside the body of the worm [...] Every granule is composed of two substances, which are both necessary for luminescence. We may suppose that these two substances represent luciferin and luciferase separated from each other by a film [...] When the cell breaks up and water is absorbed, [...] the film may be destroyed and the fluid mixture glows till the whole material is used" (cf. [82] for *Diplocardia*). Edmund Newton Harvey [83] showed that worms shaken in pure hydrogen gave no luminescence, but produced a slime which luminesced immediately when air was admitted (cf. [22] in Section 2.1). Harvey [84], however, failed to demonstrate a luciferin-luciferase reaction in *M. phosphoreus*,[17] nor did he obtain a cross-reaction with *Cypridina* luciferin or luciferase.

On rediscovering the bioluminescence of the lumbricid *Eisenia lucens*, [18] Julius Komárek [39] demonstrated that also in this species the source of light was not bacteria but refractile granules contained in the lymphocytes (=coelomocytes). These appeared identical to those of *E. fetida*, but the latter normally does not luminesce, either because of a different chemical composition of the granules or the lack of some components of the oxydative system. The granules had the appearance of yellow fatty globules but showed negative reactions to osmic acid and stained faint pink with Sudan [40]. However, the luminescence reaction definitely implied the oxidation of a thermostable luciferin mediated by a thermolabile luciferase. Considering that the coelomocytes in *Eisenia* and many other lumbricids are rich in riboflavin and fluoresce yellow-green, but that in *E. lucens* they turn to blue fluorescence after bioluminescence has ceased, Komárek and coworkers

[17] Harvey would later explain his negative results (with *Microscolex* and other organisms) hypothesizing the destruction of luciferin or luciferase during preparation of extracts, or the loss of other accessory factors necessary for light production.

[18] The synonymy of *E. submontana* with *E. lucens* was established by Plisko in 1961 [85].

postulated riboflavin as playing the role of luciferase in the luminous species and being changed into lumiflavin during the luminescence reaction [41].

5. North-American studies on *Diplocardia* unravel the chemistry of earthworm bioluminescence

After the 1960s, advances in chemical and physical knowledge of bioluminescence systems became pursuable thanks to new, more powerful instruments. The 1966 Proceedings of the Luminescence Conference held in Kanagawa in Japan hosted contributions from the majority of the world's leading scientists active in the field at the time. Two papers touched upon luminous earthworms. Frank H. Johnson and coworkers [50] attempted to develop what had been achieved over the past three decades, and found that, as in the case of *Eisenia lucens*, *Octochaetus multiporus* fluoresces under UV with the same colour of bioluminescence (orange-yellow in this species; Fig. 2), but changes to blue as luminescence ceases (i.e. some product of the luminescence reaction affects the colour of fluorescence). Placed on dry ice, live specimens exuded copious amounts of luminous fluid which continued to luminesce for a while after it was frozen. Fragments of the frozen worms and the frozen exudate itself began again to luminesce on thawing. The frozen exudate retained a capacity for luminescence after more than a year in dry ice, and remained active when dehydrated in cold acetone, stored in a desiccator, ground up in a mortar, and having water added to it. The luminescence system appeared to fit a luciferin-luciferase reaction requiring O_2. Eight different types of cofactors were assessed, including those functioning in photobacteria, fireflies, etc. but none was found to be active in the *Octochaetus* luminescence reaction.

The second paper, by Milton Cormier and coworkers [86], introduced the native North-American species, *Diplocardia longa* (up to 60 cm long, 1 cm wide; Fam. Acanthodrilidae), as an example of the peroxidase type of bioluminescence, that is, stimulated by H_2O_2, with luciferase strongly inhibited by KCN. This paper was a real kick-start to the unravelling of earthworm bioluminescence. Bellisario and Cormier [87] and Bellisario et al. [88] went into more detail: *Diplocardia* displays a classical luciferin-luciferase reaction, but requires H_2O_2 instead of molecular oxygen. The dominant type of coelomic cells, about 40-50 μm in diameter and filled with granules, are the site of both luciferin and luciferase activity: only 1% of the luciferin or luciferase activity remained in worms that had been depleted of their coelomic fluid, and by decanting the exuded coelomic fluid, nearly all the luminescent activity was found in the sediment of the coelomic cells, with less than 2% remaining in the supernatant. A negligible amount of light emission occurred before the cells

were osmotically lysed with distilled water or mechanically disintegrated. Luciferase made up about 5% of the total extractable protein from coelomic cells. *Diplocardia* coelomocytes are not fluorescent.

The purified *Diplocardia* luciferase is relatively unstable even at cool temperatures, but is somewhat stabilized when stored frozen at -80°C. It was identified as a 300 kDa protein, highly asymmetrical and consisting of three pairs of non identical subunits. The enzyme does not contain a heme or a flavin group but copper, and it is not itself a peroxidase, even though horseradish peroxidase does react with *Diplocardia* luciferin to produce light. Organic peroxides do not replace H_2O_2 in the light reaction, and H_2O_2 has a destructive effect on luciferase, so that the total light emitted is proportional to the luciferase, not the luciferin, concentration. *In vitro* the presence of O_2 inhibits light emission. *In vivo* H_2O_2 must not be present in the coelomic cells until they lyse in the presence of O_2. Thus it was postulated that the crude coelomic fluid contains an oxidase, which acts as a peroxid-generating system, which also explains the oxygen requirement *in vivo* of other worms (*Microscolex*, *Octochaetus, Parachilota*, etc.) [87, 88].

A few years later, with the contribution of John E. Wampler, *Diplocardia* luciferin was purified, identified, and synthesized [89]. It turned out to be a simple aldehyde, N-isovaleryl-3-aminopropanal, with an amide functional group. At room temperature this compound behaves like a clear, odourless, non-volatile oil, and exhibits no near-UV-visible absorption or fluorescence. The *in vitro* luminescence with partially purified luciferin and luciferase produced an emission spectrum (λ_{max} 503 nm) similar to the *in vivo* luminescence from freshly exuded slime (λ_{max} 507 nm) [88], but shifted to λ_{max} 490 nm when a pure sample of luciferin was used [89].

Subsequently Jamieson and Wampler [51, 59] conducted comparative studies testing the cross reactivity of the various components of *Diplocardia* system on other bioluminescent earthworms, and found that N-isovaleryl-3-aminopropanal, or a close analogue of it, is the common substrate for bioluminescence in 13 earthworm species belonging to six genera from the southern US (3 spp. of *Diplocardia* and *Pontodrilus bermudensis*), eastern Australia (*Diplotrema*, Fam. Acanthodrilidae; 4 spp. of *Spenceriella,* 2 spp. of *Fletcherodrilus*, Fam. Megascolecidae; and *Pontodrilus*) and New Zealand (*Octochaetus*). These species have emission spectra with λ_{max} ranging from 500 nm (*Diplocardia*, blue-green) to greater than 570 nm (*Octochaetus multiporus*, orange-yellow) (Table 1). Non-luminescent congeneric species differed in having coelomocytes that are less numerous, more finely granular, or less granular with more internal structures. The *Diplocardia* luciferase was shown to be quite specific, being active with only a few simple analogues of luciferin and none of the straight chain aldehydes [51, 59].

Table 1. Characteristics of bioluminescence in the Oligochaeta.

Species*	Spontaneous light emission log (photons/sec)	λ max (nm)	Site of light production (mean diameter in μm)	Components of bioluminescence system	Cross reactivity with *Diplocardia longa* system components
MEGADRILI					
Superfam. MEGASCOLECOIDEA					
Fam. ACANTHODRILIDAE					
Diplocardia alba	9-11	501	coelomocytes (31)	luciferin/luciferase/H_2O_2	+
D. eigeni	9	505	coelomocytes (18)	id.	+
D. longa	12	500	coelomocytes (30)	id.	+
Diplotrema heteropora	10	545	coelomocytes	id.	+
Microscolex phosphoreus	10	538	coelomocytes (27)	id.	+
Fam. OCTOCHAETIDAE					
Octochaetus multiporus	?	>570	coelomocytes	id.	+
Fam. MEGASCOLECIDAE					
Fletcherodrilus fasciatus	7	?	coelomocytes (23)	id.	+
F. unicus	8	?			+
Pontodrilus bermudensis	9	540	coelomocytes (14.4)	id.	+
Spenceriella cormieri	11	?	coelomocytes (26)	id.	+
S. curtisi	8-9	535	coelomocytes (24)	id.	+
S. minor	9-10	531	coelomocytes	id.	+
S. noctiluca	9	?	coelomocytes (18)	id.	+
Superfam. LUMBRICOIDEA					
Fam. LUMBRICIDAE					
Eisenia lucens	?	550	coelomocytes	?	?
MICRODRILI					
Fam. ENCHYTRAEIDAE					
Henlea sp.	?	464	coelomocytes ?	luciferin/luciferase/Ca^{2+}	no
Fridericia heliota	?	478	body wall	luciferin/luciferase/Mg^{2+}/ATP	no

*Bioluminescent species for which none of the listed characteristics have been investigated are omitted.

Wampler and Jamieson [51] also established that cross reactions with *Diplocardia* luciferin do not cause changes in the specific spectral distribution of bioluminescence (uncorrected spectrum). Instead, cross reactions with *Diplocardia* luciferase lead to an emission spectrum shifted toward the colour of *Diplocardia*. Thus, luciferase or other solute components control the position of the emission spectrum. The fact that the various taxa show different spectra possibly implies energy transfer to various fluorescent acceptors for subsequent emission.

Rudie et al. [90] again focused on *Diplocardia* and confirmed that luciferase contains firmly bound, but EPR-silent copper as a functional part. Luciferase was found to contain carbohydrate (6%), lipid (2%), copper (up to 4g-atom per mole), and an unusually high content of proline (5.3%) and hydroxyproline (5.8%). By protecting the enzyme from denaturation by H_2O_2, turnover could be demonstrated. The light yield and kinetics of luminescence *in vitro* varied with the order of the addition of components. The light yield and initial rate were highest when luciferin and H_2O_2 had been preincubated and the reaction was initiated by the injection of luciferase. This demonstrates that the true substrate of bioluminescence is a luciferin peroxide adduct: 3-(isovalerylamino)-1-hydroxypropane hydroperoxide, which is decomposed in a light-emitting reaction catalysed by the copper-containing *Diplocardia* luciferase (Fig. 4).

A following paper by Wampler [91] reinvestigated with modern techniques the bioluminescence of *Microscolex phosphoreus*. The results did not confirm statements made by Bersis [92], but agreed with observations by Skowron [35, 81]: this species glows brightly, with no sign of periodic variations,

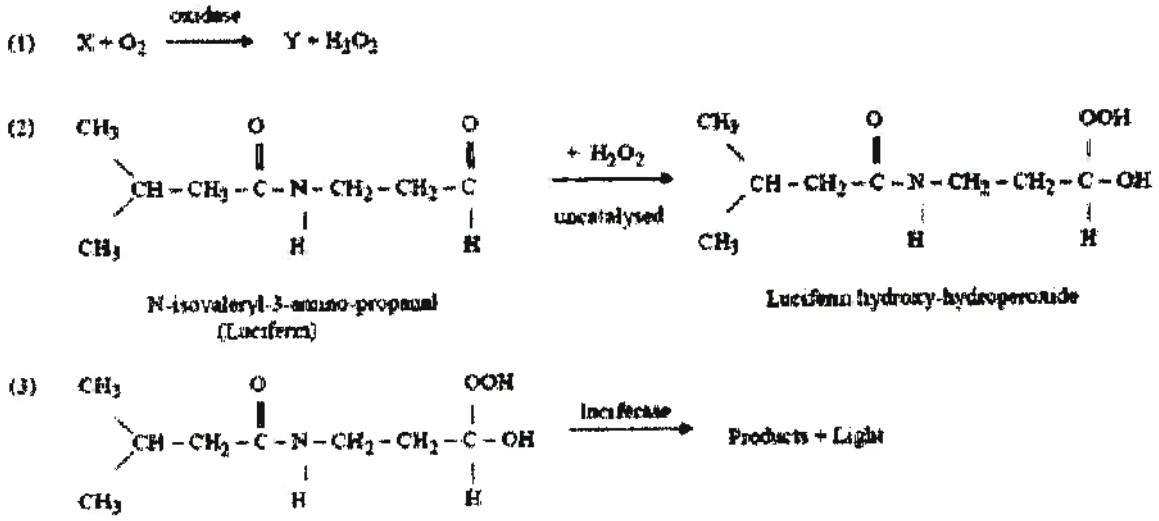


Figure 4. Scheme of the overall bioluminescence reaction in the earthworm *Diplocardia longa*. (Adapted from [103]).

but with random, fairly sudden increases due to movements of the worm (shearing of granules). Maximal emission occurs at 538 nm (yellow-green). Luminescence comes from granule-filled coelomic cells, not from epidermal cells, and these coelomic cells are fluorescent, with a chromophore [=fluorophore] having an emission spectrum nearly identical to the bioluminescence spectrum, but which is drastically altered with the disruption of cells and solubilization of the luminescence system.

Lastly, Wampler and Jamieson [93] reinvestigated the luminous system of *Pontodrilus bermudensis*, and found it very similar to that of the other megadriles. The involved coelomocytes are smaller (14.4 μm) but of the same kind as those previously studied. Luciferin is sequestered in a subcellular component, which is little affected by osmotic shock, to the point that it was formerly thought that the system was not cell bounded. As in *M. phosphoreus*, the bioluminescent cells are fluorescent and match the bioluminescent spectrum (λ_{max} 540 nm, yellow-green). The fluorophore is not associated with luciferin, whose heterospecific origin does not alter the emission spectrum, but with luciferase. In *Microscolex* this association is loose, and after cell lysis the emission spectrum shifts to reflect the primary excited state species with a spectrum near to that of *D. longa*. In *Pontodrilus* the association with luciferase is stronger and the emission spectrum does not change with cell lysis.

6. Siberian studies disclose the components of bioluminescence in Enchytraeidae

After the early reports summarized in section 3, no further study of luminous Enchytraeidae was made for nearly a century. Not surprisingly, reviewers dealing with oligochaete bioluminescence regarded the evidence in this family as uncertain [94] or too inadequate: there was no mention of these worms in Herring's [95] revised list of luminous organisms. In 1990 Zalesskaja et al. [69] published a note in Russian announcing the discovery (by Valentin Petushkov) of a new luminous enchytraeid, *Fridericia heliota*, from the taiga near Krasnoyarsk, east Siberia (Fig. 5). Only a brief taxonomic description and few details of the bioluminescence were provided, pointing out, however, that, unexpected for an oligochaete, the new species upon stimulation emitted a continuous, bright glow not involving discharge of luminous slime. The luminosity seemed to be confined to the body wall and its pattern seemed to correspond to that of the epidermal gland cells. Zalesskaja et al. [69] also mentioned that in the same forest soil, in addition to *F. heliota*, lived another luminous species, preliminarly identified as *Fridericia ratzeli*, whose luminescence showed different properties: tactile

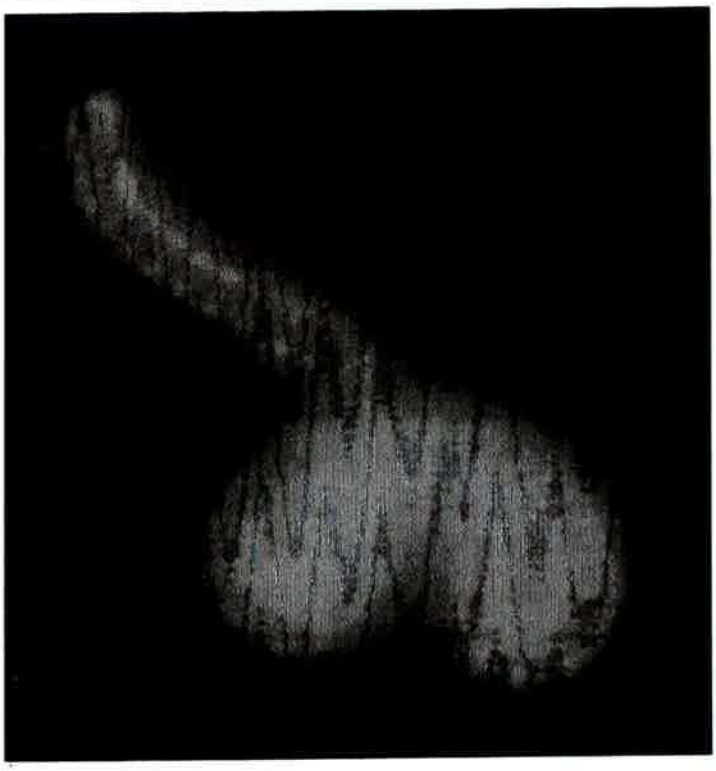


Figure 5. The glowing enchytraeid *Fridericia heliota* Zalesskaja, 1990. (Photograph taken by direct contact printing worm-to-film through thin polyethylene film in the dark room. Courtesy V. Petushkov).

stimulation produced only a weak luminosity whereas electrical stimulation elicited short flashes of light (5 min^{-1}).

In 2001, I gladly accepted the invitation from the Siberian team [V. Petushkov and N. Rodionova] to verify the validity of *F. heliota* as a distinct enchytraeid species and received towards this aim a number of live specimens in Italy for microscopical examination. That study [33] not only yielded an expanded morphological description of *F. heliota* (partly amending the original diagnosis) and more comprehensive taxonomic and physiological considerations (see below), but also stimulated my approach to the historical study of bioluminescence in the oligochaetes. Among the live material sent to me from Krasnoyarsk, some specimens belonged to the second luminous taxon, the putative *F. ratzeli*, whose identification I helped to correct in *Henlea*; those worms indeed produced a weaker, bluish light, and some quick flashes accompanied by slime discharge at the body ends (see Section 7.1). A taxonomic description of this *Henlea* species, which is different from *H. ventriculosa* or *H. nasuta*, although the type of bioluminescence recalls that ascribed to *Henlea* by Walter [66], is under way. The general lighting behaviour of *F. heliota* was summarized in [33] as follows: There is no difference between the adults and the juveniles, even the

earliest life stages being luminescent. Following (even slight) tactile, chemical or electrical stimulation, each specimen produces a continuous bright glow for 1-3 min, then the light fades off with an exponential decay of about 1 min^{-1} (i.e., brightness decreases by 2.7 times in a minute). The parietal luminescence is scattered throughout the body length, even though generally more abundant on the prostomium and the pygidium. Its light is sufficiently strong to be visible through a (turned off) transmission microscope. If the worms are strongly squeezed and the coelomic fluid is completely discharged to the exterior, the body wall will continue to emit light for some time. No fluorescence is associated with the luminous structures. Prolonged immersion in water, such as during extraction from soil with the wet funnel method, affects the luminescent capacity of worms, and a few hours may be required for a full recovery. In these last years Petushkov and coworkers [96-102] have in depth and admirably investigated the chemical and physical properties of bioluminescence of the two Siberian enchytraeids.

Their main results can be summarized as follows: In *F. heliota* the *in vivo* bioluminescence is blue-green (λ_{max} 478 nm). Considering that an adult *F. heliota* is 15-20 mm long and weighs about 2 mg and each worm contains only 1 ng of luciferin, about 2300 individuals were necessary to prepare a sufficient biomass for extraction of the reaction components. The system involves a luciferin-luciferase reaction which is not H_2O_2 dependent and does not cross-react with *Diplocardia* luciferin. *F. heliota* luciferin is a stable compound of 0.5-0.7 kDa. Luciferase is a dimer of about 60 kDa, active in the dissociated state as well. The system requires O_2 as well as ATP and Mg^{2+}. ATP acts as a cosubstrate of luciferase. *In vitro* the temperature optimum for the reaction is 33°C, the pH optimum is alkaline: 8.2. These two aspects of *F. heliota* bioluminescence (ATP-dependence and alkaline pH) are reminiscent of fireflies, dipterans and millipeds, which all are terrestrial and have intracellular luminescence systems. However, *F. heliota* luciferin and luciferase do not cross react with the reaction components from *Photinus pyralis*. Cross reactions with luciferins from *Henlea* and hydroid polyps also give negative results.

In *Henlea* sp. (whose adults are up to 30 mm long and weigh up to 10 mg) the *in vivo* bioluminescence is blue (λ_{max} 464 nm; the shortest of all known luminous oligochaetes). This system also involves a luciferin-luciferase reaction which is not H_2O_2 dependent and does not cross-react with *Diplocardia* luciferin. *Henlea* luciferin is an extremely unstable compound, inactivated by exposure to direct light or room temperature. Luciferase is a homodimer of 72 kDa. The system requires O_2 and Ca^{2+} as an activator of luciferase. *In vitro* the temperature optimum for the reaction is 20°C, the pH

optimum is neutral: 7.2 (in the habitat where *Henlea* sp. and *F. heliota* live, soil pH is 6.1-6.4). *Henlea* luciferin and luciferase do not cross react with the reaction components from *Fridericia*.

Thus, not only the luminous structures and the method of lighting but also the chemistry of bioluminescence of the two studied enchytraeids has turned out to be different, both from each other and from the one shared by the megadriles [100].

7. Morphology, evolution and use of bioluminescence

7.1. Site of light production

In the Oligochaeta, the anatomy of the worm does not restrict the seat and physiology of the luminescence. In megadriles, whether endowed with dorsal pores (e.g. *Eisenia, Octochaetus, Spenceriella, Fletcherodrilus*) or not (e.g. *Microscolex, Parachilota, Pontodrilus*), with coloured teguments or with transparent body walls, with small or large body sizes, bioluminescence originates from discrete subcellular loci within free cells suspended in the coelomic fluid.[19] The bioluminescent cells have been alternatively identified as eleocytes (i.e. cells containing refringent oil globules; [40, 49]), free chloragogen cells [51, 52, 59, 82], or mucocytes (i.e. acidophilic cells, containing mucopolysaccharidic inclusions; [93, 103, 104]). Whatever their nature and derivation,[20] these cells are repleted with granular particles of two or three types, but contain little other structures. Luminescence, however, comes not from the granules themselves, but from low-density (membrane or other lipid) components, possibly activated by rupture of the granules [82].

In the enchytraeid *Fridericia heliota*, which has segmental coelomic pores, luminescence is parietal, presumably intracellular, not associated with the coelomic fluid: the body wall remains alighted after the worm has been emptied of all coelomic fluid [33]. On the other hand, *Henlea* sp. is devoid of segmental coelomic pores, but, like all enchytraeids, possesses a head pore and coelo-rectal openings which also permit the discharge of coelomic fluid to the exterior. Intense bursts of light, localized at both ends of the body, occur in *Henlea* sp. upon strong stimulation, when the coelomic fluid flows out through the head pore and the coelo-rectal pores, suggesting that the coelomocytes are photogenic. But even at the onset of stimulation *Henlea* sp.

---

[19] According to [82], luminescence can also be elicited from cells attached on the gut wall and near the dorsal blood vessel, whereas the septal tissue, the intestinal epithelium or the epidermis contain none of the components of the system.

[20] Coelomocyte classification based on electron microscopy does not always correspond to the types recognized in light microscopy, nor it is certain whether given cell types represent distinct lineages or are stages of the same lineages [103].

shows a generalized, weak, internal luminescence, which photographs [96: fig. 2] have shown to originate from an irregular pattern of dot-like sources. It is thus possible that in this oligochaete coelomocytes may light up even when still inside the body.[21]

7.2. Colours of bioluminescence

The light emitted by oligochaetes ranges from orange-yellow to bluish, according to species (Table 1). It is orange-yellow in *Octochaetus multiporus* (Fig. 2), yellow-green in *Eisenia lucens*, *Diplotrema heteropora*, *Microscolex phosphoreus* and *Pontodrilus bermudensis*, blue-green in *Diplocardia* spp., blue in *Fridericia heliota* and *Henlea* sp. In the megadriles spectral differences between the various species do not appear to be caused by different luciferins, but by the presence of different fluorescent entities, which act as the emitter [93].

Bioluminescence and fluorescence are not always correlated: *Diplocardia* coelomocytes, unlike those of *Microscolex* and *Pontodrilus*, have no fluorescent component that matches the bioluminescent spectrum. On the other hand, the coelomocytes (eleocytes) of many lumbricid species, whether bioluminescent (*E. lucens*) or not (*E. fetida, Allolobophora chlorotica, Dendrobaena veneta, Dendrodrilus rubidus, Octolasion cyaneum, O. tyrtaeum*), are autofluorescent, with emission spectra peaking at 522 nm. This, as anticipated by [40, 41] can be explained with an accumulation of riboflavin (vitamin B2), albeit its amount varies intra- and interspecifically.[22] It has been postulated in *Eisenia* [40] that the flavins participate in the luminescence reaction. In *Diplocardia* no role for flavin-like compounds in the luminescence reaction has been found; however, a role for flavin in the generation of peroxide is still possible [82].

7.3. Evolution

The available evidence indicates that at least three bioluminescent systems evolved independently in the Oligochaeta (Table 1). But when and why did this property first appear and how many times was it lost, in the

---

[21] A comparable structural difference is found in polychaetes among different families: Polynoidae have intracellular parietal luminescence, whereas Syllidae and Chaetopteridae have extracellular light production with discharge of luminous mucus.

[22] Interestingly, no species of *Lumbricus* does accumulate riboflavin in the coelomocytes or has autofluorescent coelomic cells (four species tested by [105-107]). Such variation in the cytology of the coelomic fluid of Lumbricidae was first discovered by Rosa [108] who noticed that eleocytes were absent in *Lumbricus*, their place being taken by "vacuolated lymphocytes".

various lineages? What did it evolve from? Why did bioluminescence persist in certain species and not in others, with so few members in many genera showing this property and so many not? And also how did bioluminescence change over time? The data obtained on megadriles allow some discussion.

In contrast with enchytraeids, megadriles (at least those representing three different families in the Superfam. Megascolecoidea)[23] have conserved the capacity to luminesce using one and the same substrate (an aldehyde compound contained in the coelomocytes). The first step in their acquisition of bioluminescence must have been the production of this potentially luminescent agent (luciferin). It is noteworthy that some non-luminescent earthworms contain a luciferin which luminesces in the presence of luciferase of *Diplocardia longa* (Wampler, in [58]). Either these species were formerly luminous and have secondarily lost the luciferase while retaining the luciferin,[24] or earthworm luciferin has a more general but unidentified function in the non-luminous species [109]. A subsequent step must have been the development of an additional factor (luciferase), whose primary function may not have been the production of luminescence, but rather, for instance, to catalyse a final transformation in a detoxification process [103]. This same enzyme, however, happened to catalyse the degradation of the H_2O_2 adduct of earthworm luciferin, increasing the quantum yield from 3% to 63% [90].

7.4. Use of bioluminescence

Regarding the use of bioluminescence, there have been some suggestions but little or no experimentation in the oligochaetes. The earliest hypothesis of a function in recognition or sex attraction (Flaugergues, Friend, etc.), formulated in analogy with firefly behaviour, must be dismissed in view of the lack of adequate sight organs and because the light production occurs throughout the life span of the worms. However, Matzdorff [28] reported that *M. phosphoreus* is more prone to luminesce spontaneously in summer and autumn, and Komárek and Wenig [40] observed the greatest emission of light and production of granules in the coelomocytes of *E. lucens* in summer. We know today that earthworm coelomocytes are more active in certain seasons and the proportion of particular coelomocyte types changes with the seasons [110]. Thus, seasonal fluctuations in the light production capability could be

[23] To my knowledge, nobody has ever tried yet a cross reaction between *Eisenia lucens* and *Diplocardia* components.

[24] One species of *Digaster* (*D. keasti*) emits coelomic fluid that luminesces (weakly) on addition of H_2O_2, the only lacking ingredient; a closely related species (*D. brunneus*) does not luminesce on addition of H_2O_2 [58].

a secondary effect of rhythmic changes of primary physiological processes. Different biochemical activities of coelomocytes could also reflect the level of genetic differentiation among populations. This could be the case in species, which only rarely have been noticed as bioluminescent, e.g. *Eisenia fetida*, which according to Pérez-Losada et al. [111] is composed of reproductively isolated populations.[25]

Michaelsen [113] proposed to interpret the production of light as a deterrence factor (*Abschreckungsmittel*), acting to discourage visually guided predators. If so, the deterrent strategy would not be an aposematic warning of distastefulness (*Microscolex* is normally eaten by arthropods [114], as *Octochaetus* is by chicken [115]; and kiwi birds: Meyer-Rochow, pers. comm.), but a display aimed at frightening or blinding or distracting the predator (the discharge of luminous secretions is somehow analogous to defensive self-amputation against diurnal predators, if co-ordinated with escape behaviour), or at attracting a higher-rank predator that may attack the primary aggressor in the so-called 'burglar alarm effect', leaving the victim free to crawl away in the darkness. In the marine polynoid polychaete *Acholoe*, bioluminescent flashing dorsal scales are released to distract the predator [116].

Bioluminescence in the oligochaetes can either be produced immediately and intensely on stimulation or tardily and weakly. Readiness to luminesce is obviously a pre-requisite for this property to become usable for defence purposes, and the brighter and shorter-lived the burst of luminescence the more effective the defensive tactic. In some megadrile species, bioluminescence is combined with a complex defensive behaviour. Like *Octochaetus multiporus*, when first dug up *Parachilota* shams death, assuming a rigid, motionless attitude. But while *Octochaetus* remains generally quiescent, *Parachilota*, after a time, begins to show very lively movements, and by throwing the body in a series of strong flexures, scatters masses of coelomic fluid in all directions. In a dark room, spots of light appear unexpectedly in various places. Gilchrist [52] described the worm as very reactive, giving off its luminous fluid if the head or tail were slightly touched by a needle, and judged it capable of scaring or distracting predacious animals by throwing slime from the burrow or by leaving a luminous mass behind while moving away. Some non-luminescent earthworms, such as the African *Dichogaster jaculatrix*, or the Australian *Megascolides australis* and *Didimogaster sylvaticus*, are also known to eject

[25] Gadeau de Kerville [112] speculated that the luminescence in *Eisenia fetida*, recorded only very exceptionally, might represent a case of atavism, suggesting that this property was once present in the species.

spouts of coelomic fluid through the dorsal pores in response to stress or irritation, even to a height of 30 cm; their fluid is believed to be toxic or repellent [117]. In squids the luminous secretion in darkness has the same purpose as does the ink in daylight [116]. Yet, the function of a stable glow such as that of the enchytraeid *F. heliota* (Fig. 5) remains mysterious.

Acknowledgements

I wish to thank Mikaela Bernardoni (Library of the Faculty of Science, University of Siena); the Central National Library of Florence; and the St-Petersburg University Scientific Library, for their assistance with finding relevant literature. Photographs were kindly provided by Jirí Novák, V. Benno Meyer-Rochow, and Valentin Petushkov.

References

1. Grimm, H.N. 1683, Misc. Curiosa sive Ephem. Medico-phys. Germ. Acad. Naturae Curiosorum, dec. 2, ann. 1, obs. 172, 406-407.
2. Milne Edwards, H. 1863, Leçons sur la physiologie et l'anatomie comparée de l'homme et des animaux. Paris.
3. Leuckart, R. 1873, Wiegm. Arch. f. Naturg., 39, 2 Bd., 413-567.
4. Vejdovský, F. 1884, System und Morphologie der Oligochaeten. Franz Rivnac, Prague.
5. Friend, H. 1893, Nature, 47, 462-463.
6. Flaugergues, H. 1780, Obs. Phys., 16, 311-315.
7. Bruguière, J.G. 1792, J. Hist. Nat., 2, 267-268.
8. Bosc, L.A.G. 1802, Histoire naturelle des vers, contenant leur description et leur mœurs. Deterville, Paris.
9. Cloquet, H. 1830, Encyclopédie méthodique. Systême anatomique. Tome 4. Reptiles, poissons, mollusques, crustacés, annélides, arachnides, insectes, radiaires. Agasse, Paris.
10. Vallot, D.-M. 1832, Mém. Acad. Sci. Arts et Belles-Lettres Dijon, Sci., 1832, 175-192.
11. Reiselius, S. 1675, Misc. Curiosa sive Ephem. Medico-phys. Germ. Acad. Naturae Curiosorum, dec. 1, ann. 6-7, obs. 193, 302-303.
12. Maillard de Chambure, C.H. 1832, Mém. Acad. Sci. Arts et Belles-Lettres Dijon, Sci., 1832, 173-174.
13. Phipson, T.L. 1862, Phosphorescence or, the emission of light by minerals, plants, and animals. Lovell Reeve & Co., London.
14. Macartney, J. 1810, Phil. Trans. Roy. Soc. Lond, 100, 258-293.
15. Morren, C.-F.-A. 1829. De lumbrici terrestris historia naturali necnon anatomia tractatus. H. Tarlier, Bruxelles.
16. Dugès, A. 1837, Ann. Sci. Nat., s. 2, 8, 15-35.
17. Audouin, V. 1840, C. R. Acad. Sci., 11 (13), 747-749.
18. Giard, A. 1887, C. R. Acad. Sci. Paris, 105, 872-874.

19. Moniez, R. 1889, Rev. Biol. Nord France, 1, 197-200.
20. Barrois, T. 1891, Rev. Biol.Nord France, 3, 117-119.
21. Legros, C.V. 1913, Fabre, poet of science. Fisher Unwin, London.
22. Fabre, J.-H. 1856, Ann. Sci. Nat., Bot., s. 4, 4, 179-197.
23. Johnston, G. 1865, A catalogue of the British non-parasitical worms in the collection of the British Museum. British Museum, London.
24. Allman, G.J. 1844, Rep. Brit. Assoc. Adv. Sci., 13th Meeting, 76.
25. Cox, H. 1854, Proc. Lit. and Phyl. Soc. Liverpool, 8, 57.
26. Higgins, H.H. 1862, Proc. Lit. and Phyl. Soc. Liverpool, 16, 109-110.
27. von Stein, F. 1883, Der Organismus der Infusionsthiere, Abt. 3, II Halfte, Die Naturgeschichte der arthrodelen Flagellaten. W. Engelmann, Leipzig.
28. Matzdorff, C. 1893, S. B. Ges. naturf. Fr. Berlin, 1893, 19-24.
29. Giard, A. 1891, C. R. Soc. Biol., s. 9, 3, 252.
30. Rosa, D. 1887, Boll. Mus. Zool. Anat. Comp. Torino, 2, 1-2.
31. Rosa, D. 1890, Ann. Mus. Civ. St. Nat. Genova, s. 2, 9, 509-521.
32. Beddard, F.E. 1899, Nature, 60, 52.
33. Rota, E., Zalesskaja, N.T., Rodionova, N.S., and Petushkov, V.N. 2003, J. Zool., London, 260, 291-299.
34. Rota, E. 2004, Fauna Europaea: Terrestrial Oligochaeta, Aphanoneura and Polychaeta, http://www.faunaeur.org
35. Skowron, S. 1928, Biol. Bull., 54, 191-195.
36. Csuzdi, Cs. 1986, Opusc. Zool. Budapest, 22, 63-66.
37. Pawlowski, J. 1990, Prądnik. Prace Muz. Szafera, 1, 9-17.
38. Waga, A. 1857, Bibl. Warsz., n.s., 2, 161-227.
39. Komárek, J. 1934, Bull. Int. Acad. Sci. Bohème, 35, 63-64.
40. Komárek, J., and Wenig, K. 1938, Vestn. Kral. ceske Spol. Nauk., 12, 1-12.
41. Bačkovský, J.M., Komárek, J., and Wenig, K. 1939, Vestn. Cs. Zool. Spol., 7, 1-10.
42. Plisko, J.D. 1965, Fragm. Faun., 12, 57-108.
43. Csuzdi, Cs., and Zicsi, A. 2003, Die Bedeutung der Regenwurmart *Eisenia lucens* bei der Zersetzung von Holzabfällen. In Advances in Management and Conservation of Soil Fauna, G.K. Veeresh, D. Rajagopal, and C.A. Viraktamath, (Eds.). Oxford & IBH Publ. Co., New Delhi, 547-551.
44. Cohn, F. 1873, Z. wiss. Zool., 23, 459 -461.
45. Friend, H. 1919, Nature, 103, 446.
46. Friend, H. 1924, The story of the British Annelids. Epworth Press, London.
47. Miller, D. 1952, J. Polynes. Soc., 61, 1-61.
48. Benham, W.B. 1899, Nature, 60, 591.
49. Benham, W.B. 1901, Q. J. Micr. Sci., 44, 565-590.
50. Johnson, F.H., Shimomura, O., and Haneda, Y. 1966, A note on the large luminescent earthworm, *Octochaetus multiporus*, of New Zealand. In Bioluminescence in Progress, F.H. Johnson, and Y. Haneda, (Eds.). Princeton University Press, Princeton, N.J.,'385-390.
51. Wampler, J.E., and Jamieson, B.G.M. 1980, Comp. Biochem. Physiol., B: Biochem. Mol. Biol., 66, 43-50.
52. Gilchrist, J.D.F. 1919, Trans. Roy. Soc. S. Africa, 7, 203-212.

53. Pickford, G.E. 1937, A monograph of the acanthodriline earthworms of South Africa. Heffer, Cambridge.
54. Gates, G.E. 1925, Rec. Ind. Mus. Calcutta, 27, 471-473.
55. Gates, G.E. 1944, Curr. Sci., 13, 131-132.
56. Kanda, S. 1938, Rigagukai, 36, 1-7.
57. Haneda, Y. 1955, Luminous organisms of Japan and the Far East. In The Luminescence of Biological Systems, F. H. Johnson, (Ed.). American Ass. Adv. Sci., Washington, D.C., 335-385.
58. Jamieson, B.G.M. 1977, Proc. R. Soc. Queensland, 88, 83-88.
59. Jamieson, B.G.M., and Wampler, J.E. 1979, Austral. J. Zool., 27, 637-669.
60. Rota, E. 2001, Organisms Divers. Evol., 1, 225-238.
61. Eversmann, E.A. 1838, Utshen. Zapiski. Imper. Kazan. Univ., 1838, 156-157.
62. Michaelsen, W. 1900, Oligochaeta. In Das Tierreich, 10, 1-575. Friedlander, Berlin.
63. Owsiannikow, Ph. 1864, Bull. Acad. Imp. Sci. St. Pétersbourg, 7, 55-61.
64. Harker, A. 1887, Rep. Brit. Assoc. (Manchester), 1887, 767.
65. Pütter, A. 1905, Zeitsch. allg. Physiol., 5, 17-53.
66. Walter, A. 1909, Trav. Soc. Imp. Nat. St. Pétersbourg, 40, 103-109, 136-137.
67. Issatschenko, B. 1911, Izv. Imp. St. Petersb. Bot. Sada, 11, 31-43.
68. Vaillant, L. 1889, Histoire naturelle des Annelés marins et d'eau douce, Vol. 3 (2). In Collection des Suites à Buffon. N.E. Roret, Paris.
69. Zalesskaja, N.T., Rodionova, N.S., and Petushkov, V.N. 1990, Dokl. Akad. Nauk SSSR (Biol.), 310, 496-498.
70. Panceri, P. 1875, R. C. Accad. Sci. Fis. Nat. Napoli, 14, 21-25.
71. Panceri, P. 1878, Atti R. Accad. Sci. Fis. Nat. Napoli, 7, 1-20.
72. Haupt, H. 1903, Naturwiss. Wochenschr., 19, 65-71.
73. Molisch, H. 1904, Leuchtende Pflanzen: Eine physiologische Studie. Gustav Fischer, Jena.
74. Mangold, E. 1910-1914, Die Produktion von Licht. In Handbuch der vergleichende Physiologie, H. Winterstein, (Ed.), 3 (2nd half). Gustav Fischer, Jena, 225-392.
75. Dahlgren, U. 1916, J. Franklin Inst., 181, 659-696.
76. Dubois, R. 1914, La vie et la lumière. Félix Alcan, Paris.
77. Linsbauer, K. 1917, Umschau, 21, 67-69.
78. Pierantoni, U. 1923, R. C. Accad. Lincei, s. 5, 32, 359-362.
79. Pierantoni, U. 1924a, Boll. Soc. Nat. Napoli, 36, 179-195.
80. Pierantoni, U. 1924b, Monit. Zool. Ital., 35, 1-10.
81. Skowron, S. 1926, Biol. Bull., 51, 199-208.
82. Rudie, N.G., and Wampler, J.E. 1978, Comp. Biochem. Physiol., A: Physiol., 59, 1-8.
83. Harvey, E.N. 1926a, Biol. Bull., 51, 89-97.
84. Harvey, E.N. 1926b, Amer. J. Physiol., 77, 548-554.
85. Plisko, J.D. 1961, Bull. Acad. Pol. Sci., Ser. Sci. Biol., 9, 101-104.
86. Cormier, M.J., Kreiss, P., and Prichard, P.M. 1966, Bioluminescence systems of the peroxidase type. In Bioluminescence in Progress, F.H. Johnson, and Y. Haneda, (Eds.). Princeton University Press, Princeton, N.J., 363-384.
87. Bellisario, R., and Cormier, M.J. 1971, Biochem. Biophys. Res. Comm., 43, 800-805.

88. Bellisario, R., Spencer, T.E., and Cormier, M.J. 1972, Biochemistry, 11, 2256-2266.
89. Ohtsuka, H., Rudie, N.G., and Wampler, J.E. 1976, Biochemistry, 15, 1001-1004.
90. Rudie, N.G., Mulkerrin, M.G., and Wampler, J.E. 1981, Biochemistry, 20, 344-350.
91. Wampler, J.E. 1982, Comp. Biochem. Physiol., A: Physiol., 71, 599-604.
92. Bersis, D.S. 1977, Folia Biochem. et Biol. Graeca, 14, 5-15.
93. Wampler, J.E., and Jamieson, B.G.M. 1986, Comp. Biochem. Physiol., A: Physiol., 84, 81-87.
94. Herring, P.J. 1978, Appendix: a classification of luminous organisms. In Bioluminescence in action, P.J. Herring, (Ed.). Academic Press, London, 461-476.
95. Herring, P.J. 1987, J. Biolumin. Chemilumin., 1, 147-163.
96. Petushkov, V.N., Rodionova, N.S., Purtov, K.V., and Bondar, V.S. 2002, Dokl. Biol. Sci., 385, 310-312.
97. Petushkov, V.N., Rodionova, N.S., and Bondar, V.S. 2003, Dokl. Biochem. Biophys., 391, 204-207.
98. Rodionova, N.S., Bondar, V.S., and Petushkov, V.N. 2002, Dokl. Biochem. Biophys., 386, 260-263.
99. Rodionova, N.S., Bondar, V.S., and Petushkov, V.N. 2003, Dokl. Biochem. Biophys., 392, 253-255.
100. Petushkov, V.N., Rodionova, N.S. 2005, Dokl. Biochem. Biophys., 401, 115-118.
101. Rodionova, N.S., and Petushkov, V.N. 2006, J. Photochem. Photobiol., B: Biol., 83, 123-128.
102. Petushkov, V.N., Rodionova, N.S. 2007, J. Photochem. Photobiol., B: Biol., 87, 130-136.
103. Jamieson, B.G.M. 1981, The ultrastructure of the Oligochaeta. Academic Press, London.
104. Jamieson, B.G.M., Wampler, J.E., and Schultz, M.C. 1981, Preliminary ultrastructural description of coelomocytes of the luminescent oligochaete, *Pontodrilus bermudensis* (Annelida). In Bioluminescence and Chemiluminescence, M. Deluca, and W.D. McElroy, (Eds.). Academic Press, New York, 543-559.
105. Cholewa, J., Feeney, G.P., O'Reilly, M., Stürzenbaum, S.R., Morgan, A.J., and Plytycz, B., 2006. Folia histochem. et cytobiol., 44, 65-71.
106. Plytycz, B., Homa, J., Koziol, B., Rózanowska, M., and Morgan, A.J. 2006, Folia histochem. et cytobiol., 44, 275-280.
107. Kurek, A., Homa, J., Kauschke, E., and Plytycz, B. 2007, Eur. J. Soil Biol., 43, 121-126.
108. Rosa, D. 1896, Mem. R. Accad. Sc. Torino, s. II, 46, 149-178.
109. Ward, W.W. 1985, General aspects of bioluminescence. In Chemi- and Bioluminescence, J.G. Burr, (Ed.). Marcel Dekker, New York, 321-358.
110. Kurek, A., and Plytycz, B. 2003, Pedobiologia, 47, 689-701.
111. Pérez-Losada, M., Eiroa, J., Mato, S., and Dominguez, J. 2005. Pedobiologia, 49, 317-324.
112. Gadeau de Kerville, H. 1890, Les animaux et les végétaux lumineux. J.-B. Baillière, Paris.

113. Michaelsen, W. 1928, Oligochaeta. In Handbuch der Zoologie, W. Kükenthal, and T. Krumbach, (Eds.), 2. De Gruyter, Berlin, 1-118.
114. Sivinski, J., and Forrest, T. 1983, Florida Entomol., 66, 517.
115. Pierantoni, U. 1922, Gli animali luminosi. Sonzogno, Milano.
116. Harvey, E.N. 1952, Bioluminescence. Academic Press, N.Y.
117. Edwards, C.A., and Lofty, J.R. 1977, Biology of Earthworms. Chapman and Hall, London.
118. Meyer-Rochow, V.B., 2000, BioSpektrum, 6, 220-223.

Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 139-146
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

8 Luminescent myriapoda: A brief review

J. Rosenberg[1] and V.B. Meyer-Rochow[2]
[1]Central Animal Laboratory, University Duisburg-Essen, Medical School D-45122 Essen, Germany; [2]Faculty of Engineering and Science, Jacobs University, D-28759 Bremen, Germany and Department of Biology University of Oulu, SF-90014 Oulu, Finland

Abstract

Considering the total number of myriapod species described to date, bioluminescence is rare in this taxon. However, there are several species of geophilomorph Chilopoda worldwide that are capable of producing luminescent secretions, most likely as a defence reaction to ward off attackers. The biochemistry and the structures involved have not been studied in sufficient detail to allow us to present a definitive description or explanation for the luminescence. The same holds true for the only bioluminescent millipedes

Correspondence/Reprint request: Dr. J. Rosenberg, Central Animal Laboratory, University Duisburg-Essen Medical School, D-45122 Essen, Germany. E-mail: sommerhaus-rosenberg@t-online.de

known to date, namely eight species that belong to the genus Motyxia and are apparently restricted to California. The aims of this chapter are to summarize what is known about myriapod luminescence and to stimulate further research into it.

1. Introduction

1.1. Luminescent centipedes

The two most important subclasses of the Myriapoda, known as Chilopoda (centipedes) and Diplopoda (millipedes) both contain some luminescent species. In the Chilopoda biological light is only known from a few geophilomorph species. The light of one species, most likely that of *Orphnaeus brasilianus* according to Haase [1], must have impressed Oviedo [2], one of Christopher Columbus' men, sufficiently much that he wrote these lines into his diary in 1547, quoting from Newport [3]: "There are on this island (St. Domingo) many kinds of scolopendra or hundred-legs.... There are other of these worms about half the length of the finger, and slender, with many feet, and these shine much by night, and leave a light where they go, and many be seen fifty or even of hundred paces off; yet the whole animal does not shine, but only the joints where the legs spring from the body, and the light is very bright".

In Europe Linné and Gmelin [4] described two bioluminescent geophilids as *Scolopendra electricus* and *S. phosphora*, which according to [1] probably represented *Himantarium subterraneum* and *Orphnaeus brevilabiatus*, respectively. Several reviews on bioluminescent chilopods have appeared over the years [5-11] and a list of supposedly luminescent species based on reports by numerous authors can be found in Table 1. Since the name *Geophilus electricus* has frequently been used in connection with a variety of species, the true identity of such species requires further study [12].

1.2. How do Chilopoda produce their luminescent secretions?

For a long time it was not clear from where the luminescence in geophilid centipedes came. Dubois [13,14], examining *Orya barbarica*, believed the luminescence originated from the intestine and was released as a liquid from the anus. Macé [15] at first held hypodermal cells near the stigmata responsible for the luminescence, but later after dissecting species like *G. simplex* and *G. flavus* identified coxal organs ("glandes préanales") as the places of the origin of the light [16]. Pores oozing out a brightly luminescing greenish-blue liquid on the sternal and episternal plates were described by Gazagnaire [17] from male and female *O. barbarica* and Mohnike, cited in [18], saw the liquid appear from pores at the base of the extremities. Ludwig [7] suspected that the light was caused by the luminescent mycelia of certain

Table 1. Bioluminescent Myriapoda and their distribution according to different sources.

Chilopoda; Geophilomorpha	**Distribution**	**Author**
Geophilus carpophagus	Europe, N.Africa, W.Asia	[9, 29, 36 - 38]
Geophilus electricus	Europe	[23, 39 - 48]
Geophilus flavus	Europe	[3, 16]
Geophilus vittatus	North America (USA)	[21]
Orphnaeus brevilabiatus	Africa, S.E.Asia, Central and S.America, Hawaii	[1, 4, 18, 20, 28, 49, 50]
Orphnaeus brasilianus (syn. *O. lineatus*)	Central and South America	[1]
Orphnaeus sp.	Eritrea	[51]
Orya barbarica	N. Africa, Europe	[17, 25, 26, 52]
Stenotaenia linearis (syn. *G. simplex, G. subterraneus*)	Europe	[7, 15, 16]
Himantarium subterraneum	Europe	[1, 4]
Stigmatogaster subterraneus	Europe	[23, 24]
Strigamia crassipes	Europe	[5, 8, 13, 14, 53 - 59]
Undetermined geophilomorphs	Various places worldwide	[5, 60 - 65]
Chilopoda; Scolopendromorpha		
Otostigmus aculeatus	S.E. Asia	[19]
Diplopoda; Polydesmida		
Motyxia kerna, M. monica, M. sequoiae, M. tularea, M. sequoia, M. pior, M. porrecta, M. tiemanni	California	[30]
Spirobolellus phosphoreus, S. takakuwai, Dinematocrius sp.	Australo-Pacific region	[66-68]

luminescent fungi, but the puzzle was finally solved when Brade-Birks [9] and Koch [8] demonstrated that the luminescent liquid came exclusively from the pores of the sternal glands, which function as defence glands. Houdemer [19] observed that poking caused the scolopendromorph *Otostigmus aculeatus* to produce from tiny pores on the sternites or intersegmental regions a luminescent secretion that smells of phosphorus and induces erythemata and blisters on the human skin.

1.3. Properties and biochemistry of the luminescent liquid in Chilopoda

According to [17] the secreted liquid is yellow in *Orya*, but Brade-Birks [9] describe it as colourless in *S. crassipes*. Koch [8] reports the luminescence to be weakly yellow and Anderson [20] talks of a clear light in *O. brevilabiatus*. The liquid has an acidic pH and a fruity smell [8, 9]. The duration of the luminescence according to [21] lasts a few seconds, but others have reported durations of up to 110 second [1, 8, 9, 13] or even longer [22]. In nature the animals are recognized by their luminescent trails on the soil, which are perceivable in the dark from as far as 10 paces [13]. The intensity of the light has been compared to that of moonlight [23] and Brodhurst [24] reported that he could read a letter with the light, while Dubois could read the time on his pocket watch with it [13]. The light is certainly strong enough to blacken photographic plates [8, 9] and the liquid forms crystals when dried [8, 25, 26].

Already in 1904 Dubois (26) suspected that the crystals of the dried up material secreted by geophilids contained the photogenic substance 'matière photogène' that emitted light upon oxidation, but Koch [8] did not agree and pointed out that non-luminescent crystalline material also occurred in non-glowing defence liquids of the sternal glands *Himantarium gabriele* [27]. Chloroform vapour enhances the glow and following treatment with $(NH_4)_2S$-vapour a delayed reaction of the luminescence liquid has been described [8, 28]. It was postulated that "proto-luciferin", mucin, and some acids formed an important part of the composition of the luminescent secretions [8, 9].

Anderson's research [20] on *Orphnaeus brevilabiatus* from the Moluccan Islands revealed that tactile, thermal, chemical or electrical stimulations led to the secretion of the luminescent material and that the emission spectrum displayed two peaks, one at 480 nm and a second at 510 nm. Dilution with water prolonged the duration of the luminescence. Although similar to bioluminescent reactions described from other animals, the luminescence of *O. brevilabiatus* occurs at the rather low pH of 4.6.

1.4. Possible function of chilopod bioluminescence

In most species of geophilomorph centipedes males and females possess sternal glands that are able to produce a slime that is sticky. Following an attack on a geophilid the mouthparts of a spider or those of insect predators would become unusable until thoroughly cleaned. Even larger predators like, for instance, toads might find the sticky goo unpleasant to handle. However, in only a few geophilid species the defence liquid is luminescent. Nevertheless, since attacks by ants and beetles cause luminescent species to produce the

shiny liquid [23], an aposematic defence function of the luminescence in nocturnally-active geophilds seems likely.

Since most of the geophilid luminescent activity (of at least the palaearctic species) appears to occur between the months of September to November, Gazagnaire [6] suspected that the increase in light emissions had some connection to the reproductive behaviour of these chilopods. Indeed, reproduction peaks in autumn, but the fact that geophilids also produce luminescent slime during the rest of the year suggests that the luminescent liquid cannot be used, if at all, exclusively for reproductive or courtship purposes. Moreover, since no geophilid has eyes one would have to assume that it is the chemical message in the slime that is of importance in courtship and not the light associated with the mucus. Any connection between bioluminescence and reproduction was categorically rejected by [9, 29] and since both sexes are capable of secreting luminescent liquids the latter rather seemed to be a component of a defensive behavioural response to an attack [8].

2. Luminescent millipedes

2.1. *Motyxia* spp. and their lights

In Diplopoda bioluminescence is so far known only from Western North America and from some islands of the Australo-Pacific region, e.g. Truk Island (*Spirobolellus phosphoreus* [66]), New Caledonia (*Dinematocrius* sp. [67]), and Taiwan as well as Okinawa (*S. takakuwai* [68]). All Western North American luminescent diplopods belong to the polydesmid family Xystodesmidae, of which the species originally known as *Luminodesmus sequoiae*, but now termed *Motyxia sequoiae* [30], is the most thoroughly examined out of the 8 currently accepted species of luminescent millipedes in the genus *Motyxia* (cf., Table 1). Luminescent individuals of *M. sequoiae* are large and handsome millipedes, measuring 40 mm in length and 8 mm in width when adult. In captivity they are easy to culture [31] and their light has been described as extraordinarily bright, neon-white, continuous for hours at night, and seemingly inexhaustible [30]. Yet, peak spectral emission has been given as 495 nm [32], based on a porphyrin luciferin with a luciferase of molecular weight 104 kDa [33]. Since *Luminodesmus* (the outdated name for the genus *Motyxia*) is listed under "*Geophila*" in [33], some confusion regarding geophilid and millipede luminescence could have been involved. It was shown that isolated pieces of *Lumidesmus sequoiae* continued to emit light, which decreased to about half of its original value in 8 hours, and that the light is optimally bright at a temperature of 31.5°C [32]. ATP, added to extracts while the latter are luminescing, leads to an increase in the light intensity [32].

In luminescing *Motyxia sequoia* "the entire dorsum, legs, and antennae" glowed evenly with a greenish-white hue, resembling that of a commercially available light stick [30]. According to [34] individuals vary with regard to their readiness to emit light and the intensity of the light emitted by them. Moreover, a circadian component with brightest glows seen during the night hours, seems involved in the control of the light emission, but, which according to [30] is "not under voluntary control". On the other hand, handling for 15-20 minutes, even during the day, would eventually result in the emission of a faint light [30].

2.2. Possible function of millipede lights

As to the function of the light of the bioluminescent millipedes, there is no consensus or definitive answer yet. However, photoreception, if at all developed in polydesmid millipedes, would almost certainly not be involved, and this should rule out a role of the light in intraspecific communication. The most likely explanation is that the light acts as a replacement of the aposematic coloration, which is known from non-luminescing xystodesmids [35]. For nocturnal predators, having 'tasted' the luminescing millipedes once, the animal's nightly glow could, indeed, possess the function of a highly visible warning light. Haneda [67] reached the same conclusion for the New Caledonian luminous millipede *Dinematocrius* sp., which started to emit light for maximally 20-30 seconds when continuously irritated by chemical or physical stimulation.

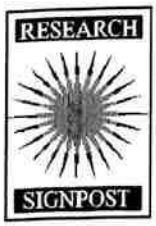
References

1. Haase, E.1889, Tagebl. Versammlg. Dtscher. Naturforsch. u. Ärzte 61, 48-49.
2. Oviedo y Valdez de, F.H. 1547, Coronica de las Indias: La hystoria general de las Indias agora nueuamente impressa corregida y emendada. Junta, Salamanca.
3. Newport, G. 1845, Trans. Linn. Soc. London 19, 265-302, 349-439.
4. Linné von, C., and Gmelin, J.F. 1788, Caroli a Linné systema naturae, 13th Edition. G.E.Beer Publ., Lipsiae.
5. Richard, J. 1885, Ann. Soc. Entomol. Belgique 29 (2è. partie), 15-20.
6. Gazagnaire, J. 1890, Mém. Soc. Zool. France 3, 136-146.
7. Ludwig, F. 1901, Zb. Baktkde. 27, 270-274.
8. Koch, A. 1927, Z. Morph. Ökol. Tiere 8, 241-270.
9. Brade-Birks, H.K., and Brade-Birks, S.G. 1920, Ann. Mag. Nat. Hist. 9, Ser. 5, 1-30.
10. Harvey, E.N. 1952, Bioluminescence, Academic Press, New York.
11. Minelli, A. 1978, Arthropod venoms: Handbook of experimental pharmacology, Vol. 48, G.V.R Born, O. Eichler, A. Fahrah, H. Herken and A.D. Welch (Eds.), Springer Verlag, Berlin, 73-85.
12. Lewis, J.G.E. 1981, The biology of centipedes, Cambridge Univ. Press, Cambridge.

13. Dubois, R. 1886, C. R. Soc. Biol. (Paris) 8. Sér. 3, 518-522.
14. Dubois, R. 1887, C. R. Soc. Biol. (Paris) 8. Sér. 4, 6-8.
15. Macé, M. 1886, C. R. Acad. Sci. (D) (Paris) 103, 1273-1274.
16. Macé, M. 1887, C. R. Soc. Biol. (Paris) 4, 37-39.
17. Gazagnaire, J. 1888, Bull. Soc. Zool. France 13, 182-186.
18. Haase, E. 1881, Z. Entomol. 8, 66-92.
19. Houdemer, M.E. 1926, Bull. Mus. Hist. Nat. Paris 32, 213-214.
20. Anderson, J.M. 1980, Photochem. Photobiol. 31, 179-182.
21. Jones, T.H., Conner,W.E., Meinwald, J., Eisner, H.E. and Eisner, T. 1976, J. Chem. Ecol. 2, 421-429.
22. Thomas, R.H. 1902, Nature, London 65, 223.
23. Adams, A. 1881, Sci. Gossip 17, 68.
24. Brodhurst, B.E. 1880, Nature, London 23, 99.
25. Dubois, R. 1893, C. R. Acad. Sci. (D), (Paris) 117, 184-186.
26. Dubois, R. 1904, C. R. Soc. Biol. (Paris) 56, 442-444.
27. Passerini, N. 1882, Boll. Soc. Entomol. Ital. 14, 323-328.
28. Haneda, Y. 1955, Luminescence of Biological Systems, F.H.Johnson, F.H. (Ed.), Am. Assoc. Adv. Sci., Washington DC, 335-385.
29. Brade-Birks, H.K. and Brade-Birks, S.G. 1918, Lancs. Chesh. Nat. 11, 152-165, 186-192.
30. Shelley, R.M. 1997, Insecta Mundi 11, 331-351.
31. Davenport, D., Wootton, D.M., and Cushing, J.E. 1952, Biol. Bull. 102, 100-110.
32. Hastings, J.W., and Davenport, D. 1957, Biol. Bull. 113, 120-128.
33. Viviani, V.R. 2002, Cell. Molec. Life Sci. 59, 1833-1850.
34. Causey, N.B., and Tiemann, D.L. 1969, Proc. Am. Phil. Soc. 113, 14-33.
35. Whitehead, D.R., and Shelley, R.M. 1992, Proc. Entomol. Soc. Washington 92, 177-188.
36. Duboscq, O. 1898, Arch. Zool. Exp. Gen. 3 (Sér. 6), 481-650.
37. Brade-Birks, H.K., and Brade-Birks, S.G. 1917, Lancs. Chesh. Nat. 10, 113-122.
38. Jeekel, C.A.W. 1964, Abh. Verh. Naturwiss. Ver. Hamburg (NF) 8, 111-153.
39. De Geer, C. 1778, Mémoires pour servir à l'histoire des insectes, Grefing, Stockholm.
40. Fabricius, J.C. 1781, Betrachtungen über die Systeme der Entomologie. Berlin.
41. Macartney, J. 1810, Phil. Trans. Roy. Soc. Lond. B 100, 258-293.
42. Audouin, M.V. 1840, C. R. Acad. Sci. (D) (Paris) 11, 747-749.
43. Erskine Greville, E. 1875, Entomologist (London) 8, 115-117.
44. Mac Leod, J. 1880, Feuille Jeune Naturalistes Naturelles 10, 38.
45. Landois, H. 1895 Jahresber. Westf. Provinz.-Ver. 22, 54-55.
46. Haupt, H. 1903, Naturwiss. Wochenschr. NF 3, 65-71.
47. Ridley, H.N. 1936, Proc. Roy. Ent. Soc. Lond. (A) 11, 48.
48. Allan, P.B. M. 1966, Entomol. Rec. J. Var. 78, 104.
49. von Porath, C.O. 1894, Svensk. Vetenskaps-Akad. Handl. 20, 1-90.
50. Brölemann, H.W. 1926, Arch. Zool. Exp. Gen. 65, 1-159.
51. Lewis, J.G.E. 1969, J. Nat. Hist. 3, 461-470.
52. Blanchard, M.R. 1888, Bull. Soc. Zool. France 13, 186.
53. Arndt, W. 1924, Schlesische Insektenkde. 14, 31-33.

54. Gadeau de Kerville, H. 1893, Die leuchtenden Tiere und Pflanzen, Weber, Leipzig.
55. Mailles, M. 1888, Bull. Soc. Zool. France 13, 186-187.
56. Bertkau, P. 1891, Arch. Naturgesch. Z. Syst. Zool. 57/2, 69-73
57. Pocock, R.J. 1895, Nature, London 53, 131.
58. Pocock, R.J. 1896, Nature, London 53, 223.
59. Stammer, H.-J. 1936, Jber. Schlesische Ges. Vaterländ. Cultur 108, 51-52.
60. Houlbert H1879, Feuille Jeune Naturalistes Naturelles 109, 14.
61. Huet, M. 1886, C. R. Soc. Biol. (Paris) 8. Sér. 3, 523-524.
62. Thomas, R.H. 1895, Nature, London 53, 131.
63. Brockhausen, H. 1903, Jahresber. Westf. Provinz.-Ver. 31, 163-164.
64. Fearnehough, T.D. 1980, Entomol. Rec. J. Var. 92, 53.
65. Bozward, J.L. 1896, Nature, London 53, 223.
66. Takakuwa, Y. 1941, Trans. Nat.Hist. Soc. Formosa 31, 84-87.
67. Haneda, Y. 1967, Sci. Rep. Yokosuka City Mus., 13, 1-4.
68. Shinohara, K., and Higa, Y. 1997, Edaphologia 59, 61-62.

Research signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India



Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 147-159
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

9 Bioluminescence and sexual signaling in fireflies

Sara M. Lewis
Department of Biology, Tufts University, Medford, MA 02155, USA

Abstract

Firefly beetles are highly charismatic insects whose spectacular bioluminesecent courtship displays have long mesmerized people around the world. The ~ 2000 extant firefly species employ a remarkable diversity of sexual signals, including pheromones, glows and discrete flashes. Phylogenetic analyses suggest that firefly luminescence originated as warning glows used by firefly larvae to deter potential predators, and was only later exapted for use in sexual communication. Thus, luminescence in fireflies appears intimately associated with the presence of predator-deterrent chemicals. In extant diurnal lampyrids (and presumably in ancestral species) adults are not luminescent, and sedentary

Correspondence/Reprint request: Dr. Sara M. Lewis, Department of Biology, Tufts University, Medford MA 02155, USA. E-mail: Sara.Lewis@tufts.edu

females produce pheromones to attract flying males. In transitional species, sedentary females use a combination of glows and possibly pheromones to attract their mates. The evolution of discrete flashes as sexual signals for both sexes is coupled to males switching into a primary signaling role. This is the most derived signaling system, and it appears to have evolved independently multiple times. There have also been several reversions back to pheromonal signals. Further consideration of what ecological factors select for particular signal types will enhance our understanding what might drive these evolutionary changes in firefly signaling systems.

1. Introduction

Although bioluminescence abounds on both land and sea [1], few creatures can rival fireflies (Coleoptera: Lampyridae) in captivating the human imagination. Firefly beetles are highly charismatic insects that have long mesmerized artists, children, and scientists with their spectacular bioluminesecent courtship displays. With over 2000 species in 100 genera [2,3], worldwide lampyrid biodiversity is impressive and also encompasses a wide diversity of signaling modalities. Substantial contributions to our understanding of firefly signaling systems have been made within the past decade. This review aims to outline these advances, including new phylogenetic analyses and better understanding of the benefits and costs of firefly courtship signals. Together these advances have provided new insights into the evolution of firefly signaling systems. When viewed from this evolutionary perspective, firefly light splendidly illuminates how the dual processes of natural and sexual selection jointly shape animal communication systems.

2. Firefly luminescence originated through natural selection

Among beetles, several phylogenetic analyses suggest there have been multiple independent origins of luminescence [4-6]. Within the Lampyridae in particular, the original function of luminescence was not as an adult courtship signal, but rather light was a warning signal used by larvae to deter potential predators [3,4,6-10]. Evidence supporting this hypothesis includes the observation that firefly larvae glow when disturbed, and that larval light-producing organs are present even when the corresponding adults lack such lanterns. Light-producing lanterns in firefly larvae may have developed from local concentrations of luciferase-containing fat body,

which in the larval and pupal stages of some fireflies has been shown to produce diffuse luminescence [11].

In addition, the demonstration that lampyrid larvae possess defensive chemicals supports the idea that luminescence originated in ancestral firefly larvae as an aposematic (warning) signal (see also [12]). Many lampyrid beetles have been shown to be distasteful and sometimes toxic to a variety of generalist vertebrate and invertebrate predators [13-20]. Such unpalatability is associated with secretions that fireflies produce when disturbed; larval fireflies evert glands that release deterrent substances [20,21], and adult fireflies produce repellant secretions through reflex bleeding [22-24] or eversible abdominal organs [25]. Lucibufagins, a class of steroidal pyrones, have been identified as defensive toxins present in adults of the North American fireflies *Photinus* [14], *Photuris* [15] and *Lucidota* [26], and in the larvae of the European glow-worm, *Lampyris noctiluca* [27]. However, considering their likely importance in the ecology and evolution of firefly signals, remarkably little is known about the distribution and diversity of chemical defenses in this group.

As pointed out by Sagegami-Oba et al. [10], many firefly relatives in the cantharoid families Cantharidae (soldier beetles), Lycidae (net-winged beetles), and Phengodidae (glow-worm beetles) all secrete toxic or distasteful chemicals when disturbed. While adults in the former two families exhibit typical visual aposematism in the form of body coloration (brightly colored or black bodies often with contrasting red, orange or yellow pigmentation), phengodids join fireflies in having additional, luminescent signals to augment their visual warning display. The adaptive value of aposematic displays depends on potential predators learning to associate warning signals with distasteful prey. Firefly larvae glowing in dark environments should be highly conspicuous to visually orienting predators, and there is good evidence that mice [16] and toads [17,19] learn to avoid glowing prey after only a few encounters.

Thus, luminescence in fireflies appears intimately associated with the presence of defensive chemicals that allow these beetles to repel potential predators. While natural selection therefore provides a likely explanation for the evolutionary origin of luminescence ability in fireflies, once this ability had evolved it was later exapted as an adult courtship signal through sexual selection [7,8], as discussed below. A rather ironic testament to the inexorable power of natural selection is the evolution of visually-orienting predators that are undeterred (in fact, are attracted) by chemical defenses; these predators target prey by eavesdropping on their luminescent sexual signals.

3. Diversity of firefly sexual signals

Adult fireflies use diverse signaling systems for sexual advertisement and mate attraction (Figure 1). These involve not only various signaling modalities, but also differences in which sex is the primary signaler and searcher [7,28-30]. Although numerous exceptions and modifications exist, most lampyrids fall into four major signaling systems described below, which are based on classifications of firefly signaling modes suggested by Lloyd [28,31], Ohba [30,32,33], and Branham & Wenzel [7].

1. Female-produced pheromonal signals

In this signaling system, sedentary females release volatile chemical signals (pheromones) to attract non-signaling flying males (Figure 1). These

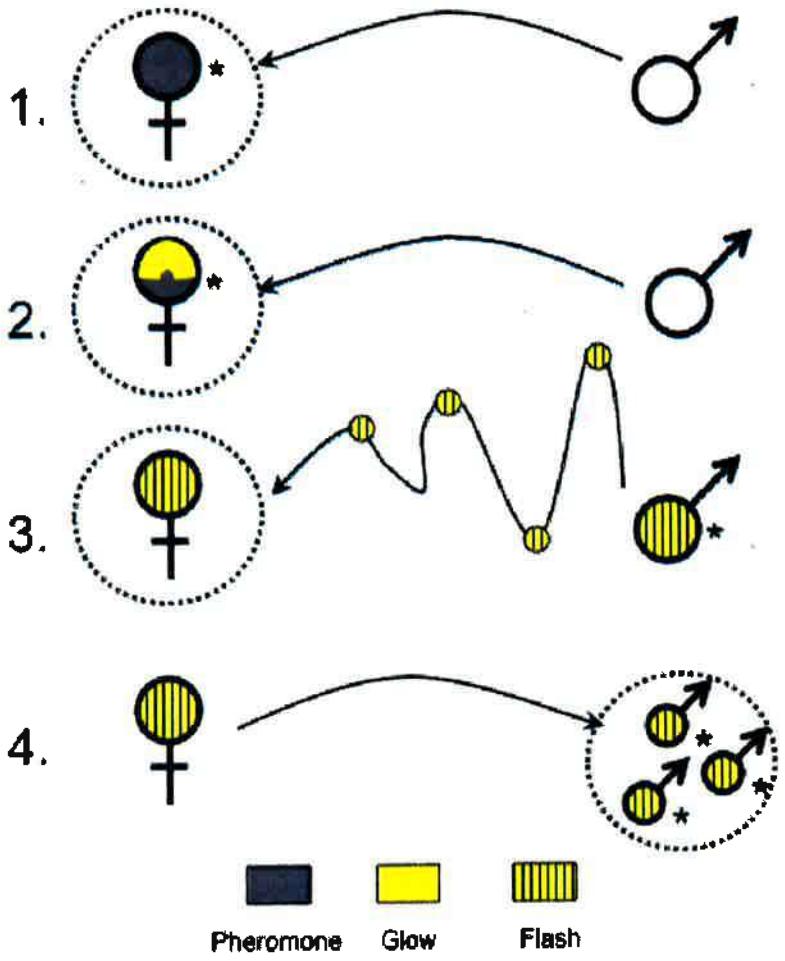


Figure 1. Sexual signaling systems in fireflies, which are based on different signal modalities (pheromones, glows, discrete flashes) and whether females or males are the primary signaler (indicated by *; primary signalers emit signals before receiving signals from the opposite sex). Arrows indicate which sex travels to mating locations (indicated by dotted circles).

species mainly signal during the day and neither sex uses light as a sexual signal, although some adults are capable of luminescence. Many (but not all) of these species show varying degrees of sexual dimorphism, with females having shortened or no wings and thus being incapable of flight. In most cases, pheromonal signals are assumed but have not been conclusively demonstrated. However, direct evidence for pheromonal sexual attraction exists for *Lucidota atra, Pyropyga nigricans,* and *Photinus indictus* in North America [34], *Phosphaenus hemipterus* in Europe [35], and *Lucidina biplagiata* in Japan [33]. Surprisingly, no lampyrid pheromones have yet been isolated or characterized.

2. Female-produced continuous light signals (glows) coupled with pheromones

In these taxa, sedentary females produce continuous glows, sometimes coupled with pheromones, to attract flying males, which generally do not signal (Figure 1). In this category, females again are often flightless, having shortened or no wings. Pheromones might serve to attract males over relatively long distances, while light signals permit close-range mate localization or mate choice [7,30,31]. Although again direct evidence for pheromones is scarce, *Pyrocoelia* fireflies appear to use a combination of glows and pheromonal signals to attract flying males [30]. Variations on this signal system include females that glow to attract searching males that also glow (e.g. *Lamprohiza splendidula* and *Phausis reticulata* [12]).

3. Both sexes produce discrete light signals (flashes)

In many nocturnally-active lampyrids the primary signaling role switches to males, which emit discrete flashes during their searching flight and courtship (Figure 1). Females in these species sometimes remain stationary, although they generally have normal wings and presumably are capable of flight. In some groups, females signal only in response to male signals, while in others females flash spontaneously. Such groups include *Photinus, Pyractomena,* and *Photuris* in North America [31,36,37], *Hotaria parvula, Luciola lateralis* and *L. cruciata* in Japan [30], and *Luciola lusitanica* in Europe [12]. North American *Photinus* fireflies engage in reciprocal courtship dialogs in which both sexes produce flash signals that are precisely-timed and encode species identity and sex [31,36]. Other species in this category have considerably more complex courtship signals: for example males in *Luciola aquatilis* alter their flash patterns depending on different courtship contexts [38].

4. Stationary males flash in synchrony to attract flying females

Several *Pteroptyx* species in southeast Asia have communal sexual displays in which males congregate in leks and emit highly synchronous advertisement flashes that attract flying females [33,39-41]. Within these aggregations, males and females both emit flash signals eventually leading to copulation [41,42]. Flash synchrony among searching males in flight (signal system 3) also occurs in a few species (e.g. *Photinus carolinus* [43] and *Luciola cruciata* [44]), although it is likely that flash synchrony in these two contexts arose through different selective mechanisms.

4. The sexy and the dead: Sexual signaling in *Photinus* fireflies

Among all firefly signaling systems, those of North American *Photinus* fireflies have been deciphered in the greatest detail. This is partly due to the relative simplicity of their courtship signal dialogs, as well as to the pioneering work of McDermott [45] and Lloyd [28,29,31,36,46,47] in decoding the information content of these signals. In *Photinus,* flying males produce species-characteristic sexual advertisement flashes to locate sedentary females by their response flashes (signal system 3 in Figure 1). Sexual selection and mate choice in *Photinus* fireflies have been examined in depth [48]. Photic playback studies indicate that females preferentially respond to male flash signals based on variation in pulse duration or pulse rate, and females generally prefer more conspicuous courtship signals (faster pulse rates and longer pulse durations [49-51]).

Photinus fireflies show broad geographic overlap with *Photuris* and *Bicellychonia* fireflies, some of which consume other fireflies [28]. Females in the *Photuris pennsylvanica-versicolor* complex are visually-orienting, specialist predators that target *Photinus* fireflies through aerial attacks [52,53] and aggressive flash mimicry [54-56]. These *Photuris* fireflies appear incapable of synthesizing lucibufagins *de novo*, but instead must acquire these defensive compounds by consuming *Photinus* prey [15].

Although males that produce more conspicuous flash signals gain a clear advantage through higher mating success, such signals might also carry costs in terms of increased energy expenditure or predation risk. The metabolic cost of producing luminescent signals measured using open-flow respirometry was surprisingly small; flashing increased metabolic rates only 37% above resting values, compared to an increase of 57% during firefly walking [53]. In controlled field experiments the predation costs of firefly signaling were quantified by measuring *Photuris* attraction to signals that

simulated the sexual signals of *Photinus greeni* males [53]. This study demonstrated that *Photinus* flash signals attract predators, and faster flash repetition rates significantly increase predation risk. Thus, the luminescent sexual signals used by male *Photinus* fireflies represent an evolutionary compromise between eliciting responses from potential mates (sexual selection) and avoiding being eaten by predators (natural selection).

5. Evolution of firefly sexual signals

Recent phylogenetic analyses suggest that the sequence of sexual signal evolution within the Lampyridae may have been from pheromones alone, to pheromones coupled with light signals, and then to light signals alone [7-10]. In basal taxa, which are presumed to show ancestral characteristics, adults are active during daytime and normal-winged females produce pheromones as sexual signals. In transitional species, females remain as primary signalers but use light sometimes coupled with pheromones. The evolution of luminescent sexual signals is associated with a temporal shift to nocturnal activity periods. The most recently evolved signaling system involves males as primary signalers producing discrete flashes, and this system has evolved independently in many lampyrid clades [7]. However, even after adults evolved the ability to produce discrete flashes, these sexual signals were replaced at least once by luminescent glows, and also occasionally reverted back to only pheromonal signals [8]. Additionally, Asian *Luciola* fireflies, which mainly show variations on signal system 3, are basal to *Pteroptyx* fireflies which exhibit the well-developed flash synchrony characteristic of signal system 4 [3,7]).

Although phylogenetic analyses have thus elucidated a likely sequence for the evolution of firefly signaling systems, the selective forces driving these evolutionary changes remain entirely unknown. Based on the diversity of signaling systems within this family, and the occurrence of what appear to be bidirectional evolutionary transitions, the selective benefits and costs associated with different signaling systems merit some speculation.

Signal System 1 → 2: Pheromones → Glow (± pheromones)

The relative advantages of pheromonal vs. visual animal signals have been extensively discussed [57,58]. Pheromones provide a persistent, long-range, and energetically efficient signal while visual signals are more easily located (although visual signal propagation requires open habitat without obstacles). Locating the source of a pheromone signal in a moving fluid can be difficult [59], particularly in the absence of visual cues (e.g. at night). The temporal shift from female signaling with pheromones during the day to

signaling by glowing at dusk or at night could potentially have been driven by selection for greater mate search efficiency and/or by diurnal predation pressure. In these darkened environments, the addition (or retention from larval stages) of luminescent glows would complement a female's pheromone signal by improving mate search efficiency once males were within visual range. An alternate explanation for the evolution of the adult female glow is that it originated as an aposematic warning to potential predators, signaling that these often flightless, and thus highly vulnerable, females are chemically defended.

Signal System 2 → 3: Glow (± pheromones) → Both sexes flash

The evolutionary transition between signaling systems 2 and 3 involved fundamental changes, the first of which was the switch from females being the primary signalers (as is the case in signaling systems 1 and 2) to males becoming primary signalers (as in signaling systems 3 and 4) [7]. As a general rule, sexual selection results in males investing more than females in mating effort, which includes both mate search & signal production [60,61]. However, which sex plays the role of primary signaler often changes with different sexual signaling modalities [59,61]. When signaling involves high energetic expenditure or heightened predator risk, as in visual or acoustic sexual advertisements, males are usually the primary signaler. In contrast, when sexual advertisement relies on olfactory signals it is usually the female who signals, and the males who search for and travel to stationary females [59]. Compared with acoustic or visual displays, pheromonal signals are less costly; they not only require less energy to produce but are also less susceptible than other signals to eavedropping generalist predators [57]. Thus, it may be due to the relatively low costs associated with olfactory signals that females are the primary signalers when this is the main signaling modality.

For fireflies, the predation costs associated with luminescent signals are likely to outweigh the energetic cost of producing flash signals. As discussed above, the metabolic cost of flashing is quite low [53], and compared with the presumed high cost of flying, signal production likely represents a minor component of the total energy expended during mate search. However, heightened predator risk has been documented for firefly males when they search and are primary producers of luminescent signals [13,28,53]. Although firefly light production likely evolved as an aposematic signal to warn off generalist predators, some fireflies are specialist predators of other fireflies. *Photuris* females are visually-orienting predators that locate prey males via their flash signals and target them in aerial attacks [52] and by

aggressive flash mimicry [28,54-56]. These *Photuris* "femmes fatales" attract prey by responding to *Photinus* males' courtship signals with flashes resembling those of prey females. Thus, predation by signal-intercepting predators may have selected for males rather than females becoming primary signalers once light came to be used as sexual signals. Additionally, it has been suggested that evolutionary reversions to pheromonal signals and diurnal activity in some North American fireflies may be related to the nocturnal activity space of *Photuris* predators [28]. This seems particularly likely for *Ellychnia*, a diurnal, non-luminescent genus that recent molecular phylogenetic analysis [8] suggests has evolved quite recently from nocturnal, luminescent *Photinus* ancestors.

The second fundamental change that occurred during the transition between signaling systems 2 and 3 was that both sexes evolved the ability to produce discrete flashes of light. The transition from the slowly modulated glows characteristic of signal system 2 may have been selected because intermittent signals are more difficult for predators to locate, or because information can be encoded in temporal features of the signal. Precise on-off control of luminescence required to produce such flash signals appears to rely on several anatomical and physiological specializations. The lanterns of adult fireflies such as *Photinus*, *Photuris, Pteroptyx,* and *Luciola* that use rapidly-modulated flash signals for sexual communication exhibit distinctive anatomical features [62]. Flashes are initiated by synaptic release of the neurotransmitter octopamine, but synapses are not located directly on the photocytes (light-producing cells). In flashing fireflies, nitric oxide (NO) appears to play a role in transmitting signals from nerve synapses to the photocytes [63,64]. Studies of whole fireflies, isolated lanterns, and firefly mitochondia demonstrate that nitric oxide synthase (NOS) is present near nerve synapses, that exogenous NO induces luminescence while NO scavengers block light production, and that NO reversibly inhibits respiration by photocyte mitochondria. Thus, the expression of NOS within the firefly lantern may represent a key physiological innovation that allowed the precise on-off timing characteristic of fireflies that use discrete light flashes as sexual signals.

Signal System 3→ 4: Both sexes flash → Stationary males flash synchronously to attract flying females

This transition involves the evolution of taxa in which males gather in groups and produce synchronous flashes to attract flying females. The evolution of firefly flash synchrony and other communal sexual displays is particularly puzzling because males generally compete for access to females, but little

progress has been made in understanding the phenomenon of synchronous fireflies [65]. Several explanations have been proposed [39,40,65], including that synchrony allows males to maximize their aggregate signal intensity to attract females or to confuse predators or parasites. Alternatively, it has been proposed that synchrony may be an epiphenomenon that arises from female sensory bias toward leading signals [65]. Additional work is needed to differentiate among these hypotheses for the evolution of synchronous flashing.

Other signal system transitions

Phylogenetic analysis suggests that use of discrete flashes as sexual signals by nocturnally active species was followed by loss of light signals and presumed reversion to pheromonal signals by some diurnally active species [8]. It has been proposed [66] that low night-time temperatures in some areas may restrict male flight, resulting in selection for a shift to daytime activity, with consequent selection for pheromonal sexual signals to replace light signals that were no longer effective.

There are also some interesting geographic trends that remain unexplained. For example, signal system 3 is prevalent in most North American fireflies (e.g. *Pyractomena*, *Photinus*, *Photuris*), where flying males use discrete flash signals to search for stationary (but capable of flight) females that respond with flash signals. In contrast, signal system 2 appears prevalent among European lampyrids (e.g. *Lampyris, Lamprohiza*), with flightless females producing glows to attract flying males [12]. It is plausible that the presence of specialist *Photuris* predators in North America imposes a high cost to light signals; this could select for males as primary signalers and select against glowing and flightless females.

6. Conclusions

Clearly we have made considerable progress in our scientific understanding of the evolutionary origins, as well as the costs and benefits of firefly luminescent signals. Phylogenetic analyses suggest that firefly luminescence originated as a warning signal used by firefly larvae to deter potential predators, and was subsequently exapted as an adult sexual signal. Ancestral lampyrids likely relied on pheromones to attract mates, and the ~ 2000 extant firefly species use diverse sexual signals that include pheromones, glows and flashes. Research on *Photinus* fireflies indicates that lampyrid sexual signals are shaped by the dual evolutionary processes of sexual selection through female choice acting in combination with natural selection through predation.

To enhance our understanding of the evolution of firefly signal evolution, much additional work is needed to identify the factors that select for different signaling systems. This includes learning more about factors that can influence signal production (temperature effects on light and pheromone signals), signal reception (light environment, vegetation structure, population density), signal costs (effects of predators and parasites), and flight ability (temperature, iteroparous vs. semelparous reproduction).

Based on the key role that chemical defenses appear to have played in the origin of firefly luminescence, it will be important to learn more about the distribution and diversity of defensive compounds across lampyrid species and life stages. For example, it is plausible that the remarkably similar color patterns and luminescence exhibited by many lampyrids could represent a mimicry complex in which some palatable species rely on signals shared with unpalatable lampyrids to deter their own predators. Much remains to be learned, and future studies will undoubtably shed light on many of these mysteries.

Acknowledgements

I am indebted to Adam South and Raphael De Cock for their insightful comments on an earlier draft of this chapter.

References

1. Meyer-Rochow, B. 2009, Bioluminescence through the ages, In: *Bioluminescence in focus: a collection of illuminating essays*, B. Meyer-Rochow (ed.), Research Signpost, Kerala.
2. Lloyd, J.E. 2002, *American Beetles, Vol. 2*, R.A. Arnett, M.S. Thomas, P.E. Skelley, and J.H. Frank (eds.), CRC Press, Boca Raton, 187.
3. Jeng, M.L. 2008, Ph.D. thesis, University of Kansas USA.
4. Branham, M.A., and Wenzel, J.W. 2001, *Fla. Ent.*, 84, 565.
5. Hunt, T., Bergsten, J., Levkanicova, Z., Papdopoulou, A., St. John, O., Wild, R., Hammond, P., Ahrens, D., Balke, M., Caterino, M., Gomez-Zurita, J., Ribera, I., Barraclough, T., Bocakova, M., Bocak, L., and Vogler, A., 2007, *Science*, 318, 1913.
6. Oba, Y. 2009, Evolution of beetle luminescence, In: *Bioluminescence in focus: a collection of illuminating essays*, B. Meyer-Rochow (ed.), Research Signpost, Kerala.
7. Branham, M.A. and Wenzel, J.W. 2003, *Cladistics*, 19, 1.
8. Stanger-Hall, K., Lloyd, J.E., and Hillis, D.M. 2007, *Mol. Phylog. Evol.*, 45, 33.
9. Bocakova, M., Bocak, L., Hunt, T., Teravainen, M., and Vogler, A.P., 2007, *Cladistics*, 23, 477.
10. Sagegami-Oba, R., Takahashi, N., and Oba Y. 2007, *Gene*, 400, 104.

11. Viviani, V.R., Okawachi, F.M., Scorsato, V., and Abdalla, F.C. 2008, *Photochem. Photobiol. Sci.*, 7, 448.
12. De Cock, R. 2009, Biology and behavior of European lampyrids, In: *Bioluminescence in focus: a collection of illuminating essays*, B. Meyer-Rochow (ed.), Research Signpost, Kerala.
13. Lloyd, J.E. 1973, *Coleopt. Bull.*, 27, 91.
14. Eisner, T., Wiemer, D.F., Haynes, L.W., and Meinwald, J. 1978, *Proc. Nat. Acad. Sci.* USA, 75, 905.
15. Eisner, T. Goetz, M.A., Hill, D.E., Smedley, S.R., and Meinwald, J. 1997, *Proc. Nat. Acad. Sci. USA*, 94, 9723.
16. Underwood, T.J., Tallamy, D.W., and Pesek, J.D. 1997, *J. Insect Behav.*, 10, 365.
17. De Cock, R., and Matthysen, E. 1999, *Evol. Ecol.*, 13, 619.
18. De Cock, R., and Matthysen, E. 2001, *Ethology*, 107, 1019.
19. De Cock, R., and Matthysen, E. 2003, *Behav. Ecol.*, 14, 103.
20. Fu, X., Vencl, F., Ohba, N., Meyer-Rochow, B., Lei, C. and Zhang, Z. 2007, *Chemoecology*, 17, 117.
21. Tyler, J., 2001. *The Coleopt.*, 10, 38.
22. Blum, M.S., and Sannasi, A. 1974, *J. Insect Physiol.*, 20, 451.
23. Ohba, N., and Hidaka, T. 2002, *Sci. Report Yokosuka City Mus.*, 49, 1.
24. Fu, X. 2006, *Coleopt. Bull.*, 60, 207.
25. Tyler. J. 2002, *The Glow-worm*, Lakeside Printing, Sevenoaks, UK.
26. Gronquist, M., Schroeder, F.C., Ghiradella, H., Hill, D., McCoy, E.M. et al. 2006, *Chemoecology*, 16, 39.
27. Tyler, J., McKinnon, W., Lord, G., and Hilton, P. 2008, *Physiol. Entomol.* 33, 167.
28. Lloyd, J.E. 1997, *The Evolution of Mating Systems in Insects and Arachnids*, J. Choe and B. Crespi, (eds.) Cambridge University Press, Cambridge UK, 184.
29. Lloyd, J.E. 2002, *American Beetles*, R.A Arnett, M.S. Thomas P.E. Skelley, and J.H. Frank (eds.) CRC Press, Boca Raton, USA, 187.
30. Ohba, N. 2004, *Integr. Comp. Biol.*, 44, 225.
31. Lloyd, J.E. 1971, *Ann. Rev. Entomol.*, 16, 97.
32. Ohba, N. 1983, *Sci. Report Yokosuka City Mus.*, 30, 1.
33. Ohba, N. 2004, *Mysteries of Fireflies* [in Japanese]. Yokosuka City Museum, Yokosuka, Japan.
34. Lloyd, J.E. 1972, *Environ. Entomol.*, 1, 265.
35. De Cock, R., and Matthysen, E. 2005, *Anim. Behav.*, 70, 807.
36. Lloyd, J.E. 1966, *Univ. Mich. Misc. Publ.* 130, 1.
37. Lloyd, J.E. 1979, *Sexual Selection and Reproductive Competition in Insects*, M.S. Blum and N.A. Blum (eds.), Academic Press, New York, 293.
38. Thancharoen, A. 2007, Ph.D. thesis, Mahidol University, Bangkok, Thailand.
39. Buck, J. and Buck, E. 1978, *Am. Nat.*, 112, 471.
40. Buck, J. 1988, *Q. Rev. Biol.*, 63, 265.
41. Lloyd, J.E. 1973, *Nature*, 243, 268.
42. Case, J.F. 1980, *Bio. Bull.*, 159, 613.
43. Copeland, J. and Moiseff, A. 1995, *J. Insect Behav.*, 8, 381.
44. Ohba, N. 1984, *Sci. Report Yokosuka City Mus.*, 32, 23.
45. McDermott, F.E., 1917, *Can. Entomol.*, 49, 53.

46. Lloyd, J.E. 1978, *Bioluminescence in Action,* P.S. Herring (ed.), Academic Press, New York, 241
47. Lloyd, J.E. 1983, *Annual Review of Entomology,* 28, 131.
48. Lewis, S.M. and Cratsley, C.K. 2008, *Annual Review of Entomology,* 53, 293.
49. Branham, M.A. and Greenfield, M.D. 1996, *Nature* 381, 745.
50. Cratsley, C.K. and Lewis, S.M. 2003, *Behav. Ecol.,* 14, 135.
51. Michaelidis, C., Demary, K., Lewis, S.M. 2006, *Behav. Ecol.,* 17, 329.
52. Lloyd, J.E., and Wing, S. 1983, *Science,* 222, 634.
53. Woods, W.A., Hendrickson, H., Mason, J., and Lewis, S.M. 2007, *Am. Nat.,* 170, 702.
54. Lloyd, J.E. 1965, *Science,* 149, 653.
55. Lloyd, J.E. 1975, *Science*, 187, 452.
56. Lloyd, J.E. 1984, *Fla. Entomol.,* 67, 368.
57. Andersson, M. 1994, *Sexual Selection*, Princeton University Press, Princeton USA.
58. Sebeok, T.A. 1977, *How Animals Communicate*, Indiana University Press, Bloomington USA.
59. Greenfield, M.D. 2002, *Signalers and Receivers: Mechanisms and Evolution of Arthopod Communication*, Oxford University Press, New York.
60. Trivers, R.L. 1972, *Sexual Selection and the Descent of Man,* B. Campbell, (ed.), Aldine, Chicago.
61. Thornhill, R., and Alcock, J. 1983, *The Evolution of Insect Mating Systems*, Harvard University Press, Cambridge USA.
62. Ghiradella, H. 1998, *Microscopic Anatomy of Invertebrates. Vol 11: Insecta,* F.W. Harrison and M. Locke (eds.), Wiley-Liss, New York, 363.
63. Trimmer, B.A., Aprille, J.R. Dudzinski, D.M., Lagace, C.J., Zayas, R., Lewis, S.M., and Michel, T.M. 2001, *Science,* 292, 2486.
64. Aprille, J.R., Lagace, C.J., Modica-Napolitano, J., and Trimmer, B.A. 2004, *Integr. Comp. Biol.,* 44, 213.
65. Greenfield, M.D., 2005, *Adv. Stud. Behav.* 35, 1.
66. Lloyd, J.E. 1977, *How Animals Communicate*, T.A. Sebeok (ed.), Indiana University Press, Bloomington, 164.

Research signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 161-200
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

10 Biology and behaviour of European lampyrids

Raphaël De Cock
Associate Researcher, Evolutionary Ecology Group, University of Antwerp
B-2610 Antwerp, Belgium

Abstract

Firefly research already started a couple of centuries ago in Europe. Nevertheless recent research on the behaviour and the ecology of European species seems to leap behind compared to the other continents. Possible explanations for being less studied may be that European fireflies are just less eye-catching. This may be due to a more northern latitudinal position by which Europe has a relative lower biodiversity compared to other continents. Also because of this more northern position, sunset occurs late in summer and firefly displays are only seen late at night. The high degree of urbanisation, industrialisation and intensive agriculture may have

Correspondence/Reprint request: Dr. Raphaël De Cock, Associate Researcher, Evolutionary Ecology Group
University of Antwerp, B-2610 Antwerp, Belgium. E-mail: rdecock@hotmail.com

had negative impacts on numbers of fireflies making them relatively rare in many European countries. Last but not least, the far less spectacular bioluminescent displays of European fireflies may explain their lower success as a study species. The information about European fireflies is quite scattered and within this chapter I try to summarize what is known about behaviour, ecology, some peculiarities and especially about bioluminescence and latest discoveries. A most updated overview of European firefly taxa and a general review about the types of communication systems used by these taxa are presented. This resulted in a presumption that many sympatrically living species of some taxa may experience quite some confusion in recognising a right mate resulting in the possibility of multiple hybridisations (in the past), which in turn may explain the problem of classifying and species description without the involvement of genetic markers. Also other topics are discussed. Synchronous flashing is known from American and Asian species but does it also occur in Europe? What types of bioluminescent displays do the larvae show and why do they glow? Over the whole, a lot of questions remain unresolved and I conclude with several topics that may deserve research in some near future.

1. Introduction

In general few Europeans can tell they witnessed fireflies in their surroundings. Mediterraneans usually have dinner when the adult insects show their bioluminescent displays, while more to the North where the sun sets at about 10 or even 11.30 pm people usually prepare to go to bed when the glow-worm starts to shine [1]. Yet glow-worms and fireflies play some role in the mind of European people. They are often associated with remembrance of childhood, romantic summer evenings, mystic legends, holidays, or in the past even with Saints, more in particular Saint John whose holyday (24th June), the longest day of the year when in many European countries at midsummernight fires are lit, falls in the peak season of some firefly species. Probably because of this association of fire and light the shiny insect was named after Saint John (or St. Jan, Johan, Johannis, Hans) in many countries: Sankthansorm (Danish, Norwegian), Szent-Jánosbogár (Hungarian), Świetlik świętojański (Polish), Lu mohe de Sint Tch'han (Walloon: Belgium), Jonvabalis (Estonian), Johanniskäfer (German). During the last few decades, a lot of effort has been spent in some European countries and regions (UK, Portugal, Switzerland, Germany, Normandy, Benelux, Denmark, Zurich, Turin, etc.) in popularising fireflies and making them known to the general public [2], in order to organise volunteer surveys and monitor glow-worm populations. Indeed, more and more anecdotal evidence suggests a decline in populations [1-6]. Possible factors for a decline are

habitat destruction and decline as well as fragmentation due to intensified agriculture, industrialisation and urbanisation, drainage and overconsumption of water, light pollution, and pesticides [1,6]. Recently also climate change and global warming have come up as likely future threats.

Depending on the geographical region there seems to exist quite some misunderstanding and confusion about the use of the english terms "glow-worm" and "firefly". In Europe, The term "glow-worm" is commonly used in connection with the flightless larviform females of lampyrid fireflies and with lampyrid larvae. In other parts of the World however, a glow-worm might belong to a totally different taxon (Table 1). For instance, in some parts of the United States, New-Zealand and Austalia, "glow-worms" are luminous larvae or even the adults of certain fungus gnats beloging to the subfamilies Arachnocampinae, Keroplatinae and Macrocerinae [7] or the bioluminescent larvae and larviform females of phengodid beetles. Therefore one often has to specify "lampyrid glow-worms" or "firefly glow-worms" in order to prevent misunderstandings when addressing an international audience. Further down this text we will always refer to lampyrid species when mentioning "glow-worms". Fireflies then, are the winged and flying bioluminescent forms, i.e., exclusively males on our Continent. As a matter of fact, using these terms, the British Isles are only home to glow-worms!

Europe is not particularly famous for its high biodiversity of firefly species. It lies almost entirely in the temperate climate zone where biodiversity is generally lower than in more (sub)tropical regions. In the early eighties only about 35 species were known [8], but at present about 64 European lampyrid taxa, divided over only 8 genera (see Table 2), have been described in the literature [9]. Recent fieldwork proves that yet many more new species are to be discovered [10,11] especially in the southern and eastern parts of the continent where many species seem to have been misidentified in the past or stayed overlooked up till now.

In contrast to the Americas [12-18] and Asia [19-39], research on the ecology, bioluminescent behaviour and communication systems of European lampyrids in mate location, attraction and identification has been rather scarce

Table 1. Regional distribution of fireflies and glow-worms in English terminology.

Region	**Lampyridae**	**Phengodidae**	**Keroplatidae (Diptera, Mycetophilidae)**
Europe	Firefly & Glow-worm	absent	No common name (*Keroplatus* sp.)
USA	Firefly & Lightning bug	Glow-worm	Glow-worm (*Orfelia* sp.)
Australia & New-Zealand	Firefly	absent	Glow-worm (*Arachnocampa* sp.)

Table 2. Updated list of European Lampyrid species and subspecies (with the kind permission of Dr. Michael Geisthardt,[9]).

SUBFAM.	TRIBE	GENUS	SPECIES/ SUB-SPEC	AUTHOR	GEOGRAPHIC DISTRIBUTION
Lampyrinae	Lampyrini	*Lampyris*	*ambigena*	Jacquelin du Val, 1860	Italy, Sicily
			angustata	Motschulsky, 1854 (Lampronetes)	Georgia; Caucasus
			angustula	Fairmaire, 1895	Syria; Asia Minor: Turkey, Anatolia; Mesopotamia
			brevicollis	Motschulsky, 1854	Georgia, Caucasus
			brutia	Costa, 1882	Italy, Calabria
			caspica	Motschulsky, 1854 (Lampronetes)	Georgia, Caucasus; Turkey?; Iran?
			costalis	Motschulsky, 1854	Armenia
			depressiuscula	Motschulsky, 1854	Georgia, Caucasus
			fuscata	Geisthardt, 1987	Italy, Abruzzi
			fuscata subsp. apuliae	Geisthardt, 1987	Italy
			germoriensis	Jacquelin du Val, 1860	Jugoslav countries
			helienica	Geisthardt, 1983	Greece
			lareynii	Jacquelin du Val, 1859	France, Corsica
			lareynii subsp. maculata	Geisthardt, 1987	Italy, Giglio
			limbata	Motschulsky, 1854	Georgia, Caucasus
			membranacea	Motschulsky, 1854 (Lampronetes)	Georgia, Caucasus
			monticola	Geisthardt, 2000	Greece
			noctiluca	Linnaeus, 1758	Europe
			orientalis	Faldermann, 1835	Georgia, Caucasus; Turkmenistan
			pallida	Geisthardt, 1987	Malta, Gozo
			pseudozenkeri	Geisthardt, 1999	Turkey, W-Turkey
			raymondi	Mulsant & Rey, 1859	France, West-Alps; Italy; Spain
			sardiniae	Geisthardt, 1987	Italy, Sardinia
			sardiniae subsp. brunnea	Geisthardt, 1987	Italy, Asinara
			vesuvius	Geisthardt, 2007	Italy, Campania
			vesuvius subsp. insularis	Geisthardt, 2007	Italy, Pontic Isles
			zenkeri	Germar, 1817	Jugoslav countries, Kroatia; Greece; Bulgaria; Romania
			zenkeri subsp. liebegotti	Geisthardt, 1985	Greece, Kyra Panagiá
			zenkeri subsp. sulpuri	Geisthardt, 1999	Greece
		Nyctophila	*anatolica*	Geisthardt, 1982	Turkey, Anatolia
			bonvouloirii	Jacquelin du Val, 1860 (Lampyris)	Italy, Sicily
			calabriae	Geisthardt, 1983	Italy, Calabria
			caucasica subsp. lenkorani	Geisthardt, 1982	Caucasus, Azerbaijan
			colorata	Geisthardt, 1983	Greece, Santorini
			graeca	Geisthardt, 1990	Greece
			heydeni	E. Olivier, 1884 (Lampyris)	Spain, Balearic Isles
			libani	Laporte, 1833 (Lampyris)	Syria; Turkey, Anatolia; Cyprus (?); Libanon
			maculicollis	Fairmaire, 1866 (Lampyris)	Turkey, Anatolia; Iran; Persia; Syria
			molesta	Jacquelin Du Val, 1860 (Lampyris)	Italy, Liguria
			pseudocaucasica	Geisthardt, 1982	Caucasus
			reichii	Jacquelin Du Val, 1859 (Lampyris)	France; Spain; Jugoslav countries; Turkey, Anatolia Portugal
			reichii subsp. brullei	Reiche, 1863 (Lampyris)	Greece
			riegeri	Geisthardt, 1990	Greece
			scabripennis	Olivier, 1907	Asia Minor
		Pelania	*mauritanica*	Linnaeus, 1767 (Cantharis)	S-Europe, France, Portugal, Spain; N-Africa, Algeria, Morocco, Tunisia
	Photinini	*Lamprohiza*	*boieldieui*	Jacquelin Du Val, 1859	France
			delarouzei	Jacquelin Du Val, 1859	France
			foliacea	Baudi, 1871	Italy, Sardinia
			germari	Küster, 1844 (Lampyris)	Croatia, Dalmatia
			morio	Baudi, 1875: (Lamprorhiza)	Italy, Etruria
			mulsanti	Kiesenwetter, 1850	Pyrenees (France, Spain)
			paulinoi	Olivier, 1884	Portugal
			splendidula	Linnaeus, 1767 (Lampyris)	Europe
		Phosphaenopterus	*metzneri*	Schaufuss, 1870	France; Portugal
			montandoni	Bourgeois, 1900	Romania
		Phosphaenus	*hemipterus*	Goeze, 1777 (Lampyris)	Europe; North Italy; Spain; Portugal; Baltic states; Latvia; Denmark; Slovenia; Croatia;

Table 2. Continued

					Bosnia; France; Sweden; Finland; Denmark; England; Belgium; Netherlands; Switzerland; Poland, Hungary; Check Rep.; Slovakia; West Russia, Karelia; Canada, Nova Scotia [49]
Luciolinae	Luciolini	*Lampyroidea*	*achaiaca*	Geisthardt, 1999	Greece
			dispar	Fairmaire, 1857 (Luciola)	Turkey; Bulgaria
			greaca	Laporte, 1833 (Luciola)	Greece, Naxos; Turkey (?)
			quadrinotata	Wittmer, 1935	Greece
			quadrinotata subsp. bimotata	Wittmer, 1935	Greece
		Luciola	*italica*	Linnaeus, 1767 (Lampyris)	Europe; Italy; Romania; Jugoslav countries, Croatia, Slovenia; Turkey; Switzerland
			lusitanica	Charpentier, 1825 (Lampyris)	Greece; Bulgaria; France; Corfu; Italy; Turkey; Caucasus; South Russia; Portugal
			novaki	Müller, 1946	Jugoslav countries

and restricted to few species. Geisthardt [9], who seems to be the only taxonomist working on European lampyrids over the last few decades, has described and (re-)classified most of our species (see Table 2). The behaviour and ecology have been studied more or less thoroughly only in four lampyrid species, i.e. *Lampyris noctiluca*, *Lamprohiza splendidula*, *Phosphaenus hemipterus* and *Luciola lusitanica* [5-6,40-55]. Probably this is also due to the fact that these are the most widely distributed and most common species in Europe. The first three species are as a matter of fact the only species occurring above a latitude of 48°N (north of the line defined by southern France, Switzerland, Austria, Slovenia).

In this chapter we will focus on the bioluminescent behaviour, sexual communication systems and some particular features of the general biology of the best-studied European species.

2. General aspects and peculiarities of European firefly biology

None of the adults of European fireflies are able to feed and few will survive for more than a week or two. Instead they must rely on the internal fatbody, built up during their larval stage, which can last from one to three years depending on the species region and/or climate zone. The larvae differ in their tastes. As with the majority of fireflies, most species actively hunt slugs and snails [6,41]. The tiny larvae are capable of overcoming prey a hundred times larger than themselves by using their sharp, hollow jaws to inject a powerful toxin that paralyses the victim and digests it from within [41]. O'Donald [56] set up an experiment to test for any preference of larval *Lampyris noctiluca* for particular phenotypes of the snail *Cepaea nemoralis* (brown, banded yellow, unbanded yellow) as he observed lower frequencies

of the brown variant in part of a population where also glow-worms occurred. And indeed, the preference for brown and unbanded yellow to banded snails was statistically highly significant. Up till now it stays unclear why *Lampyris noctiluca* shows this strong preference for certain prey phenotypes. *Phosphaenus hemipterus*, in many features an exception to the rule (read further below), prefers a diet of earthworms [49], a habit which it shares with just a handful of its American (e.g. *Photinus* spp.) and Oriental (e.g. *Stenocladius* spp., *Lucidina* spp.) relatives. The larvae spend their entire life hunting and eating prey and after about six to eight moults, most species (*Lampyris noctiluca, L. sardiniae, Nyctophila reichii, Phospaenus hemipterus, Luciola lusitanica*) pupate under leaf litter, stones, pieces of bark, in cracks in the soil or in moss [6,41,49]; pers. obs. in captvity), or even in ants nests (*Pelania mauritanica*, [57]). Except for *Luciola* species that build pupal mud chambers in the soil, and *Lamprohiza* species that seem to make a cell of little pieces of dead leaf litter (pers. obs., unpublished), other European lampyrids do not seem to make any special structure in which they spend pupation; they just shed skins in a hiding place with the right ambient conditions. No aquatic larvae are known to Europe. Semi-aquatic larvae are known from the New World [58] and species with fully aquatic larvae seem to be exclusively limited to the Asian region while some uncertain cases were reported from Africa [36].

The larvae of some species show some conspicuous colour patterns, usually combinations of whitish, pinkish or yellowish-orange lateral spots on a jet-black velvety background (Figure 1; *Lampyris* spp. [10,53]; *Pelania mauritanica* [56]; *Phosphaenopterus metzneri* [59], or a jet-black velvety background with almost fluorescent-like magenta or fuchsia lateral sides (*Lampyris sardiniae* [10]; *Nyctophila reichii*, pers. obs.). Similar dotted colour patterns are also known from genera in Asia (e.g. *Pyrocoelia* spp. [33]; *Diaphanes* spp. (pers. obs.)) or even more consipucous colour patterns combining white and black with orange or even red stripes or dots (e.g. *Stenocladius* spp. [34]). Such conspicuous colour patterns often seem to occur in species with so-called "walkabout larvae" that exhibit a change to diurnal activity and in mature larvae display exposed behavioural patterns, which probably accompany the search for a suitable pupation site [6,10,50,53]. "Walk-about" larvae are know in *Lampyris noctiluca, Lampyris sardiniae, Phosphaenus hemipterus* and *Pelania mauritanica* [6,10,50,53,57]. It seems that glow-worm larvae evolved such colour patterns to advertise to diurnal and visually guided predators that they are distasteful or even toxic [53]. This defensive or anti-predator strategy is better known under the name colour aposematism. Yet the other European firefly taxa in general have a more cryptic lifestyle and show duller and usually brown camouflage colours

Figure 1. Larvae of different species of European firefly; *Luciola lusitanica* (top left; photo YA), *Nyctophila reichei* (top right; photo YA), *Lampyris sardiniae* (middle left; photo YA), *Lampyris noctiluca* (middle right photo; JM), *Lamprohiza splendidula*, (down left; photo RV), *Phosphaenus hemipterus* (down right; photo JM). YA: Yves Adams; RV: Rollin Verlinde; JM: Jeroen Mentens.

blending with the leaf litter or soil backgrounds (*Phosphaenus hemipterus* contrary to its walkabout behaviour; *Lamprohiza* spp; *Luciola spp.* Figure 1).

2.2. Introduction to European species

Details of the adult bioluminescent behaviour will be dealt with further below. Since reviews about European fireflies are rather scarce I will first shortly introduce the best-known species.

Lampyris noctiluca, the Common Glow-worm, one of the first lampyrid species described by Linnaeus [60], is without doubt the best-studied European species. Studies have been performed on its life cycle and general biology [6,41], sexual development [61,62], adult and larval anatomy [63,64], larval ecology in the lab [41] and in the field [65], feeding preferences [10,41,56], onset of circadian bioluminescent activity in adults [43,46], sexual communication [41], system of colour vision in males and evidence for a green-blue chromatic mechanism [66], larval bioluminescent displays and activity period [44], the use of aposematic coloration and bioluminescent defensive displays [52-54], its defensive chemicals [67], light emission spectrum [41,51,68], the structure of its luciferase gene [68], and its distribution worldwide and within countries and regions ([3,10] and ongoing glow-worm surveys). If no misidentifications have been made, this species is the most widespread lampyrid with an almost complete Palaearctic distribution, occuring from Portugal to Northern China from West to East, and from halfway Scandinavia to the Caucasus from North to South [10,8].

Pelania mauritanica has been reported from Southern parts of Europe, like South France, South Portugal and Spain [57], but recent sightings are lacking in spite of renewed search actions for instance in Portugal [11]; pers. comm. Gonçalo Appleton Figueira). The species seems to be more typical of North-African Maghreb countries [57,69]. These authors are the only sources about this species' behaviour and ecology, which is quite unusual for European species. Previously, only normally winged males were known, since they were caught using light traps which are often the standard method to hunt for lampyrid males. However, both Cros [57] and Lhéritier [69] discovered males with shortened wings and wingcases (brachelytrous males) in copula when collecting glowing females. These brachelytrous males also seem to show some other adaptations compared to the normally winged ones, such as shortened legs, more distant coxae, less developed eyes and they are vaguely reminiscent of *Phosphaenopterus* or *Phospaenus* males (see further below) or the American *Pyropyga nigricans* [17]. The female always resides in a burrow and only comes out for a short period during the night in order to attract males with a constant glow [57,69]. This behaviour is also known from *Photinus collustrans*, a Florida firefly with females that reside in burrows [70].

Yet more remarkable is that *Pelania* larvae and females not only like to hide in burrows and cracks in the soil just like many other species but often reside inside an ant's nest. This turns them into non-obligatory myrmecophiles. The frequency of myrmecophile individuals seems to depend on the region as Cros [57] found 77% associated with ants nests in Mascara, Algeria, and Lhéritier [69] 14% in Chella, Morocco. They usually seem to

associate with "friendly" granivorous ants (*Messor barbara* L., *Messor instabilis* var. *maroccana*, *Pheidole sinaitica* Mayr). All life stages are left unharmed and probably some chemical defence protects *Pelania* from being removed or even touched by the ants. Lhéritier [69] noticed a strong smell vaguely reminiscent to elder (*Sambucus nigra*) and this might be associated with toxicity and chemical aposematism [50]. Probably the species prefers ant's nests as the temperature and humidity are highly regulated here and being protected from ant attacks by their chemical defenses the nests are the best choice in the arid conditions of the outside world [71]. Moreover this makes it an ideal environment for the development of eggs, larvae and pupae and even food sources seem to be available (snails reside inside the nests as well: *Rumina decollata*; [69]) Myrmecophile or ant-associated fireflies are also known from other continents, i.e. *Pleotomodes needhami* from Florida, North America that shows a very similar lifestyle and inhabits equally harsh and dry environments like *Pelania mauritanica* [71]. It is yet unclear if these are cases of convergent evolution or if continental drift separated a myrmecophile common ancestor into Old and New World demes [71]. Then recently, Fu & Ballantyne [19] discovered a possibly new and unusual species, *Pygoluciola qingyu,* from mainland China which is not only remarkable for its semi-aquatic mode of life and luminous activity including synchronous flashing and sexual dimorphism in adult colour of bioluminescence, but even more because its larval predacious activity on large mandibulate ants.

The fact that females, after their nightly glow activity, always find the 20 to 30 cm way back to their own preferred burrow suggests that they use some kind of pheromone track [69]. This author also supposed that especially the flightless males use olfactory cues in order to locate females, but rough, on the spot experiments with crushed females were fruitless.

Lamprohiza splendidula is the best studied European species after *Lampyris noctiluca.* It has a more central European distribution. Larval and adult morphologies were compared with those of *L. noctiluca* [72]. Schwalb [41] studied its larval behaviour and ecology and the courtship behaviour and importance of the light organ patterns in the adults. *Lamprohiza delarouzei* is described thoroughly by Bugnion [40] with notes on its general biology and behaviour.

Phosphaenus hemipterus - also known as the Lesser Glow-worm -, is apart from its appetite for earthworms quite exceptional. In fact at first sight it hardly looks like a glow-worm at all. The males are tiny, no more than 10 mm from head to tail, and have relatively large antennae, running about half the length of the body. What makes the males particularly unusual - and possibly unique – amongst fireflies is that they are always flightless: there are plenty of firefly species in which the female is wingless but as yet the Lesser

Glow-worm is the only known species in which neither sex is able to fly. The wings are reduced to small vestiges covered by equally small wing-cases. This makes the male similar in appearance to numerous species of common rove beetles (Staphilinidae) and, together with its diminutive size, causes the Lesser Glow-worm to be easily missed [49]. Being also present in Nova Scotia, Canada (Table1,[50]), it seems to be the only European firefly that, probably occasionally, got imported into another continent [6].

The poorly documented genus ***Phosphaenopterus*** counts two species, *Phosphaenopterus montandoni* from Romania [73] and *Phosphaenopterus metzneri* from Portugal and the French Pyrenees [74]. Since their descriptions these species have not been reported again (except for some recent and vague rumours from Portugal; Gonçalo Appleton Figueira pers. comm.). Mikšić [8] suggests they might just be macropterous forms of their short-winged look-alike *Phosphaenus hemipterus*. Interestingly these species occur at the outer borders of the distributional range of *Phosphaenus* which in turn suggests that the latter species possibly evolved from a winged *Phosphaenopterus*-like ancestor that spread back to North and Central Europe from refugia in the Pyrenees and Portugal, as well as the Balkans after the last glaciation. Assuming such a scenario there could have been strong selection for flightless forms through isolation of founder populations and/or habitat fragmentation (e.g. [17]). Further genetic analysis and study of male genitalia may reveal the phylogenetic relationship between these taxa but first we will have to wait for new specimens. Yet, one fact differentiating *Phosphaenopterus metzneri* from *Phosphaenus* is that -at least if no misidentification happened-, its larva seems to look quite different with a dull brown-blackish colour and orange spots on the hind corner of each segment, (i.e. this sounds more like the description of larval *L. noctiluca*).

Luciola lusitanica is the third best-studied European firefly after *L. noctiluca* and *L. splendidula*. Especially Bugnion [40] delivered excellent descriptions about the adult and larval morphology. Papi [42] and Bialdaccini et al. [75] cautiously analysed the rather complicated courtship behaviour and flash dialogues between and within sexes (details see below). I did not find any studies about the behaviour or ecology of *Luciola italica*. Also, there seems to exist some unreliability of the taxonomic characters commonly chosen for discriminating between these *Luciola* species and Bonaduce & Sabelli [76] retain some doubts regarding the real status of *L. italica* and *L. lusitanica* (often still called *L. mingrelica* in Russia), a doubt expressed by other authors who have stated "that *L. italica* and *L. lusitanica* in reality form two quite distinct geographic races of one unique species" [42,47]. The coastal Montenegro endemic *Luciola novaki* has never been caught again since its description [8] and no information is available on its flash behaviour.

3. Bioluminescent displays & signalling systems in European fireflies: Perfumes, glow-shows and flashing

Table 3 summarizes the bioluminescent displays, light organ patterns and signalling systems used by European lampyrid taxa.

3.1. Who is who? Glow confusions!

Europe harbours mostly lampyrids that rely on the simplest bioluminescent courtship signals, involving flying males attracted to the seemingly continuous (but see below) glow emitted by flightless sedentary females. These generally have enlarged abdomens, but may be larviform, brachypterous, or apterous; [77]. Typical genera that use this signalling system are *Lampyris, Nyctophila, Pelania* and *Lamprohiza*. Lloyd [15] classifies it as System I and Ohba [30] as the (modified) PR system (Table 3).

The onset of adult activity is induced by a reduction of ambient illumination under ca. 1 lux (for *L. noctiluca*: [43,45,46]. The females leave their hiding places around the end of sunset, choose a display spot –either climbing in the vegetation or sitting on the ground or on the litter-, and start to glow continuously [43,45]; Figure 2). The males begin to fly and when they spot a female's light they just fly to her, hover above her and then simply drop down right near her to begin with the copulation [41]. Females stop to glow when mated, so the female glow activity in the field stops earlier, depending on the density of males around [43]. Male activity occurs during the first part of the activity period of the females and lasts about 1 hour, while unmated females continue to glow for 3 to 4 or even more hours. The activity of the males, and therefore indirectly that of females, depends on environmental factors, especially temperature and wind [43].

There is good evidence that location of mates by lampyrid beetles is achieved by a single spectral class of photoreceptor, whose spectral sensitivity is tuned to the bioluminescent spectrum emitted by conspecifics, and is achromatic [35,78,79,80]. However, two spectral classes of photoreceptor seem to be involved in male *Lampyris noctiluca* phototaxis to their bioluminescent mates [66]; binary choice experiments with artificial light stimuli showed that the normal preference for a green stimulus (λmax=555 nm), corresponding to the female bioluminescence, was significantly reduced by adding a blue (λmax=485 nm) component to the signal. This implies an antagonistic interaction between long- and short-wavelength sensitive photoreceptors, suggesting colour vision based on chromatic opponency [66]. Cryosections also showed a band of yellow (blue-absorbing) filter pigment in

Table 3. Summary of adult and larval light organ patterns, bioluminescent diplays and sexual communication systems in European lampyrid taxa (based on [8,40-42,57,88,141] and pers. obs.).

Species group	Sexual communication system	Colour of bioluminescence	Female light organ	Female bioluminescent displays	Male light organ	Male bioluminescent displays	Larval light organ	Larval bioluminescent displays
Lampyris spp.; Nyctophila spp.; Pelania mauritanica*	System I [15] or modified PR system [30]: Sedentary flightless females attract males with continuous glow display	Yellow-green (λmax = 546-550 nm: [41,51,68])	transversal band on sternite VI & second band one on VII + 2 larval dots on sternite VIII; yellow-green light	Sexual display continuous glowing & induced glowing	Larval light organs	induced glowing (spontaneous glows are extremely rare)	Paired, lateroventral ovally shaped dots on sternite VIII	Spontaneous glows & induced glowing
Lamprohiza splendidula	System I [15] or modified PR system [30]: Sedentary flightless females attract males with continuous glow display	Yellow-green (λmax = 546-550 nm: [41,51])	Broken transversal band on sternite VI & transversal band on sternite VII + larval light organs	Sexual display continuous glowing & induced glowing	transversal band on sternite VI & second band one on VII + larval light organs in freshly pupated individuals only	Spontaneous continuous glowing & induced glowing	Highly variable, often asymmetric number and pattern; usually bright lateral spots in abdominal segments II and VI + lateral smaller dots in the segments in between (all dorsally translucent)	induced glowing
Lamprohiza mulsanti, L. paulinoi, L. delarouzei & other Lamprohiza spp.	System I [15] or modified PR system [30]: Sedentary flightless females attract males with continuous glow display	Yellow-green; most probably like *Lamprohiza splendidula*	transversal band on sternite VI & second band one on VII + larval light	Sexual display continuous glowing & induced glowing	Vestigial or no light organs or larval light organs in freshly pupated individuals only	No glowing (needs confirmation for *L. foliacea*)	Highly variable, often asymmetric number with pattern depending on the species; usually lateral spots from abdominal segments I to VI or even VIII	induced glowing

Table 3. Continued

Phosphaenus hemipterus	LB system [30]: chemical communication by pheromones (males are diurnal)	Yellow-green (λmax = 546 nm: [51])	Larval light organ	induced glowing	Larval light organ (dorsally translucent)	induced glowing	(dorsally translucent) Paired, laterventral ovally shaped dots dots on sternite VIII; yellow-green light	Spontaneous glows & induced glowing
Phosphaenopterus	Unknown; probably like *Phosphaenus*	Unknown; most probably like *Phosphaenus*	unknown	unknown	unknown	unknown	Unknown; most probably like Phosphaenus sp.	unknown
Luciola spp.	HP system [30]; Flash dialogues between male and female;	All stages yellow (pers. obs.)	Transversal more or less broken band on sternite VI (= ventrite V)	Sexual display flashes & induced glowing	Sternite VI + VII (= ventrite V+VI)	Sexual display flashes & induced glowing	Paired, laterventral ovally shaped dots dots on sternite VIII	Spontaneous glows & induced glowing
Lampyroidea spp.	Unknown; probably a form of flash dialogues between male and female	unknown	unknown	Unknown; probably sexual display flashes & probably induced glowing	Sternite VI + VII (= ventrite V+VI) some species lack light organs [72]	Unknown; probably sexual display flashes & probably induced glowing	unknown	unknown

Figure 2. Displaying *Lampyris noctiluca* female. Note the turned upward abdomen tip with the segments containing light organs (photo: Marek Kozlowski).

the fronto-dorsal region of the male compound eye [66]. This presents an intriguing paradox because the resultant reduction in photon catch would tend to restrict green-blue colour vision still further. Although their precise flight patterns have not been studied, male glow-worms are believed to mate-search by flying low over the vegetation [6], so the ventral retina probably plays a vital role in the initial stage of mate location. The primary function of these filter pigments in nocturnal lampyrids may therefore be more concerned with shielding the sensitive eye from skylight than with signal discrimination [66]. In this regard the distinction should be made between nocturnal species, such as *L. noctiluca*, and crepuscular fireflies: the latter possess filter pigments that are quite different in both spectral absorbance properties and location within the eye [81], and it has already been suggested that the role played by filter pigments may differ between nocturnal and crepuscular species [80].

From what is described in literature, the general aspects of glow and courtship behaviour of *Pelania mauritanica* seem very similar, if not identical, to that of *Lampyris noctiluca* [57,69]. The same is true for *Nyctophila reichii* (pers. obs., [11]). Pheromones are probably not involved at larger distances. However, when the male arrives at the female contact pheromones may be important [41], maybe in the form of cuticular hydrocarbons [82]. However, from personal observations in Portugal in 2007, it seems that species recognition is not only difficult for taxonomists, but even for males belonging to different taxa. On several occasions males of *Nyctophila reichii* were seen trying to copulate with *Lampyris sp.* females, and vice versa. Such observations suggest that contact pheromones are quite inefficient as species discriminatory cues – at least in nocturnal species [82] -, and their purpose may rather be finding the right copulatory position, as proposed by Schwalb [41]. Moreover, the female (and male) light organ patterns and the spectral emissions of *Lampyris, Nyctophila* and *Pelania* are as good as identical (see Table 3, Figure 4; [10,57,69]. All these factors suggest that species recognition is very poor within and amongst these genera.

Currently, there is no information whatsoever on whether sympatric species evolved, adapted certain behaviours (e.g. female choice), selected different activity periods at night or shifted their appearance during the season in order to prevent mistakes and such forms of interspecific male competition. Such misidentifications by the males themselves may also form the basis for numerous hybridisations. The possibility of multiple hybridisations may in turn explain why the identification of European species within these taxa is so difficult, based on the high levels of variation in coloration and morphology as testified by taxonomists [57,83].

Sometimes *Lampyris* males, but only very rarely, also glow continuously and spontaneously in flight from tiny light organs they inherited from the larval stage [84]; pers obs. only once). Why would they do so? Adult lampyrids, as well as the other life stages, almost always react with distress-glowing (or flashing) when handled or when disturbed. This suggests a defensive function. Maybe the spontaneous glowing indicates stress, either in the form of disease or possibly as an anti-predator reaction if they were able to perceive bat echolocation (either adaptive as "facultative aposematism" or "startle signal")? In that case we would expect to see more of such behaviours in the field, especially in areas with high bat densities, unless the glows are to weak to notice down below from the ground. The hypothesis, "do male lampyrids react with bioluminescent displays when stimulated with infrasonic impulses" could be easily tested.

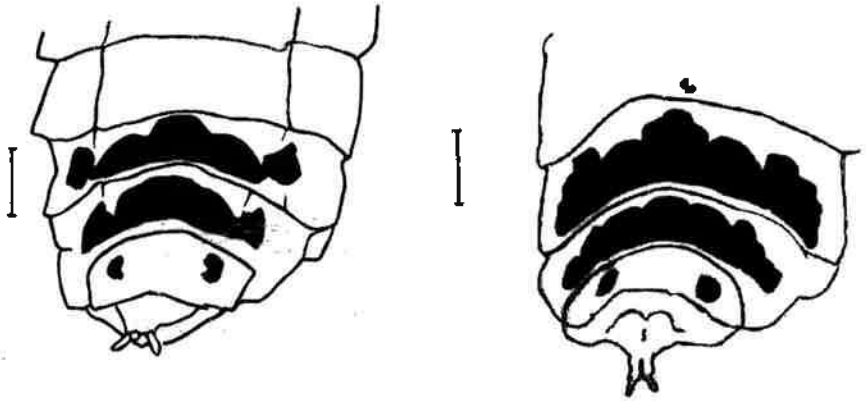


Figure 3. Female light organ patterns of *Lampyris sardiniae* (left) and *Lampyris noctiluca* (right) (from [10]).

3.2. Nature's mini disco light shows: Selection for more spots?

The female light organ in *Lamprohiza* species (and their North-Amercan close relatives of the genus *Phausis* from the Appalachian Mountains; [13] is completely different from that of other lampyrid genera and is somewhat reminiscent of Phengodid or Rhagophtalmid species. It consists of quite a normal looking adult lampyrid-like light organ in ventral segments V and VI, also called "ventrites" (Tergites VI+VII) and depending on the individual or species an additional number of 4 to 12 smaller lateral light spots in the abdominal segments that are "inherited" from the larval stage (details check Table 3). Females of the genus *Lamprohiza* often show a great deal of individual variation in the number of lateral light organs (pers. obs.; [41]). In contrast to females of the other continuously glowing genera, they rarely climb up plants or grasses to broadcast their glow-display sites but rather stay on the ground or on dead leaf litter. They also do not curl up their abdomen sideways in order to let the light shine upward, but rather raise their abdomen, fully equipped with light organs, dorsally upward (Figure 4 & 5).

In his lab experiments with models of female light organ patterns Schwalb [44] discovered that *Lamprohiza splendidula* males are attracted to any colour of light he presented (red, yellow, green, blue), but with a supernormal preference for blue light. Interestingly, *Lampyris noctiluca* males showed a very pronounced preference for yellow shining models (ca. 571 nm) and preferred these over their own yellow-green shining females (ca. 550 nm). Schwalb [44] also found that *Lampyris noctiluca* males prefer models with similar intensity and the same light organ pattern as natural females, whereas the *Lamprohiza splendidula* males select for more intensely shining lures, be it larger scale, stronger shining models or models with more light dots regardless of the configuration. This last observation shows that there exists a strong male selection for stronger light emissions and it may explain why we

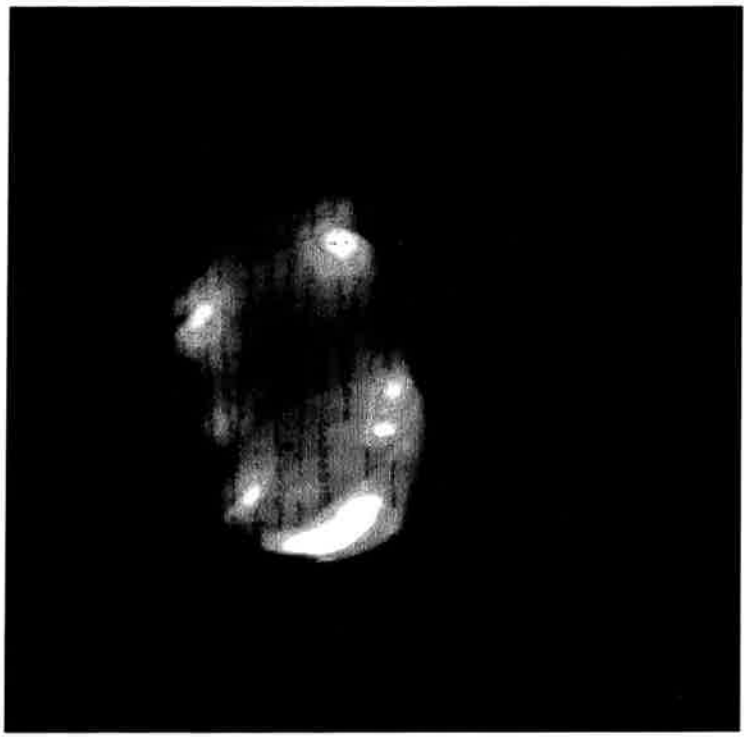


Figure 4. Female light organ pattern in *Lamprohiza splendidula.* (photo: Raphaël De Cock).

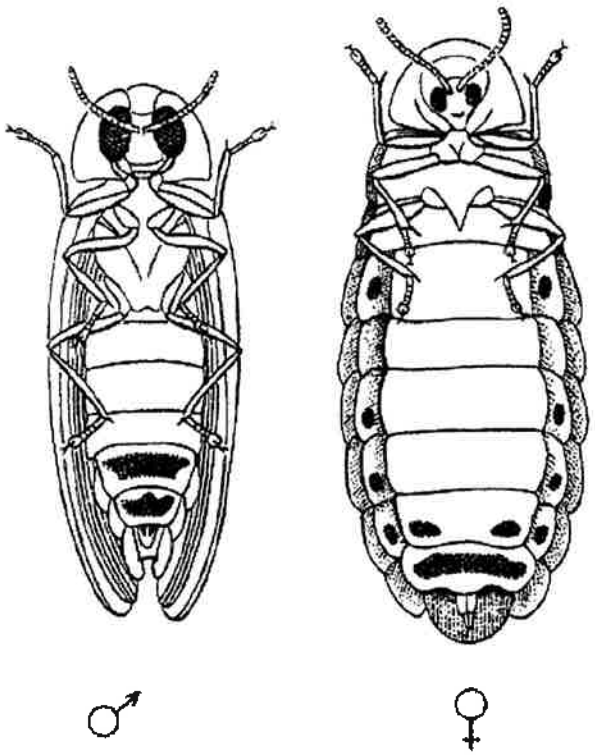


Figure 5. *Lamprohiza splendidula* male and female light organ patterns (indicated as dark areas on the abdomen).

observe such individual variation in the number of light dots and their size in *Lamprohiza* female light organs. The same is true for *Lampyris* males, yet that they select for models with a fixed light organ pattern, natural light intensity and colour of emission, and that is exactly what we see in the field.

However, own field and lab observations contradict Schwalb's [44] findings. In the field *Lampyris* males seem to be far less choosy for colour and intensity of light traps (6V light bulbs, green or yellow LED, glow-stick tubes, *Lamprohiza* females all work fine), while *Lampohiza splendidula* males (contrary to *L. paulinoi* and *L. mulsanti*: see [11]) are very difficult or impossible to lure with whatever kind of light trap (unpublished results, R. De Cock). It should be noted that although his findings look convincing, Schwalb used few males (10 to 60) in his experiments, did not repeat experiments per treatment and did not apply statistics. Another suspect fact is that the success rates of attracting males by natural females are quite low in Schwalb's experiments (*Lampyris*: 40%-65%; *Lamprohiza*: 25%-40%), while in natural conditions and high male densities it is very difficult to find unmated (and per definition glowing) females, since they are found almost immediately by the males [6,85].

An additional peculiarity of *Lamprohiza splendidula* is that also the males glow continuously or show minute-long glows of variable intensity in flight from two ventral bands in the last abdominal segments (Figure 5). They start activity at sunset and first fly short distances low over the forest floor or show short lasting glows. When it gets darker they fly higher (1.50m to just under the canopy), glow continuously and also fly in more open spaces like forest edges and over fields and orchards [41]. These displays look like fairytale sceneries full of slowly air-drifting little lights. After about 45 minutes to one hour male activity gradually decreases to stop completely two hours after sunset. Similar behaviour is seen in *Phausis reticulata* males from North-America [13]; pers. comm. Lynn Frierson Faust) and *Diaphanes* and *Pyrocoelia* species from Asia ([30,86], pers. obs.). Noteworthy is also that all these genera know species with males lacking functional light organs. For instance, the males of the other *Lamprohiza* species often show pale areas on the segments where one would expect adult light organs; yet, no luminescence is observed [40]. So why did some nocturnally active species apparently lose the male's ability to glow in the course of evolution, or why do they start glowing only when getting disturbed (e.g., the *Lampyris–Nyctophila-Pelania*-group), while other taxa glow in full glory and conspicuously in flight?

Male bioluminescence does not seem to be involved in courtship signalling, as *Lamprohiza splendidula* females glow spontaneously and do not answer to overhead flying glowing males. Closely related American

Phausis reticulata females seemingly respond to glowing males later at night; yet it remains doubtful that male glowing is evolutionary adaptive for courtship, since females glow spontaneously without the need of a male's glow during the normal male activity period [13]. *Lamprohiza splendidula* males land at a distance from the female and crawl the last few centimeters to her, without glowing or only weakly glowing [41]. Thus, the male's glowing is probably not involved in female choice either especially if several males arrive at once. So what is the adaptive value or function of male glowing in these cases? The males seem to fly quite dispersed, but it is difficult to decide if this effect or impression has to do with spacing on the basis of the light of nearby flying males. Moreover, what would be the biological significance of such behaviour? Or is it used for illumination [14] in dark environments like in the dense forests, which these species with glowing males seem to prefer as a habitat? However, most probably it is involved in sort of an anti-predator display against bats or night hawks (Caprimulgidae) as suggested earlier [16,50]? Favouring this hypothesis are anecdotal reports of bats that approach fireflies, but then turn away from the shiny target at a distance of ca. 1 m. Future experiments analysing male behaviour in relation to the glow of nearby males, or bioassays and predation experiments with bats and nighthawks might tell what is going on.

3.3. A handful of species flash simply or in synchrony!

In Europe probably only 2 genera, *Luciola*, and less certain *Lampyroidea*, counting altogether for about 8 species (12% of European taxa), show flash bioluminescence. All of these "flashy" species, occur south of the line southern France, South slopes of the Alps, Slovenia, South Ukraine, South Russia (see Table 2), and are, thus, restricted in their distribution to only about 30% of Europe. Up till now there are no published studies on the (bioluminescent) behaviour or ecology of *Lampyroidea.* From personal communication with local informants it seems they use flash communication, but this needs to be confirmed yet.

Neither the flash behaviour nor the communication system of *Luciola italica* and *L. novaki* have been described yet. Papi [42], Mikšić & Mikšić [48] and Bialdaccini et al. [75] provide the only European studies on the flash and courtship behaviour of *L. lusitanica* (Figure 6). These reports are rare and excellent examples of studies describing and analysing the flash behaviour of a species in such detail. We will summarize here most of their findings.

One of the most exciting findings for Europe is that also from this continent, more exactly from Sarajevo, Bosnia-Herzegovina (former Yugoslavia) we have reports on synchronously flashing fireflies! They probably

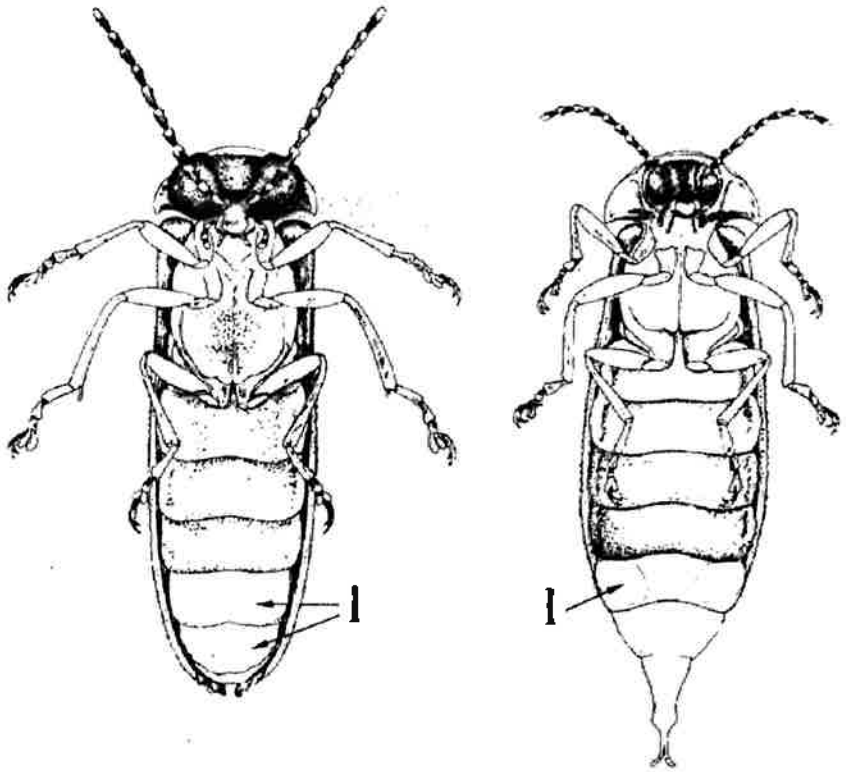


Figure 6. *Luciola lusitanica*, male (left) and female (right) light organs are indicated [42].

belong to some regional form or maybe a still unidentified sibling species of *Luciola lusitanica* since posterior wings are missing in the females [42,48,75]. After their discovery these synchronized flashing populations have not been studied again, but this could have been a side effect of the recent turbulent political situation in that region.

Papi [42] reports that there seem to be local variations or "dialects" in the flash displays (i.e flash frequencies, flash and interflash lengths and variability here within) with differences observed between populations from Nice (France), Bologna (Italy) versus Pisa and Genoa (Italy). This suggests that flash behaviour could perhaps be used as a taxonomic character in order to classify and identify species, just as was done for *Photuris* or *Photinus spp.* in the Americas where identically looking species seem to be isolated and are recognisable by their flash courtship behaviour [18]. Since for the moment we only have information about the situation near Pisa we will stick to Papi's [42] findings.

It is typical of females of many species of flashing Lampyridae to respond with steady latency to the "on" of a light stimulus and interspecific differences in latency times at the same temperature can often be used as taxonomic characters [87]. Yet, *Luciola lusitanica* communication systems

can be classified as the *HP* system of Ohba [30] or System II according to Lloyd [15]. Here males fly and flash with an unfixed frequency and females just respond to male flashes with a fixed delay response and their own peculiar and recognisable flash display which differs in flash length, form and/or rate from male flashes. However, in most (New World) species the female seems to be the discriminating partner, checking for and responding to the right male flash length, flash number and pulse interval length. In *Luciola lusitanica*, however the male is the discriminator leading the flash dialogue by changing the flash frequency and checking whether the female is able to respond to his changes with a response flash of the right length and at the right response delay.

In both sexes of *L. lusitanica* the bioluminescent displays may consist of both clear flashes as well as dim light emitted for variable time periods. Flying males emit an average 1.8 flashes per second at 17°C with occasional dim light emissions during interflash periods (Figure 7). These dim emissions might be adaptive for illumination [14] and consist of either a close sequence of short irregular flashes or a more regular flicker [42]. Females are never seen flying. They have a thicker and heavier body and shorter wings and wingcases than males.

Females regularly respond with a typical flash (see Figure 8) to each light stimulus of steeply rising intensity, regardless of stimulus intensity, length, or

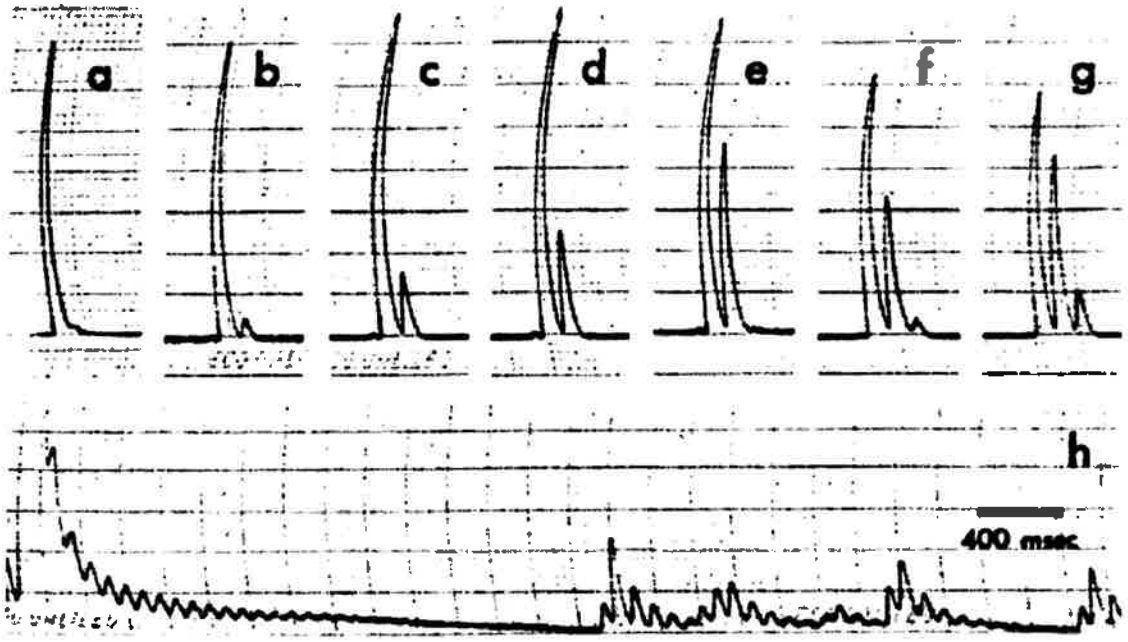


Figure 7. *Luciola lusitanica,* (a-g) male flashes of various forms, (h) a flash (with top part cut out on the left side) slowly dying out with a flicker and dim light emissions [42].

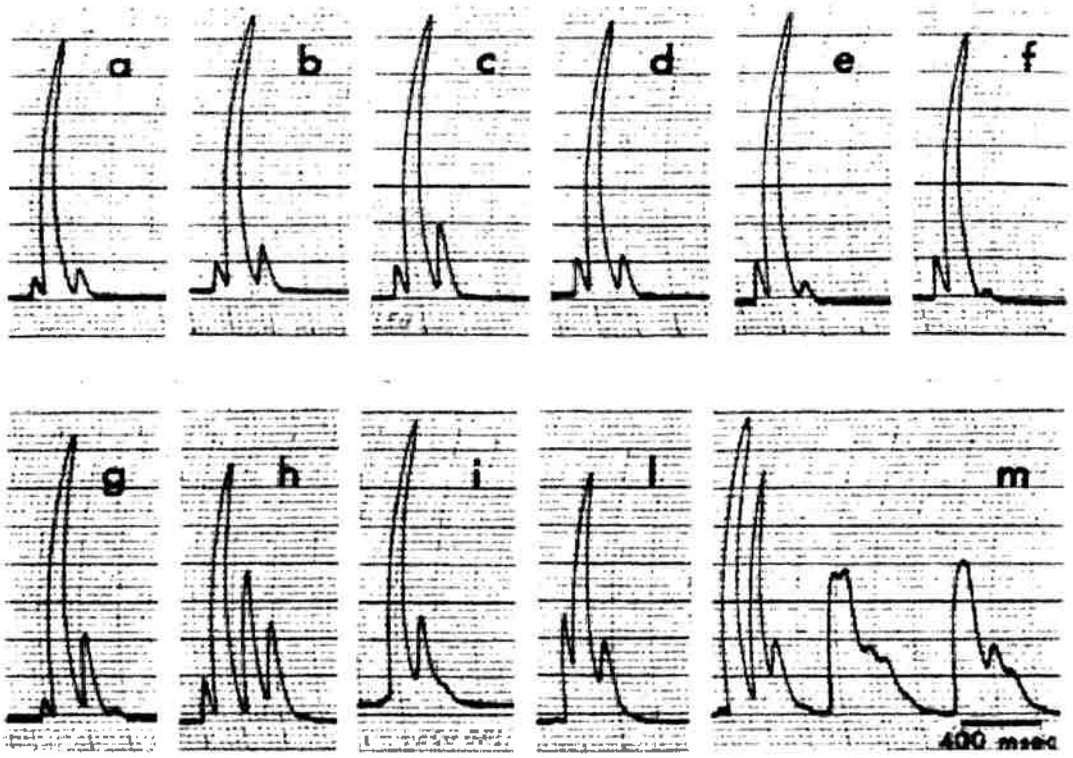


Figure 8. *Luciola lusitanica*, female flashes (a-g) dialogue response flashes, (h-m) irregular flashes when disturbed or when flash dialogues are interrupted [42].

spectral range (at least between 413 nm (violet) and 682 nm (red)). The response flash is emitted at a specific delay time after the stimulus with a delay that increases exponentially at lower temperatures. When the stimulus frequency increases progressively above a rate of 2 flashes per second, the female at first responds 1:1 for brief periods or else flashes once every other stimulus, and subsequently fails to respond regularly. In rhythmical stimulation by light pulses with slowly rising intensity females don't respond or respond irregularly, sometimes flashing at rates almost equal to the stimulus flashes but with no fixed temporal relation between stimuli and response flashes.

Mechanically stimulated males or females whose flash dialogues with males have been interrupted show irregular flashing with longer flash lengths composed of many peaks of which the flicker can be seen by the human eye.

The scheme in Figure 9 summarises the general flash communication protocol for *L. lusitanica* based on Papi [42]. Flying males upon receiving a female response to their own flashing, will fly toward the female, make an inspection-dialogue flight of variable duration, then land and approach the female by crawling on the ground. The substrate where the female is flashing seems to play a role as the inspection-dialogue phase may be lengthened depending

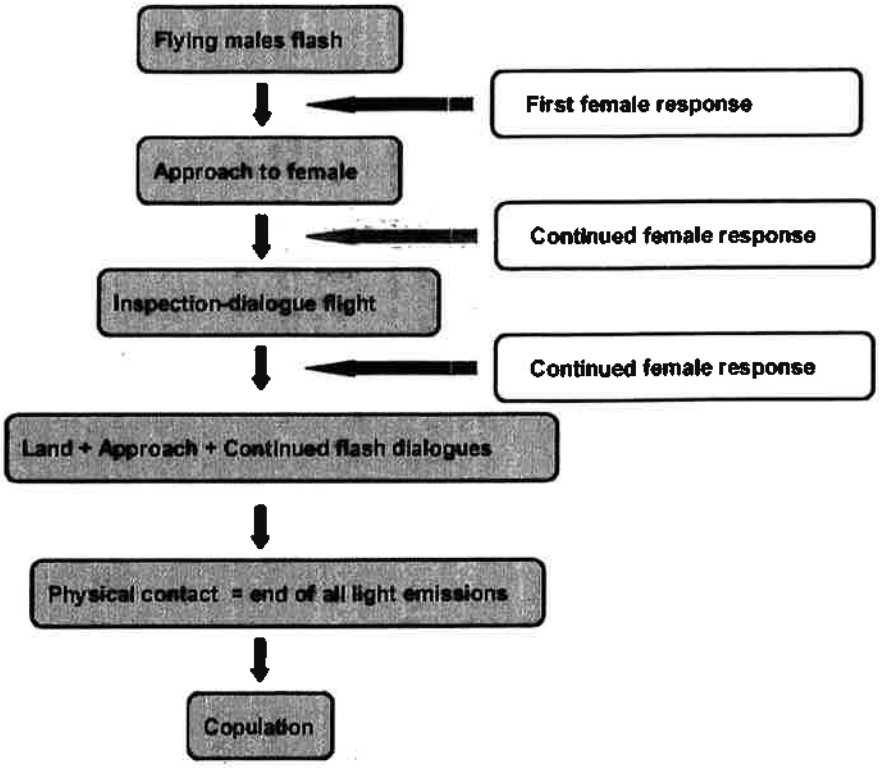


Figure 9. Generalized flash communication protocol of *Luciola lusitanica* from Pisa, Italy, based on Papi [42].

depending on the characteristics of the female surroundings. Flash dialogues take place during all phases of the approach to the female, but all, or nearly all light emission ceases immediately after the partners make physical contact. The spectral characteristics of the female light signal don't seem to play an important role as flashes of different colours ranging from 473nm to at least 644nm can attract males. Features of the female response that play an important role in male recognition and positive responses (resulting in landing or abandoning a female) are flash length and flash response latency. The female flash form (the triple flash) does not seem to have a major influence on male behaviour.

Male-to-male responses

Males on the ground flash irregularly and sometimes show female type responses to light stimuli. Such behaviour is frequently seen in males which are engaged in unsuccessful dialogues with females. With their flashes these males induce the other overhead flying males to engage in inspection-dialogues with them and occasionally even to land. Male-to-male dialogues may take several forms. Perching males may engage in flash exchanges according to certain rules. The most common type (a-b, a-b, ... type dialogues) simulates a heterosexual dialogue, with one of the two males (A,

leader) leading the dialogue and the other (B, follower) responding after a fixed delay as a female. The mimicking male very often flashes irregularly in response to a rhythmical light stimulus maintaining a frequency very close to the stimulation rate, or he even delivers regular 1:1 responses. The response latency of female simulating males is more variable than the actual female latency. In another type of dialogue the leader emits two flashes for each follower's flash (a1-b-a2, a1-b-a2, ... dialogues). The second flash seems to be a response to the follower's flash, thus the leader subsequently shifting"role", while the follower seems unable to act like a leader due to the high frequency rate of flashes. Yet in a third and less frequent type (a-b-a-b... dialogues) no role difference is discernible between the males.

According to Papi [42] the biological significance of such male-to-male flash dialogues is to induce other males to inspect an area where a difficult to approach female is to be found and as such in improving her chances for fertilization. It seems more likely that such behaviour is rather involved in complex intra-sexual competitive strategies which are yet not fully understood. Similar female-mimicking of rejected males have also been observed in the American *Photinus carolinus* (pers. comm. Lynn Frierson Faust).

Table 4. Major characteristics of bioluminescent behaviour of male and female *Luciola lusitanica*.

Male
• Light organs on entire ventrites 5 and 6 (Figure 6)
• Spontaneous flashes
• Occasional dim light emissions during interflash periods
• Flashrate: at flight 1.8 flashes/second at 17°C; decreases with temperature; variable in landed or perched males
• Flash form and length: at flight 200-250 msec, usually very intense peak accompanied by a series of minor ones (Figure 7); variability is higher in males landed or perched males than in free flying individuals.
• Disturbance flash flickers
Female
• Light organ on ventrite 5, consisting of a transversal band with highest light intensities coming from spots on the lateral sides (Figure 6)
• Occasionally spontaneous flashes
• Occasional dim light emissions during interflash periods
• Flash form and length: composed of three peaks with the interval between the first two peaks increasing exponentially at decreasing temperatures (ca. 77 msec at 26°C; 245 msec at 10°C). Second peak most intense, while first equal, smaller or larger than third one. The latter may be missing or is replaced by other peaks of decreasing intensity. Flash length depends on temperature and number of peaks, but the time interval between the first and second peak is remarkably constant at a given temperature (Figure 8).
• Response flash delay: 160-590 msec depending on temperature (respectively 26-10°C)
• Disturbance flash flickers

3.4. Day-active species

For a long time it stayed unclear whether adult *Phosphaenus* show day-active courtship behaviour [74] or rather are nocturnal [4,8] since both sexes are weakly bioluminescent. But recent evidence established that the males are indeed diurnally active and use olfactory cues to locate their mates [49,55]. Male *Phosphaenus hemipterus* like those shown in Figure 10, are extraordinary, at least within the European fireflies, not only in being flightless and active during the day, but also in using its large, sensitive antennae to sniff out the airborne scent – or pheromones -, produced by the even more inconspicuous female. No more than 13 mm long, she looks like a miniature version of a *Lampyris noctiluca,* but lacks the well-developed light organs that make the latter so visible. Whatever feeble light she can muster comes from two small dots at the tip of her abdomen which, like those of the male, are inherited from the larval stage and are switched on if she is disturbed. This makes her extremely difficult to find (at least for humans), so in most places the majority of sightings are of males, which often roam about on bare surfaces such as footpaths, pavements and the bases of walls on sultry June afternoons [49].

Figure 10. A day-active male *Phosphaenus hemipterus.* Note the large antennae, short wings and wingcases and the pale translucent dorsal spots in the last segment that permit that the light produced in the more ventrally situated light organs is also visible from above (Photo: T. Tolasch).

3.5. Larval glowing

It is a well-established fact that adults of nocturnal fireflies use light signals for sexual communication, but surprisingly little is known about the functions of glowing behaviour of the larvae. Defence is the most cited function [see 89]. Many other plausible functions of luminescence have been proposed, but most of these reflected the inventiveness of the observers rather than being based upon any supporting evidence [89,90,91,92]. Examples of prey attraction are known from Elaterid beetles like *Pyrearinus* sp. larvae that probably use luminescence to attract flying termites as prey [93], or the famous "femmes fatales" of *Photuris spp.* which mimic courtship flashes of other firefly species in order to lure males and to acquire additional chemical defences [94-97]. One may also think of the and luminous larvae of fungus gnats, Mycetophilidae, which attract insects into their sticky webs [7,98,99]. However, in all these examples the predator stays immobile whereas lampyrid larvae actively hunt for prey. Therefore, prey attraction seems an unlikely function.

Illumination and communication have been proposed as alternative possibilities [89,97,100,101]. Some phengodid beetles, like the railroad-worms, *Phrixothrix spp.* have continuously shining red headlights and a preliminary electroretinogram study (V. R. Viviani, E. J. H. Bechara, D. Ventura and A. Lall unpublished data; [100]) revealed red-shifted vision in these larvae, implying that they might use a visual channel not used by their prey.

Dreisig [44] suggests that they act as a competitive signal since lampyrid larvae seem to be evenly spaced in the field. Unfortunately, he never presented data on the spatial distribution of lampyrid larvae, nor did he test the effect of glow signals on the behaviour of the larvae to support his view. Kaufmann [102] also proposed a competitive signal in which egg-laying females use the spontaneous larval glowing as a cue to avoid densely populated areas. Even when kin selection is involved, it remains difficult to see how such complex communication could have evolved, especially when the larvae have a very poorly developed visual system of simple ocelli.

Even if bioluminescence is used for such non-defensive functions it is difficult to explain how it could have evolved without protection against visually guided predators. Moreover, Dreisig [44] argues that deterring predators is not the primary function of luminescence in lampyrid larvae because they do not only emit light when attacked but glow in a regular way for several hours in exposed places, making them more conspicuous to predators and abolishing any possible startling effect. However, Dreisig apparently overlooked the possibility of the luminescence being an aposematic display. It seems logical that visual patterns that catch the

attention of predators should have an anti-predatory function, unless the organism has other adaptations to evade predation. From the point of view of predation, light emission could have evolved under similar selective pressures as other visually conspicuous signals, such as colour patterns. Surprisingly, bioluminescence has rarely been mentioned as a warning signal in studies concerned with the principles or the evolution of aposematism. Recent evidence firmly supports the hypothesis of bioluminescent aposematism [52-54,103].

3.5.1. Induced glowing

Disturbance-induced glowing is a typical type of luminescent display in lampyrid larvae [16,58,89,104]. Hereby larvae –but also all other life stages, from hatch-ready eggs to adults- glow continuously several seconds or minutes in response to several types of disturbance and usually stay motionless or act dead [44,89,104]. In European taxa this display is known from *Lampyris, Phosphaenus, Lamprohiza, Luciola, Nyctophila* (pers. obs.) and *Pelania* [57], and probably occurs in all species. In *Lampyris*, *Lamprohiza* and *Phosphaenus* larvae the disturbance intensity threshold to respond to with induced glows varies between individuals from slight disturbance, such as weak sounds (e.g. rustling of dead leaves), over surface vibrations, to rough handling such as grasping with forceps. The disturbance-intensity threshold seems to decrease with feeding status, whereby recently moulted and slender larvae tend not to glow, whereas larvae that get ready to moult or pupate begin already to glow at sudden sounds [50]. Noteworthy is that *Lamprohiza splendidula* does not react with glowing to any disturbance except for one to two weeks prior to moulting and then usually already to acoustic stimuli. However, Portuguese *Lamprohiza* larvae (either *L. paulinoi* or *L. mulsanti*) always respond with glowing (pers. obs.). I assume that, when in danger and being less mobile and more vulnerable due to their well-fed status, larvae that prepare to moult instead of fleeing choose the best of a bad job, which is trying to deter or confuse a possible predator by showing the induced luminescence display instead, in order to startle the predator or as a facultative aposematic display.

3.5.2. Weak body glow

It should be noted that apart from light organs (at least in European taxa like *Lampyris*, *Phosphaenus* and *Nyctophila*) larvae, but also eggs, pupae and even adults, show a very faint overall body glow from all body parts that are not pigmented (pers.obs.). This is visible with dark-adapted eyes in a darkroom or even recordable with specialized light-sensitive gadgets (pers.

comm. Laurence Tisi). This effect has also been described from other species by several authors [cf.,105].

3.5.3. Spontaneous bioluminescent displays in larvae

Apart from disturbance-induced luminescence, glow-worm larvae often show spontaneous glows, emitted intermittently at night without any indicative reason. Spontaneous glowing has been observed in European *Lampyris spp., Phosphaenus, Nyctophila reichei* and *Luciola lusitanica* (pers. obs.,[44]. Although Schwalb [41] described spontaneous luminescence in *L. splendidula* from laboratory observations, it seems very doubtful that this species shows the display, since I never observed it in the field in neither this nor any other species of the genus. Schwalb's [41] recorded luminescence was probably a disturbance-induced rather than spontaneous display. The absence of spontaneous glowing behaviour is only known from some aquatic species, but these seem to show spontaneous glows once they leave the water [89,104].

The spontaneous display is especially seen in crawling larvae (Figure 11; [31,41,44,50,58,89,91,94,102,104,106]. This coincidence of spontaneous glowing and locomotion as seen in Figure 11, is one of the key predictions for the possibility of luminescent aposematism in glow-worm larvae. Obviously, locomotion renders the larvae more conspicuous to visually-guided predators that are adapted to hunt at night, such as amphibians. In that manner, the spontaneous glow display could be considered more a context-dependent or facultative form of aposematism [52,54].

Almost no detailed studies have been performed on the behavioural characteristics of the spontaneous display, i.e. the lengths of glow pulses and extinguished intervals, pulse frequencies, or the proportion of time that individuals spent glowing, and interspecific differences. Some data found in the literature suggest that the length of glow pulses and pauses between glows vary within individual larvae [44,89,102,104], but that averages may differ between species [89,102,104]. Preliminary results of ongoing analyses suggest that the duration of glow pulses and the amount of time spent glowing differ between *L. noctiluca* and *P. hemipterus*, and that ambient light suppresses the amount of time that is spent glowing (Figure 12, [50]. Kaufmann [102] also reported that ambient factors seem to influence the characteristics of the display. Further studies could test whether the perceptible interspecific variation in pulse frequency is associated with the detectability of the signal within certain microhabitats, whereby lower frequencies are expected in open habitat and higher frequencies in denser vegetation [58,89]. Sometimes *Lampyris noctiluca* larvae show periods of more intensive glowing, which gives the impression of some sort of synchrony

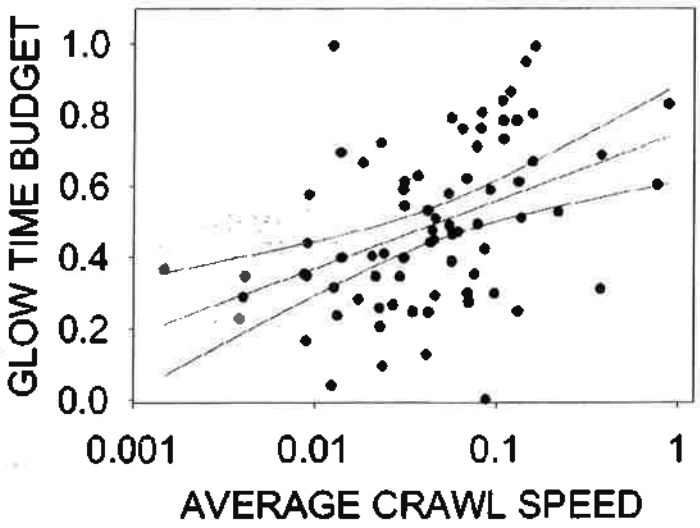


Figure 11. The amount of time spent glowing in larval *Lampyris noctiluca* increases with crawl activity (mm/sec) ($r^2 = 0.18$) [50].

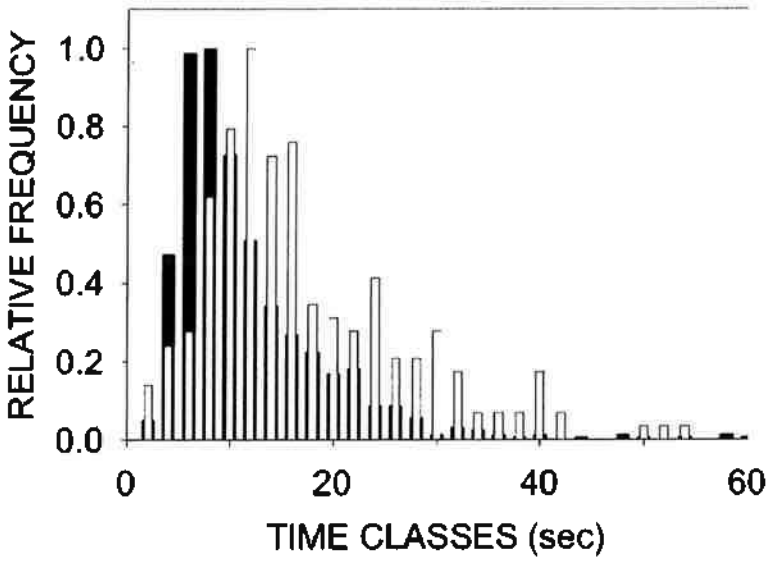


Figure 12. Preliminary data on glow pulse lengths in larvae. Histogram showing the relative frequencies of pulse lengths in *L. noctiluca* (black bars) and *P. hemipterus* (open narrow bars). [50].

of larval spontaneous glow displays [84]. Also Sivinski [89] and Viviani [58] report that in some species, larvae seemingly glow in response to the glow of nearby individuals and the possibility that larvae are able to perceive each other's light signals.

An important question that needs more attention is why the larvae signal intermittently and not continuously. Given a metabolic cost to signalling a most likely explanation is that the display is cheaper when it consists of a

series of glows rather than a sustained glow [89]. Although it is possible to calculate the biochemical cost of light production, which seems to be low indeed compared to other metabolic reactions [107], it remains difficult to evaluate the total cost of luminescent signalling. Firstly, most metabolic processes involve a more complex chain of several enzymatic reactions while only one enzyme is involved in bioluminescence (apart from the metabolic cost of the biosynthesis of luciferin). The cost of light production, therefore, may in the end be relatively low [107,108]. On the other hand, in order to control light production and signalling, glow-worm larvae invest extra metabolic costs producing specialised innervated light organs with an adapted tracheolar network for the supply of sufficient oxygen [105,109]. Species with less control over bioluminescence, glowing continuously, usually do not develop such extra costs (e.g. *Phengodes*; [105,110]. Apart from metabolic costs, there might be strategic predator related reasons to produce intermittent luminescence. Tests with LEDs have shown that continuous glows make better targets than intermittent, short flashes, and that staying dark is safest [111]. From this follows that intermittent luminescence in fireflies probably evolved under predation pressure, as the trail of a flashing prey is more difficult to track [112]. This mechanism is vaguely reminiscent of "flash coloration" [113] whereby individuals are cryptic at first, but once detected, move suddenly flashing bright colours (e.g. in moths, butterflies and grasshoppers).

It is hypothesised that predators are either startled or get distracted and search for the brightly coloured prey, which have disappeared when the prey settles again [113]. However, the larval glow is much slower and longer than a flash [104], and hence it does not seem adapted for such a tactic. Though, in addition to the warning function of luminescence, the pulsing of the signal may be selectively advantageous in another way by interfering with the predator's visual performance. Visually hunting nocturnal predators, such as toads, need to focus long at low ambient light conditions in order to receive enough information about the location of their prey [114,115]. Moreover, these predators normally snap at the prey's front and need to correct the direction of their attack for the delay in the visual information they obtain at low light levels, which they seem to learn by experience [115]. Hence it seems likely that the predator might get blinded or fooled by the afterimage of the glow pulses and snaps at these or to the rear end where the light organs are located, rather than to the exact location of the extinguished prey, which by then already has moved further. Such a tactic would obviously not work with continuously emitted light. Future experiments on the visual performance and prey catching accuracy of predators (toads/frogs), using glow-pulsing prey models, may resolve whether such anti-predator strategy

works and under what conditions (e.g. only at extreme low light levels, or depending on pulse rate and lengths). Of course experienced predators can adapt their prey catching technique and then the advantage is lost. However, the initial adaptive advantage provided by such a mechanism could have been important for the evolution of (spontaneous) bioluminescence as an aposematic signal, much in the same way as innate biases and novelty effects of birds are often cited as processes that might have facilitated the initial evolution of conspicuous warning colours [116].

In the literature no details are found about when exactly larvae show spontaneous glowing, while such knowledge may particularly explain when or why the display may be of adaptive importance. *L. noctiluca* and *P. hemipterus* glow spontaneously throughout the night, but the moments of peak activity seem to differ significantly between species, respectively at dusk and between midnight and dawn [50]. Further it seems that the amount of spontaneous glowing observed in natural populations depends on ambient factors, such as temperature, humidity, rainfall, and also on the season, with more glowing in autumn. At first sight this could be ascribed to the emergence of a new generation of larvae by the end of summer. However, at least in *Lampyris noctiluca,* it generally are later instar larvae that are seen glowing. "Autumn glowers" like *Phosphaenus hemipterus, Nyctophila reichei, Lampyris sardiniae* hardly glow spontaneously in spring, while other species like *Lampyris noctiluca, Lampyris iberica* and *Luciola lusitanica* show spontaneous glows from spring to autumn ([50,10,11], pers. obs.). Explanations for this seasonality in spontaneous larval glowing should come from further research.

3.5.4. Ecology of larval bioluminescence colours

Lampyrid larvae typically emit green coloured light [51]. However, intraspecific differences in the colour of bioluminescence between adults and larvae do exist in quite many species (e.g. orange vs. green; [58,79,108]. *Luciola* larvae seem to be an exception and produce yellowish light like the adults. Ecological reasons, more especially the spectral properties of ambient light, explain why adults produce light shifted to the yellow. The colour-shift appears to be an adaptation to overcome the noise-to-signal effect from green reflected light of foliage on courtship signalling during twilight in adults of species predominantly active shortly after sunset [81,117,118] while their larvae are active later at night. According to Viviani [58] the conservancy of green bioluminescence in lampyrid larvae agrees with the lack of an intra-specific function, for instance reproduction, and the increased importance of an interspecific function like defence. However, selection for visibility of the emitted light provides a more proximate explanation for this conservancy

[119]. This idea is supported by the fact that most terrestrial vertebrates and arthropods have eyes with highest spectral sensitivity in the green region of the light spectrum [35,118,120-126], which strongly suggests that the green colour of bioluminescence was selected for maximal visibility of the emitted light [50]. Some evidence for strong selection for the colour of bioluminescence comes from Viviani and Bechara [127], who discovered enzymes with luciferase-like action in non-luminescent beetles, which produce weak red light instead of green when firefly luciferin is added as a substrate.

3.5.5. Luminescent aposematism in a "multimodal" context

Next to warning flashes and glows, Lampyridae show several other features that support the hypothesis of protection through aposematism. The literature harbours numerous references on the unpalatability of lampyrids [16,41,89,94,103,128,129]. In addition, own experiments show that lampyrid glow-worm larvae are unpalatable to different species of lizards (*Lacerta vivipara, L. muralis, L. viridis*), frogs (*Rana temporaria* and *R. esculenta),* starlings (*Sturnus vulgaris*) and toads, and they are rejected to a high extent by insectivorous arthropods, such as carabid beetles, centipedes and spiders (R. De Cock, B. De Weirdt and E. Matthysen, unpublished graduate thesis; pers. obs.). In these and other predation experiments [95,130,131] lampyrids experience very low attack rates. Eisner et al. [95] found direct evidence for chemical defences in *Photinus* and *Photuris* fireflies, in the form of cardiotoxic steroids or so-called lucibufagins and other toxins [96]. The fact that lampyrids show chemical defences strongly suggests that their aposematism functions through avoidance learning. The numerous descriptions of plant-like, musky, cabbage-like, fungus, peppermint and resin odours in several lampyrid species [16,89] are reminiscent of the description of pyrazine smells and suggest the possibility that they may also use warning odours [132,133]. Blum and Sannasi [129] describe reflex bleeding in lampyrids and its effects on predators, which is another feature usually associated with aposematism [134].

Recently, defensive gland-like organs have been described in larval *Lampyris noctiluca,* which seem to facilitate reflex bleeding and which become exposed in certain cases of danger, e.g. in the presence of ants [67,135,136]. In Asian species with aquatic larvae similar eversible glands produce possibly defensive volatile products [20]. Yet, bio-assays should still show if these odours and volatiles function as direct predator repellents or are used as another aposematic signal to warn for toxicity. Experiments with lizards showed that the colour patterns with combinations of black, red and yellow of some species of adult lampyrids act as warning colours [131]. Many lampyrid species also have conspicuously coloured larvae that seem to

be aposematically defended against bird attacks [53,67]. In some species the larvae become diurnal before pupation [2,6,10,50,53]; Raphaël De Cock, unpublished data) and in these cases colour aposematism may be a useful adaptation. Lloyd [112] further suggested the possibility of Batesian mimicry within the Lampyridae and of mimicry complexes with moths, roaches (e.g. non-luminescent Firefly roach, *Schultesia lampyridiformis*), luminescent beetles, and soldier beetles, as these often show firefly-like colour patterns or luminescence [16,130,137]. This may also apply to the only known species of staphilinid beetle which is luminous in the larval stage [138] and emits light of the same spectral properties and from light organs in the same position as in lampyrids. There is also anecdotal evidence in support of chemical defences in other luminous beetles, Phengodidae and Elateroidea [89,100,101], which suggests the existence of Müllerian mimicry (different noxious species using similar signals to advertise their defencive abilities), or even Batesian mimicry (undefended species "lie" by copying warning signals of truly defencive species) between and within taxa of luminescent beetles. The fact that the adults of many firefly species emit yellow light as opposed to the green light produced by larval stages of all species [139], may also be an indication of mimicry in the larvae of different species.

Finally, a more physiological support that glowing may be involved in defencive activities is that the neurophysiological onset of larval glowing is controlled by specialized neurons and the transmitter octopamine, which in other insects are involved in stressful situations, e.g. encounters with predators [109]. In some species of fireflies this neural system also triggers reflex bleeding in larvae (A.D. Carlson, pers. comm.). Taken together, this knowledge not only supports the possibility of luminescent aposematism, but also suggests that lampyrids may use multimodal signals in which olfactory and visual components co-operate to enhance the aposematic signal [134,140-143].

Conclusion

From the previous paragraphs it must have become obvious that we possess quite a lot of information about European fireflies, but most of it stems from the first half of the 20th century with additional inputs from the sixties and seventies. Unfortunately numerous studies were discontinued, not followed up, or led to outcomes that contradicted what we would have expected on the basis of what we observe in the field nowadays. Many of the observations or experiments could and actually should be repeated, since we can now use our greater knowledge of experimental set-ups and statistic methods. Although the study of lampyrids started very early in Europe, especially in the Central and Northern European countries with their low

lampyrid biodiversity, it remains puzzling why so few studies have been performed as a whole and why fireflies have so seldomly been chosen as study subjects compared with other regions of the world. For instance, only one taxonomist has been specialising on European lampyrids for the last 50 years. Beyond doubt, there is still plenty more to be discovered about the ecology and luminescent behaviour of European species; details of their communication systems and especially the fine-tuning of their phylogenetic relations need to be investigated. Yet there is hope, for European fireflies lately became sufficiently "sexy" and popular enough, even to non-scientists, and are the topic of volunteer surveys and in artistic, environmental, and educational projects. The following list provides a summary of some challenging ideas and research topics, some more general and some more specifically based on European species:

- What are the effects and what is the importance of light pollution on the bioluminescent behaviour and survival of firefly and glow-worm populations? How do artificial lights and more indirectly cloud-reflected city lights interfere with courtship signals or defensive glows?
- Is the male and female partner choice based on bioluminescent displays and light organ patterns and what is the importance of pheromones and contact pheromones in our nocturnal species? What is the role of nuptual gifts (spermatophores) and multiple mating and how are these involved in sexual competition?
- To what extent is the system of sexual communication and partner discrimination related to the isolation of species and state of speciation especially in Southern European lampyrids, where males seem to have problems to distinguish their own females within and between genera, and what is the possibility of recent or past multiple hybridisations? Such questions call for multidisciplinary research, combining genetic and etho-ecologic analyses.
- Why do some species in which male bioluminescence does not seem to be involved in sexual communication, nevertheless show spontaneous and continuous glowing, while other closely related species have lost this characteristic during evolution?
- Are European *Luciola* species a complex of sibling species and how can differences in flash characteristics offer possibilities for species isolation and identification?
- What is the principle of synchronous flashing in the Bosnian *Luciola* species and what can this tell us about the system, ecology and multiple evolution of synchronous flashing? Indeed, synchronous flashing seems

to have evolved often in isolated species within certain genera (*Pygoluciola, Luciola, Pteroptyx, Photinus, Atyphella*)?

- Are *Phosphaenopterus spp.* and *Pelania mauretanica* still present in Europe?
- What about the general biology and communication systems of *Phosphaenopterus* and *Lampyroidea*?
- To what extent do the larval glow characteristics differ between species and how are they related to differences in ecology and (micro)habitat? Does the glow have an anti-predator function (e.g. relationship to the predator community, density and presence of other model species)?
- Did lampyrids evolve Müllerian or even Batesian mimicry (see text) between species? Only an international multidisciplinary approach can answer such questions by combining more detailed descriptions about bioluminescent displays, the presence of other possible aposematic signals, the performance of bioassays with analyses of chemical defences and mapping of these characteristics on the phylogeny.
- Why do many lampyrids show pink or magenta colouration on non-melanised bodyparts? Depending on their absorption spectrum, many pigments reflect the complementary colour of the wavelengths they absorb. The complementary colour of pink is yellow-green, which is exactly the colour of bioluminescence of most lampyrid species. So, did lampyrids evolve pinkish absorption pigments in order to conceal an unwanted overall weak body glow as much as possible? Is this pigment a derivative of orange and red pigments that are, or were, 'pre-adaptive' as aposematic colours?

Acknowledgements

I thank Dr. V. Benno Meyer-Rochow for inviting and convincing me to contribute to this book about one of my most favourite topics in Biology. Warm thanks go to Dr. Sara Lewis and my former PhD supervisor Dr. Erik Matthysen for their support and enthusiasm for my quest for firefly research. Dr Michael Geisthardt receives special thanks for his encouragement and the sharing and kind permission for the use of the most updated information about the current biodiversity of European Lampyrids.

References

1. Nuss, M., and Seidel J. 2008, Sächsische Entomologische Zeitschrift, 3, 30-38.
2. Tyler, J. 2004, Luminescence, 19(6), 358.
3. Wootton, A. 1971, Country Life, 150, 604-605.
4. Björck, M. 1998, Körmacken, 9, 8-15.
5. Tyler, J. 1982-1984, Alatala, 10-12, 17-19.

6. Tyler, J. 2002, The glow-worm, Sevenoaks, Lakeside Printing Ltd., 76 pp.
7. Meyer-Rochow, V. B. 2007, Luminescence, 22 (3), 251-265.
8. Mikšić, R. 1982, Acta Entomologica Jugoslavica, 17(1-2), 19-26.
9. Geisthardt, M. 2007, Lampyridae, In: Löbl, I., and Smetana, A. (Eds.): Catalogue of Palaearctic Coleoptera, Vol. 4. Stenstrup, Apollo Books, 46-47.
10. De Cock, R., and Geisthardt, M. 2007, Entomologische Zeitschrift, 117(3), 99-102.
11. Geisthardt, M., Figueira, G., Day, J. C., and De Cock R. 2008, Heteropterus Rev. Entomol., 8(2), submitted.
12. Lewis, S.M., and Cratsley, C.K. 2007, Annu. Rev. Entomol., 53, 293–321.
13. Lloyd, J. E. 1965, Ann. Entomol. Soc. Am., 58, 588-591.
14. Lloyd, J. E. 1968, Entom. News., 10, 265–268.
15. Lloyd, J.E. 1971, Ann. Rev. Entomol., 16, 97-112.
16. Lloyd, J.E. 1973, Coleops. Bull., 27, 91-106.
17. Lloyd, J.E. 1999, Florida Entomologist, 82(2), 165-179.
18. Lloyd, J.E. 2003, Florida Entomologist, 86(2), 99-113.
19. Fu, X., and Ballantyne, L.A. 2008, Zootaxa, 1733, 1-44.
20. Fu, X., Vencl, F.V., Ohba, N., Meyer-Rochow, V. B., Lei, C., and Zhang, Z. 2007, Chemoecology, 17, 117-124.
21. Fu X., Wang Y., Lei C., and Ohba N. 2005, Coleopterist Bulletin, 59(4), 501-504.
22. Fu X., Ohba N., Vencl, F.V., and Lei, C. 2005, Canadian Entomologist, 137, 83-90.
23. Ohba, N. 1980, Sci. Rept. Yokosuka City Mus., 27, 13-18.
24. Ohba, N. 1983, Sci. Rept. Yokosuka City Mus.,30, 1-62, pls.1-6
25. Ohba, N. 1983, , Sci. Rept. Yokosuka City Mus., 31,1-8.
26. Ohba, N. 1984, Sci. Rept. Yokosuka City Mus, 32, 23-33, pl.8.
27. Ohba, N. 1985, , Sci. Rept. Yokosuka City Mus., 33, 13-17.
28. Ohba, N. 1999, Sci. Rept. Yokosuka City Mus. 46, 33-40.
29. Ohba, N. 2003, SICB 2003 Annual Meeting and Exhibtion Final Program and Abstract, 264.
30. Ohba, N. 2004, Integr. Comp. Biol., 44, 225-233.
31. Ohba, N., and Sim, S.H. 1994. Sci. Rept. Yokosuka City Mus., 42, 1-11.
32. Ohba, N., and Sim, S.H. 2000, Sci. Rept. Yokosuka City Mus., 47, 23-30.
33. Chen, T.R. 1999, An Ecological Guide to Taiwanese Firelies, Field Image Publisher, Taipei, 199.
34. Ohba, N., Y. Goto, and Kawashima, I. 1996, Sci. Rept. Yokosuka City Mus., 44, 21-31.
35. Eguchi, E., Nemoto, A., Meyer-Rochow, V. B., and Ohba, N. 1984, J. Insect Physiol., 30, 607-612.
36. Jeng, M.-L., Lai, J., and Yang, P.-S. 2003, Lampyridae: a synopsis of aquatic fireflies with description of a new species (Coleoptera), In: Jäch, M.A., and Ji, L. (Eds.), Water Beetles of China. Vol.III. Zoologisch Botanische Gesellschaft in Österreich and wiener Coleopterologenverein, Vienna, Austria, 539–562 pp.
37. Suzuki, H. 1997, Tokyo met. Univ. Bull. Nat. Hist., 3, 1-53.

38. Suzuki, H., Sato, Y., Fujiyama, S., and Ohba, N. 1996, Genetics, 34(5/6), 191-200.
39. Suzuki, H., Sato, Y., Ohba, N., Base, Jin-Sik, Jin, Ryung-Raae, Sohn, Hung-Dae, and Kim, Sam-Eun 2004, Biochemical Genetic, 42(9/10), 284-300.
40. Bugnion, E. 1929, Association des naturalistes de Nice et des Alpes-Maritimes. Imprimerie "Association Typographique", Nice. pp. 131.
41. Schwalb, H.H. 1961, Zool. Jb. Syst., 88, 399-550.
42. Papi, F., 1969, Monit. Zool. Ital. (N.S.), 3, 135–184.
43. Dreisig, H. 1971, J. Zool. Lond., 165, 229 -244.
44. Dreisig H, 1974, Ent. Scand., 5, 103-109.
45. Dreisig, H. 1975, Oecologia,. 18, 85-99.
46. Dreisig, H. 1978, Behav. Ecol. Sociobiol., 3, 1-18.
47. Mikšić, R. 1969, Bollettino della Associazione Romana di Entomologia, 24(2), 43-46.
48. Mikšić, R., and Mikšić, S. 1965, Entomol. Nachr. 9, 76-77.
49. De Cock, R. 2000, Belgian Journal of Zoology, 130(2), 93-101.
50. De Cock, R. 2004, The adaptive value of bioluminescent behaviour in glow-worms (Coleoptera: Lampyridae), [Ph.d. dissertation]. University of Antwerp, Belgium, 193 pp.
51. De Cock, R. 2004, Photochemistry and Photobiology 79(4), 339–342.
52. De Cock, R., and Matthysen, E. 1999, Evol. Ecol., 13, 619-639.
53. De Cock, R., and Matthysen, E. 2001, Ethology ,107, 1019-1033.
54. De Cock, R., and Matthysen, E. 2003, Behavioural Ecology, 14(2003), 103-108.
55. De Cock, R., and Matthysen, E. 2005, Anim. Behav., 70(4), 807-818.
56. O'Donald, P. 1968, Nature, 217, 194.
57. Cros, A. 1924, Bull. Soc. Hist. Nat. Afr. Nord, 15, 10-52.
58. Viviani, V.R. 2001, Annals of the Entomological Society of America, 94, 129-145.
59. Olivier, E. *1884,* L'Abeille, 22, 1-55.
60. Linnaeus, C. 1767, Systema naturae, Tom. I. Pars II. Editio duodecima, reformata. Holmiae. (Laurentii Salvii)., 533-1327.0
61. Naisse, J. 1966, Archives de Biologie (Liège), 77, 139-201.
62. Maas, U., and Dorn, A. 2005, Gen. Comp. Endocrinol., 143(1), 40-50.
63. Vogel, R. V. 1912, Zool. Anz., 39, 515-519.
64. Vogel, R. 1913,. Zool. Anz., 41, 325-332.
65. Wunsch, E. 1995, Mitteilungen des Internationalen Entomologischen Vereins, 20(1/2), 1-14.
66. Booth, D., Stewart, A.J.A., and Osorio, D. 2004, J. Exp. Biol., 207, 2373-2378.
67. Tyler, J., McKinnon, W., Lord, G, and Hilton, P. 2008, Physiological Entomology, 33(2), 167-170.
68. Sala-Newby, G.B., Thomson, C.M., and Campbell, A.K. 1996, Biochem. J., 313, 761-767.
69. Lheritier, G. 1955, Société des Sciences Naturelles et Physiques du Maroc, 35, 223-233.
70. Wing, S.R. 1989, J. Insect Behaviour, 2(6), 841-847.
71. Sivinski, J. M., Lloyd, J.E., Beshers, S.N., Davis, L. R., Sivinski, R.G., Wing, S.R., Sullivan, R.T., Cushing, P.E., and Petersson, E. 1998, Coleops. Bull., 52, 23-30.

72. Geisthardt, M. 1979, Zool. Jb. Anat., 101, 472-536.
73. Bourgeois, J. 1900, Bull. Soc. Ent. Fr., 1900, 337-338.
74. Olivier, E. 1907, Genera Insectorum, 53, 74 pp.
75. Bialdaccini, N.E., Fiaschi, V., and Papi, E. 1970, Monitore Zool. Ital. (N.S.), 3, 239-245.
76. Bonaduce, A., and Sabelli B. 2006, Bollettino del Museo Civico di Storia Naturale di Verona, 30, Botanica Zoologia, 155-159.
77. Branham, M.A., and J.W. Wenzel. 2003, Cladistics, 19(1), 1-22.
78. Lall, A. B. 1981, J. Insect Physiol. 27, 461-468.
79. Lall, A. B., Seliger, H. H., Biggley, W. H., and Lloyd, J. E. 1980, Science, 210, 560-562.
80. Lall, A. B., Strother, G. K., Cronin, T. W., and Seliger, H. H. 1988, J. Comp. Physiol. A, 162, 23-33.
81. Cronin, T. W., Järvilehto, M., Weckström, M., and Lall, A. B. 2000, J. Comp. Physiol. A186, 1-12.
82. South, A., K. LeVan, L. Leombruni, C. Orians, and S.M. Lewis. 2008, Ethology, 114, 916–924.
83. Grandi, G. 1907, Osservazioni sulla variabilità delle Lampyris. Il Naturalisto siciliano, anno XIX, n° 9.
84. Campbell, A.K. 2008, Luminescence, 23, 187-190.
85. De Cock, R. 1997, Preliminary etho-ecological study of three glow-worm species. [in Dutch: Inleidende gedragecologische studie van drie soorten glimwormen; (Coleoptera: Lampyridae). m.s. thesis], University of Antwerp, Belgium, 99 pp.
86. Jeng, M.-L., and Yang, P.-S. 2003, Journal of the Kansas Entomological Society, 76(3), 477–483.
87. Lloyd, J.E. 1981, Scientific American, 245, 138-145.
88. Geisthard, M. 1979, Familie: Lampyridae, In: Freude, H., Harde K.W., and Lohse, G.A. (Eds.), Die Käfer Mitteleuropas, Band 6, Diversicornia, Goecke and Evers, Krefeld, pp. 14-18.
89. Sivinski, J. 1981, Coleops Bull, 35, 167-179.
90. Buck, J.B. 1978, Academic Press, London, pp. 419-460.
91. Bushman, L.L. 1988, Coleops. Bull., 42, 94-97.
92. Hastings, J.W., Morin, J.G. 1991, Bioluminescence. In: Prosser, C.L., (Ed): Neural and integrative animal physiology, Wiley Interscience, New York, 131-170.
93. Redford, *K. H. 1982,* Coleopt. Bull., 35, 167-179.
94. Carlson, A.D., and Copeland, J. 1985, Q. Rev. Biol., 60, 415-436.
95. Eisner, T., Goetz, M.A., Hill, D.E., Smedley, S.R., Meinwald, J. 1997, Proc Natl Acad Sci USA, 94, 9723-9728.
96. Gonzales, A., Schroeder, F.C., Attygalle, A.B., Svatos, A., Meinwald, J., and Eisner. T. 1999, Chemoecology, 9, 105-112.
97. Lloyd, J. E. 1983, Annu. Rev. Entomol., 28, 131-160.
98. Richards, A.M. 1960, Trans. R. Soc. N.Z., 88,559-574.
99. Sivinski, J. 1982, Ecological Entomology, 7, 443-446.
100. Viviani, V.R., and Bechara, E.J.H. 1997, Annals of the Entomological Society of America, 90, 389-398.

101. Lloyd, J.E. 1978, Insect bioluminescence. In: Herring, P.J. (Ed.), Bioluminescence in Action, New York, Academic Press, 570 pp.
102. Kaufmann, T. 1965, Ann. Entom. Soc. Am., 58, 420.
103. Underwood, T.J., Tallamy, D.W., and Pesek, J.D., 1997, J. Insect. Behav., 10, 365-370.
104. Hasama, B. 1942, Annot. Zool. Japan, 21(2), 59-76.
105. Buck, J.B. 1948, Ann. N.Y. Acad. Sci., 49, 397-482.
106. Ballantyne, L.A., and Buck, E. 1979, Trans. Am. Entomol. Soc. 105: 117-137.
107. Woods, W.A., Hendrickson, H., Mason, J., and Lewis, S.M. 2007, Am. Nat., 170(5), 702-708.
108. Wood, K. V. 1995, Photochem. Photobiol., 62, 662-673
109. Christensen, T.A., and Carlson, A.D., 1982, J Comp. Physiol., 148, 503-514.
110. Halverson, R.C., Case, J.F., Buck, J., and Tiemann, D. 1973, J. Insect Physiol. 19, 1327-1339.
111. Lloyd, J.E., and Wing, S.R. 1983, Science, 222, 634-635.
112. Lloyd, J.E. 1989, Coleops. Bull. 43, 83-91.
113. Cott, H.B.1940, Adaptive Coloration in Animals. Menthuen & Co Ltd, London
114. Aho, A.-C., Donner, K., Hydén, C., Larsen, L.O., and Reuter, T. 1988, Nature 334, 348-350.
115. Aho, A.-C., Donner, K., Helenius, S., Larsen, L.O., and Reuter, T. 1993, J. Comp. Physiol. A, 172, 671-682.
116. Lindström, L. 2000, Evolution of Conspicuous warning Signals, Academic Dissertation, Jyväskylä Studies in Biological and Environmental Science, 81, University of Jyväskylä, Finland.
117. Lall, A. B. 1993, J. Insect Physiol., 39, 123-127.
118. Seliger, H.H., Lall, A.B., Lloyd J.E., Biggley, W.H. 1982, Photochem. Photobiol., 36, 673-680.
119. Herring, P. J. 1983, Proc. R. Soc. Lond. (Ser. B), 220, 183–217.
120. Granit, R. 1943, Nature, 151, 11-14.
121. Donner, K.O., and Rushton, W.A.H. 1959, J. Physiol., 149, 303-317.
122. Koskelainen, A., Hemilä, S. and Donner, K. 1994, Acta. Physiol. Scand. 152, 115.
123. Goldsmith, T.H., and Bernard, G.D. 1974, The physiology of Insecta, Vol. 2. (Edited by R.M. Rockstein), Academic press, London, 165 pp.
124. Jacobs, G.H. 1981, Comparative Color Vision. Academic Press, New York.
125. Barth, F.G., Nakagawa, T., and Eguchi, E. 1993, J. Exp. Biol., 181, 63-79.
126. Meyer-Rochow ,V.B., and Eguchi, E. 1983, N.Z. Ent., 8, 111-119.
127. Viviani, V.R., and Bechara, E.J.H. 1996, Photochem. Photobiol., 63, 713-718.
128. Sydow, S.L., and Lloyd, J.E. 1975, Fla. Ent. 58, 312
129. Blum, M.S., and Sannasi, A. 1974, J. Insect Physiol., 20, 451-460.
130. Jones, F.M. 1932, Trans. Ent. Soc. London, 80, 345-385.
131. Sexton, O.J. 1966, Anim. Behav., 12, 101-110.
132. Rothschild, M. 1961, Trans. R. Ent. Soc. Lond., 113, 101-121.
133. Guilford, T., Nicol, C., Rothschild, M., and Moore, B. 1987, Biol. J. Linn. Soc. 31, 113-128.

134. Marples, N.M., van Veelen, W., and Brakefield, P.M. 1994, Anim. Behav., 48, 967-974.
135. Tyler, J. 2001, The Coleopterist, 10, 38.
136. Tyler, J., and Trice, E. 2001, The Coleopterist, 10, 75-78.
137. McDermott, F.A. 1964, Trans. Am. Entom. Soc., 90, 1-72.
138. Costa, C., Vanin, S.A., and Colepicolo-Neto P. 1986, Rvta. Bras. Ent., 30, 101-104.
139. Viviani, V.R., and Bechara, J.H. 1995, Photochemistry and Photobiology, 62, 490-495.
140. Marples, N.M., and Roper, T.J. 1996, Anim. Behav., 51, 1417-1424.
141. Geisthardt, M., and Day, J. 2004, Zootaxa, 427, 1-6.
142. Rowe, C., and Guilford, T. 1996, Nature, 383, 520-522
143. Roper, T.J., and Marples, N.M. 1997, Anim. Behav., 53, 1241-1250.

Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 201-228
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

11

Visual ecology of bioluminescent beetles: Visual spectral mechanisms and the colors of optical signaling in Coleoptera, Elateroidea: Lampyridae, Elateridae and Phengodidae

Abner B. Lall[1], Thomas W. Cronin[2], Etelvino J.H. Bechara[3] Cleide Costa[4] and Vadim R. Viviani[5]
[1]Department of Biology, Howard University, Washington, DC 20059, USA [2]Department of Biological Sciences, University of Maryland, Catonsville MD 21228, U.S.A.; [3]Departamento de Bioquimica, Instituto de Quimica CP 26077, Universidade de São Paulo, 05513-970 São Paulo, and Departamento de Ciências Exatas e da Terra, Universidade Federal de São Paulo, 09972-270 Diadema, SP, Brazil; [4]Museu de Zoologia, Universidade de São Paulo, CP. 42494,04218-970, São Paulo, SP, Brazil; [5]Laboratorio de Bioluminescencia e Biotecnologia, Universidade Federal de Sao Carlos Campus de Sorocaba, Sorocaba, SP, 08060-070 São Paulo, SP, Brazil

Abstract

Visual ecology of inter- and intra-specific bioluminescent communication in Coleoptera belonging to three Elateroidea families: Lampyridae, Elateridae

Correspondence/Reprint request: Dr. Abner B. Lall, Department of Biology, Howard University, Washington DC 20059, USA. E-mail: alall@howard.edu

and Phengodidae, that are either strictly vespertine and/or nocturnal species, is described. Spectral tuning between visual mechanism and bioluminescence is now established in a number of species. Broad green visual spectral sensitivity is ubiquitous among insects as an adaptation to optimize vision in green foliage habitats, following the predictions of visual ecology. The selection of green bioluminescence among nocturnal beetles would thus maximize the signal detection. During twilight, however, sunlight reflected from green foliage acts as an environmental photon 'noise' in the communication channel. The twilight-active fireflies possess narrow yellow spectral mechanisms (as predicted by the contrast sensitivity hypothesis of visual ecology) and emit yellow bioluminescent optical signals of contrast color against green background. The twilight-active fireflies' visual spectral sensitivity functions are narrowed by species-specific magenta screening pigments such that the shape of the species' bioluminescence emission and its narrow-yellow spectral sensitivity match. An optimization model of signal-to-noise ratio for the detection of bioluminescence among fireflies provides the mathematical underpinnings to show that the characteristics of the visual system present selective pressure for the color of the bioluminescent optical signals. The presence of spectral tuning of species bioluminescent emission and visual mechanism strongly suggests co-evolution of colors of bioluminescence and visual spectral mechanisms of vision in beetles.

1. Introduction

This chapter deals with the visual ecology of Elateroidea bioluminescent beetles. During the course of evolution, bioluminescence as an optical signal for inter- and intra-specific communication has appeared a number of times in varied organisms for different purposes. Among coleopterans, one of the important functions of bioluminescence is courtship signaling between the sexes. It can also be used to attract prey and to ward off predators and intruders. For effective communication to occur, characteristics of the optical signal have to be such that it evokes a significant response in the intended recipient. The bioluminescent optical signal is low in photon content, hence it can only be detected in dim illuminated environments such as deep sea, subterranean, twilight and nocturnal [1].

The bioluminescent Elateroidea beetles comprise three families: Lampyridae, Elateridae and Phengodidae. Bioluminescence emission spectra have been recorded from about 140 of the thousands of known species. It has been known for some time that the colors of bioluminescence are species-specific, ranging from green to yellow, orange and red [2] and that this color is dependent upon a species-specific luciferase enzyme in the light organ [3].

The significance of the variation in colors of BL initially eluded investigators as it was assumed that the fireflies lack color vision. Buck [4] in a behavioral experiment conducted in the field showed that firefly *Photinus pyralis* males and females responded to light stimuli of long wavelengths that ranged from green to red, and not to short wavelength light stimuli in the blue-green, even at levels of illumination 900 X brighter than the long wavelength stimuli. In order to solve the enigma of the presence of different colors of BL, the senior author initiated classical neuroethological experiments, which grew into a multidisciplinary effort involving numerous collaborators over more than three decades.

Von Uexküll [5] proposed that in instinctive behavior there is a dovetailing of the sensory receptor with the perceptual cue to facilitate unambiguous communication. The characteristics of the perceptual cue, the optical signal, can be ascertained by obtaining the bioluminescent emission spectrum. The characteristics of the sensory receptors can be obtained by determining the spectral sensitivity of the eye. The spectral sensitivity [$S(\lambda)$] functions have been obtained for twenty-six (26) species representing three coleopteran families: Lampyridae [6, 7, 8, 9, 10, 11], Elateridae [12, 13, 14], and Phengodidae [15]. Our electrophysiological and microspectrophotometric data show a correspondence between visual spectral mechanisms and bioluminescent emission in beetles that utilize optical signaling for sexual communication.

We begin by describing the structure of the eye and the photoreceptors that process the optical signals. We then present the ecological considerations and the spectral mechanisms for each family. We show that for inter- and intra-specific bioluminescent communication, the characteristics of the insect visual system are the determining factor for the selection of the color of optical signals. We extend Seliger's mathematical optimization model [16, 17], which explains the selection of color of BL in lampyrids, to elaterids and phengodids. Two principles of visual ecology, the sensitivity hypothesis and the contrast hypothesis, explain the presence of green through yellow colors of bioluminescence among beetles which inhabit diverse photic niches, twilight and nocturnal. The spectral tuning of visual mechanisms and BL emissions in different species of beetles further supports the hypothesis of co-evolution of vision and bioluminescence in the Elateroidae, Coleoptera [17]. We conclude by discussing the possibility of color vision in bioluminescent beetles.

2. Structure of the compound eyes in fireflies and click beetles

The structure of the compound eyes in *Photuris versicolor* [18] and *Photinus pyralis* [19] is now well documented. They possess superposition

optics. The eye consists of a spherical array of ommatidia. Each has an external cornea fused to a crystalline cone, below which is a proximal large clear zone region followed by a fused, hypertrophied rhabdom consisting of large retinular cells numbered 1 to 6 (R_{1-6}), R_7 on top of R_{1-6}, and a basal R_8 cell below [18]. Besides the visual pigment housed in the microvilli of the rhabdomeres, there also exist screening pigments: a yellow screening pigment in the ventral region of *P. versicolor* and an oily magenta screening pigment in *P. pyralis* [19].

Similar to the firefly visual system, the click beetles (e.g., *Pyrophorus punctatissimus*) also have typical refracting superposition compound eyes [14]. The outermost layer of the cornea, about 50 μm in thickness, overlies the ommatidia. Each ommatidium consists of a corneal lens (~ 20-25 μm in diameter and 10 μm in thickness) followed by a crystalline cone (~ 84 x 100 μm), below which lies the clear-zone region (200-250 μm wide) covering two rhabdomeric layers, each about 100 μm thick, consisting of an outer mandrel-shaped rhabdomeric layer and an inner slender rhabdomeric layer (~ 55-65 μm) with much smaller microvilli than those in the outer layer. This arrangement provides ~138% increase in the surface area of the microvillar membrane for the inner rhabdomeres compared to that of the outer mandrel-shaped rhabdomeres. Matti Järvilehto [20] proposes that the function of such an arrangement is for the outer rhabdomeres to mediate daylight vision and the inner rhabdomeres to mediate night vision. The structure of the eyes of the railroad worm species as yet has not been investigated.

3. Lampyridae: Some ecological considerations

The ecology of North American lampyrids has been studied extensively [1, 21, 23, 24] and the emission spectra ranging from yellow to orange (λ_{max} = 546 to 594 nm) [25, 26] from many species are known. There are about 1900 known species worldwide, inhabiting marshes, grasslands and forests from tropical to temperate regions. Lampyrids possess abdominal bioluminescent lanterns located in the last two segments of the body. In the Palearctic species, the glow-worm (*Lampyris noctiluca*), the mature female advertises her presence by emitting a constant greenish glow from the abdominal lantern until she is mated [27]. In most North American lampyrids, the males advertise themselves with a species-specific flash pattern that is recognized by conspecific females. The female flash response has a species-specific time delay. *Photuris versicolor* males, for example, emit three flashes in succession at regular intervals of 5 s, and the female responds with a 1 s delay. *Photuris lucicrescens* males emit a long 2 s crescendo flash every 7.5 s and females respond after 1.5 s. *Photinus pyralis*

males emit a J-shaped 500 ms flash every 3.5 - 4 s, and the female answers after a 1.5 - 2 s delay [28]. Lloyd [21, 29] demonstrated that timing factors such as the male's flash interval and duration, and the female's flash duration and latency are essential parameters for species recognition.

In the temperate zone, unlike the tropics, the length of the twilight hours during summer increases as a function of the latitude. A substantial number of North American lampyrid species inhabit the twilight photic niches. Here a correlation was observed between the colors of bioluminescence and the time of initiation of flashing activity. Those species that emit yellow to amber bioluminescence at twilight (dusk-active or crepuscular) are followed by green bioluminescence-emitting nocturnal (night-active) species [7]. Bioluminescence emission spectra of 55 firefly species were obtained in Professor Seliger's laboratory at The John Hopkins University, and the emissions of different species fell into two broad groups: "early-starting (twilight-active)" species, that is, those beginning flashing activity in advance of 30 minutes after sunset, and "late-starting (or dark-active or night-active)" species, beginning after this [7]. The determination of "early" or "late" was made from field records, and the observer (J. E. Lloyd) had no knowledge of the peak wavelengths of bioluminescence emission. Of 32 dark-active (or night-active) species, 23 emit green light ($\lambda_{max} \leq 558$ nm) while 9 species emit yellow bioluminescence. These latter species were collected (by Lloyd) in specialized habitats such as salt marshes and open prairie grassland. Of 23 dusk-active species, 21 emit yellow light ($\lambda_{max} \geq 560$ nm) and restrict their flashing activity to a short interval at twilight, while two green-emitting species initiate flashing at dusk and continue on late into the night (twi-night active species). A high degree of correlation of peak wavelengths of bioluminescence with the time of beginning of flashing activity was found such that green bioluminescence correlated with night-active species and yellow bioluminescence with twilight-active species [7].

3.1. Spectral mechanisms of vision in nocturnal (night-active) lampyrids

The success of optical signaling for mating requires visual receptors for the detection of BL signals. The spectral characteristics of the visual receptors can be easily determined by measuring the spectral sensitivity of the eye for a physiological response elicited by a light stimulus. The experimentation involves recording the electroretinogram (ERG) elicited by colored stimuli across the spectrum (360-680 nm) from the corneal surface of the compound eyes in intact preparations. The ERG is a mass response representing the summed response of all the photoreceptors stimulated by light. The spectral sensitivity of this response is governed by the absorption

characteristics of the rhodopsin(s) and associated screening pigments present in the eye. Spectral sensitivity is defined as the reciprocal of the number of photons needed to elicit a threshold ERG response across the spectrum. The ERG S(λ) functions were obtained from the compound eyes in intact specimens of 13 different lampyrids. The peaks of the green bioluminescence-emitting dark-active or nocturnal fireflies such as *Photuris versicolor* (λ_{max} = 552 nm) and *P. lucicrescens* (λ_{max} = 554 nm) matched their broad green ERG S(λ) functions, with λ_{max} = 550-555 nm [7, 8] (Fig. 1). It was established by microspectrophotometry (MSP) that vision in *P. versicolor* is mediated by a P543 rhodopsin filtered by a yellow screening pigment absorbing in the blue (λ_{max} = 456 nm) [19]. A similar situation exits for *P. lucicrescens*.

In the nocturnal Brazilian lampyrids, *Pyrogaster* sp1 emits green BL (λ_{max} = 556 nm) and *Photinus* sp1 emits lemon-yellow BL (λ_{max} = 563 nm). These species possess broad green (λ_{max} = 555-560 nm) visual spectral sensitivity functions [11]. Similarly the Japanese nocturnal lampyrids emit green BL that is tuned to their broad green visual sensitivity [10]. In general, then, nocturnal fireflies emit green BL, with a few exceptions emitting yellow,

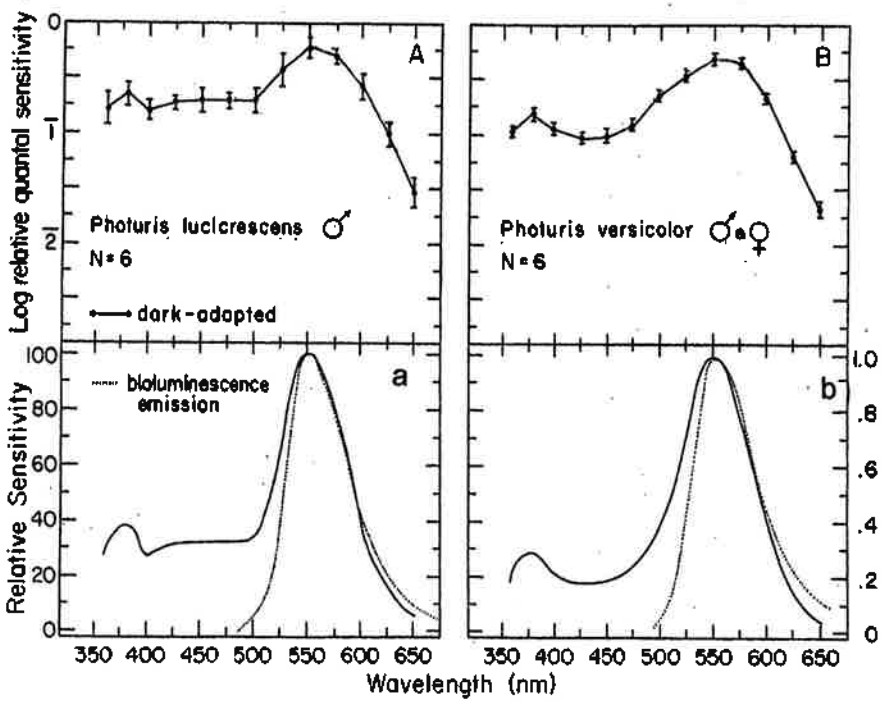


Figure 1. *Upper panels*: Spectral sensitivity of ERG response from the dorsal sector in dark-adapted compound eyes in *Photuris lucicrescens* (A) and *P. versicolor* (B). *Lower panels:* The data points for threshold curves A and B plotted on a percentage scale in panels a and b, together with the normalized species bioluminescence emission spectrum.

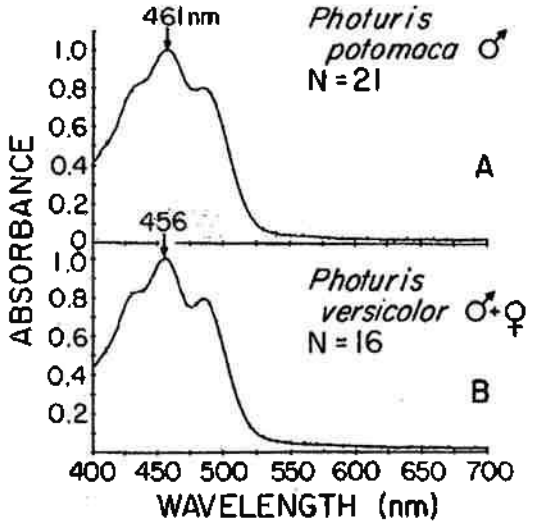


Figure 2. Normalized absorption spectrum of the yellow screening pigment from the compound eye in *Photuris potomaca* (A) and *P. versicolor* (B). Taken from Lall et al., 1988 Fig. 4. [9] With kind permission of Springer Science and Business Media.

and both green and yellow optical signals can be easily detected by their broad green visual spectral mechanisms.

The nocturnal lampyrids that emit yellow bioluminescence, as well as the twi-night species, possess broad green S(λ) functions. The twi-night active *P. potomaca* and nocturnal *P. versicolor* possess a yellow screening pigment (Fig. 2) absorbing broadly in the blue (λ_{max} = 461 nm) [9]. In both twi-night *P. potomaca* and *P. frontalis*, the yellow BL can be easily detected by their broad green ERG S(λ) functions with λ_{max} = 555 [9]. *Pyractomena barberi* also emits amber BL (λ_{max} = 563 nm) which is offset from the broad green ERG S(λ_{max} = 555 nm) functions. We propose that in the twi-night or nocturnal fireflies which emit yellow to amber BL, the species' broad green ERG S(λ) functions are mediated by P545 rhodopsin in conjunction with yellow screening pigments (λ_{max} 456-461 nm) (Fig. 2). Hence lampyrids that are twi-night active or nocturnal possess broad green vision that is optimized for detecting both green and yellow bioluminescence.

3.2. Spectral mechanisms of vision in crepuscular (twilight-active) lampyrids

An unexpected finding was the presence of narrow S(λ) functions obtained from the compound eyes in firefly species which restrict their BL flashing to a short interval (40 to 55 min) at twilight. In six North American species (*Photinus scintillans*, *P. macdermotti*, *P. collustrans*, *P. pyralis*, *P. marginellus* and *Bicellonycha wickershamorum*), the ERG S(λ) functions

were narrow, peaking in the yellow [9] with an attenuation of sensitivity in the green (λ = 500-525 nm, Fig. 3 A & B) region of the spectrum as compared with the green-sensitive nocturnal *Photuris versicolor* and *P. lucicrescens* (Fig.1). Furthermore, there was matching in shape between the species BL emission and species narrow visual S(λ) functions (Fig. 3 a & b). A similar situation was encountered in the crepuscular Brazilian firefly *Macrolampis omissa* [11].

Unique to crepuscular lampyrids is the presence of species-specific, magenta-colored screening pigments in the compound eye (Fig. 4). This pigment in *Photinus pyralis* is of oily consistency and is spread in the distal portion of the clear-zone region and in the rhabdomeric segment [19]. Absorption spectra of these screening pigments, determined by MSP, are narrow (1/2 bandwith = 50-80 nm) with species-specific differences in their peak absorption in the green, being at 525 nm, 510 nm, 512 nm, and 517 nm in *P. scintillans, P. macdermotti, P. collustrans*, and *P. pyralis* respectively [9]. In all cases, transmission increases at both long and short wavelengths, but

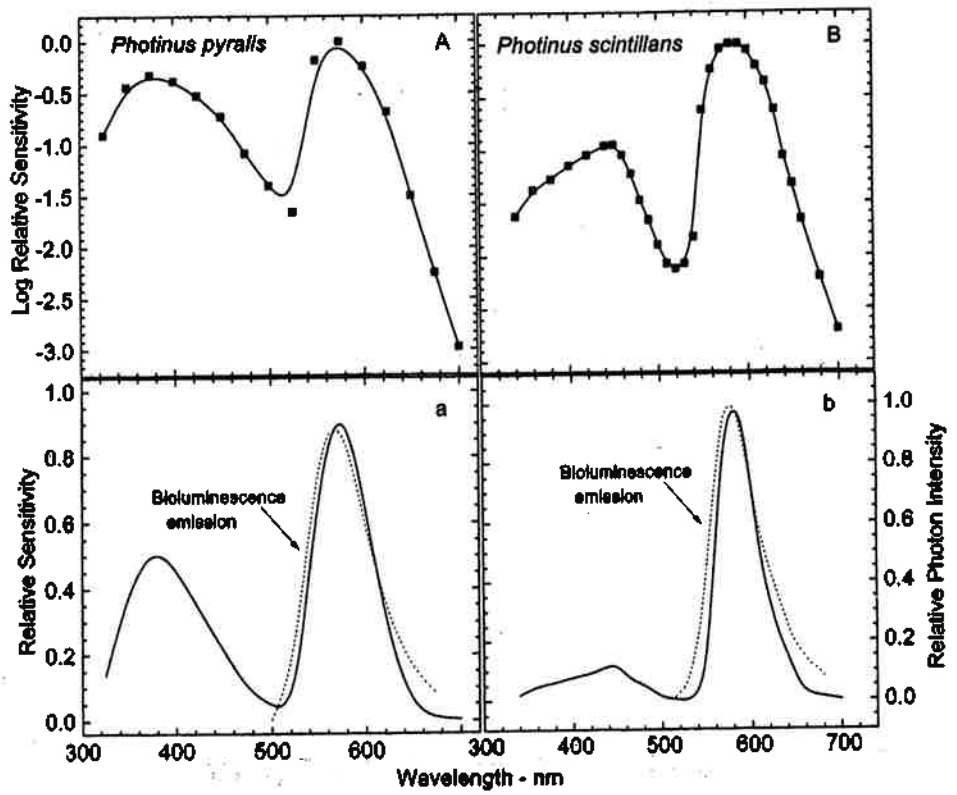


Figure 3. ERG S(λ) functions obtained from dark-adapted compound eyes in firefly species that restrict their flashing activity to twilight hours: *Photinus pyralis* (A), and *P. scintillans* (B). A comparison of the species bioluminescence emission with visual S(λ) function plotted on a percentage scale for each species is given in panels a & b, respectively.

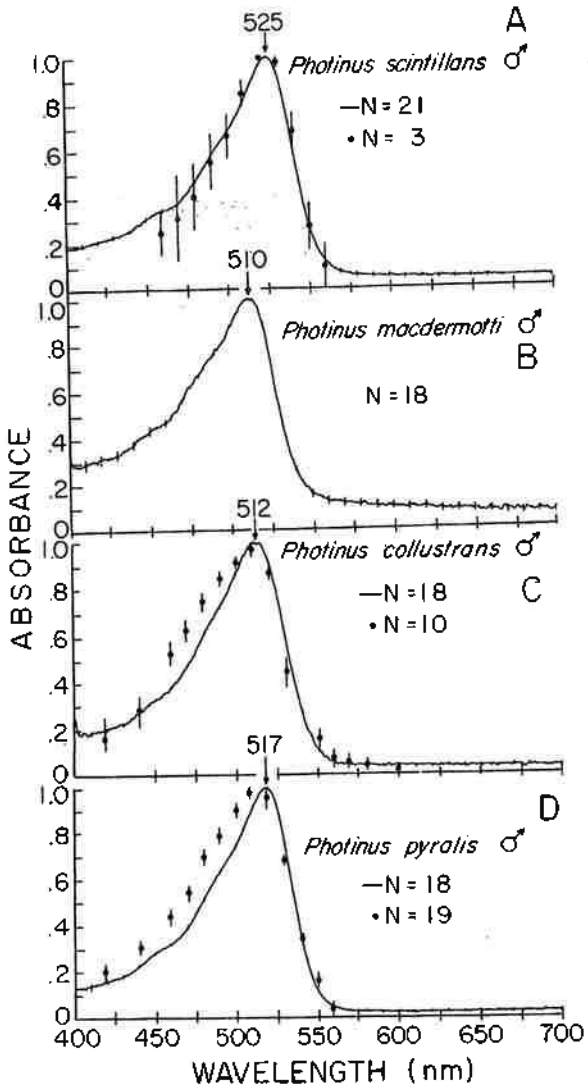


Figure 4. Normalized microspectrophotometric absorption spectra of magenta-colored screening pigment located in slices of the compound eyes in the males in four firefly species: *Photinus scintillans* (A), *P. macdermotti* (B), *P. collustrans* (C), *P. pyralis* (D). The data obtained in Cronin's lab (continuous line) are in agreement with those obtained in Strother's lab (only points are given). Bars ±1 SE. Taken from Lall et al., 1988 Fig. 3. [9] with kind permission of Springer Science and Business Media.

more sharply in the long wavelength regions. These magenta screening pigments with narrow absorption in the green modify the broad green ERG S(λ) function mediated by the P545 rhodopsin into a narrow yellow ERG S(λ) function in species which restrict their flashing to twilight hours. These screening pigments form a series of cut-off filters, graded to the photon intensity of the 'noise' (sunlight reflected from vegetation) at twilight, enhancing brightness and/or color contrast and reducing glare. Our earlier assumption was that the P545 rhodopsin occurs in all twilight-active fireflies

and that the narrowing of S(λ) function was accomplished exclusively by magenta-colored screening pigments [9]. Subsequently it was found that vision in *P. scintillans* was mediated by a P557 in conjunction with a 525 screening pigment [19].

Behavioral confirmation that the narrow yellow spectral mechanism is involved in processing the optical signal was obtained by determining the action spectrum of the female's response to a simulated male's flash in *Photinus pyralis* [31]. The threshold intensity of the female response to light stimuli across the spectrum from 420 nm to 660 nm was obtained. The females responded to stimuli exclusively from 500 to 660 nm and not to stimuli in the short-wavelength region. The shape of their action spectrum matched the shape of the species BL emission as well as the action spectrum of the intracellular response obtained from single retinular cells (Fig. 5). This further confirms that the green/yellow spectral mechanism is the receptor devoted to decoding the bioluminescent signal in lampyrids.

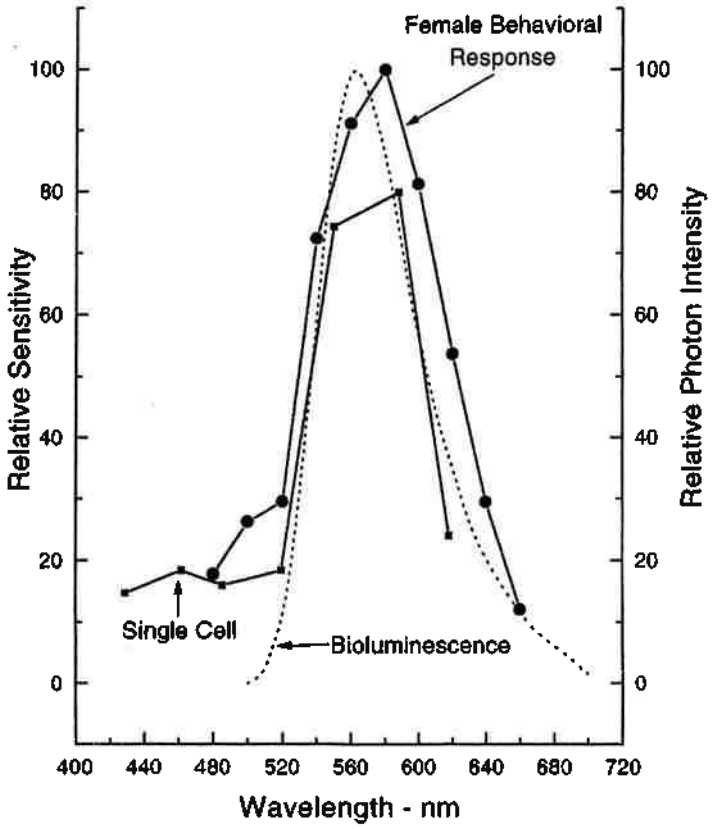


Figure 5. A comparison of the action spectrum of the *Photinus pyralis* female's response to a conspecific male's simulated flash and the species bioluminescence emission and also the spectral sensitivity of the intracellular receptor potential of single retinular cells from the compound eyes. Lall and Worthy 2000, Fig. 2 [31]. With kind permission of the publisher.

4. Elateridae: Some ecological considerations

Pyrophorini (Candèze, 1863) are widely distributed in the Americas, from the southern United States to Argentina. They comprise 144 known species and 19 genera, whose adults are easily distinguishable from other elaterids by a pair of bioluminescent oval organs (lanterns which when not lighted or in dead insects are yellowish in color) on posterior angles of the pronotum and a third luminescent organ located in the ventral surface of the first abdominal segment juxtaposed to the metathorax that is only activated during flight. This organ on the abdomen illuminates a circular field below the body. It lights up only when there is some movement of the wings. The symmetric pair of anterior thoracic organs lights up with a constant bright green emission when the beetle is disturbed, either walking or stationary. All three lanterns emit continuous light [32, 33, 34, 35, 36].

The Pyrophorini are closely related to the Heligmini (non-bioluminescent elaterids), and a recent cladistic analysis pointed out that many genera of Heligmini lie at phylogenetic placements among the descendants of ancestral species of Pyrophorini, suggesting that bioluminescence was lost more than once in the group [37]. Recently the mitochondrial genome of *Pyrophorus divergens* was sequenced [38] and the phylogenetic analysis based on this genome and data from the literature suggest that bioluminescence may have arisen three times independently in click beetles, railroad worms and fireflies.

All Pyrophorini are vespertine and/or nocturnal. In those species where BL is associated with sexual communication, such as *Pyrophorus* spp, nuptial flights last less than 30 min, during which males emit yellow/orange light from the abdominal lanterns, while the females remain on the vegetation either walking or stationary and emit green light from their pronotal organs, leading to mating. Rosa [39] observed that *Opselater pyrophanus* appear not to use their abdominal lantern for mating. Males fly, continuously emitting green BL through the pronotal organs, while the females remain on the vegetation glowing intermittently via their pronotal lanterns (duration1-2 s for 3-9 times consecutively).

In the genus *Pyrophorus*, *P. punctatissimus* and *P. divergens* are generally disjunct in geographical distribution, as they occur in the Amazon and Atlantic forests respectively. Nonetheless the two species are sympatric in the open fields ("*cerrados*") of the Brazilian state of Mato Grosso [33, 34]. Another Pyrophorinid species, *Fulgeochlizus bruchii*, is known to occur only in Argentina and in the *cerrados* of central Brazil [40]. *F. bruchii* lacks dorsal lanterns.

4.1. Chemical ecology: *Pyrearinus* spp

The genus *Pyrearinus* includes over fifty species found throughout South America. *P. termitilluminans* is endemic to the Brazilian *cerrados*, where their larvae and pupae live in shallow tunnels excavated into termite mounds [40]. During the rainy season, these larvae expose their heads and shining prothoracic lanterns in the exits of the tunnels in order to attract and catch flying prey, mostly termites and ants. This phenomenon produces an effect referred to as "luminous termite mounds" [42, 43]. The color of the larval bioluminescence is green. Since green receptors are ubiquitous among insects [44] and many other animals as well [45], these emissions from the mounds attract several opportunistic predators such as scorpions, spiders, centipedes, and frogs. The myriad of tiny green lights on the termite mound surface [41, 43] thus attracts a diverse community of animals. Nocturnal birds and bats forage on these animals, sometimes leaving feces with large amounts of seeds, which would significantly increase plant diversity around the termite mounds. The mounds also act as perches for seed dispersers like birds and bats [46]. The termites facilitate the aeration of the soil as well as recycling and accumulation of organic matter [47]. All these elements enhance diverse nucleation [48,49] and create differentiated patches with a new ecological biodiversity.

The majority of elaterids in their larval phases dwell under hypoxia in tunnels and it appears that bioluminescence plays a role in their adaptation to these conditions. Crowson [50] found larvae in tunnels in hypoxic conditions (pO_2 < 0.05 atm) which he excavated inside decaying logs. *P. termitilluminans* resides in normally aerated tunnels where pO_2 is ~.2 atm. These larvae require antioxidant defenses for survival. A plethora of data show that a small percentage of oxygen (< 1%) inhaled by aerobic organisms undergoes partial reduction by the respiratory mitochondrial chain to reactive, potentially toxic superoxide and hydroxyl radicals [51]. To protect biomolecules and cells against oxidative damage, aerobic organisms utilize enzymatic defenses, mainly superoxide dismutase (SOD), catalase and glutathione peroxidase, and low-molecular weight compounds, particularly urate and trehalose in the case of insects. An imbalance in the production and consumption of active oxygen may lead to oxidative injury to crucial cell components (membranes, DNA, proteins, carbohydrates) and impairment of their normal functions. Thus, it is not surprising to find three-fold higher activity of SOD in larval *P. termitilluminans* as compared to *P. divergens* [52], suggesting that the level of SOD responds to the pO_2 of the habitat. Accordingly, experimental exposure of *P. termitilluminans* larvae to hyperoxia induced increases in both their luciferin concentration (50%) and

luciferase activity (2-fold), with a 50% increase in total SOD activity [53]. These and other data obtained for SOD, luciferase, urate and trehalose in *P. termitilluminans* suggest that the variation of chemical and antioxidant defenses during the natural and 20-hydroxyecdysone-induced development follow a pattern that strongly indicates that bioluminescence and antioxidant systems cooperate to minimize oxidative stress [54].

4.2. Bioluminescent emission spectra

The color of the bioluminescence emission from the prothoracic lanterns in click beetles is green (λ_{max} = 548-562 nm) [25]; (λ_{max} = 538-566 nm) [54,55], and that from the ventral abdominal lantern is green, yellow or orange (λ_{max} = 543-568 nm,) [54]; (λ_{max} = 575-585 nm) [25]. The significance of the differences in the lambda maximum of BL emission among elatrids has not been fully explored. As we have seen, bioluminescence is associated with sexual communication in some species. Click-beetle larvae emit green bioluminescence from the pronotum area. In some species a pair of round light organs is situated either laterally or dorsoventrally on transverse zones in each abdominal segment. In the larval phase, besides attracting prey, BL is probably associated with defense against intruders and predators [32, 33, 35].

4.3. Spectral tuning of visual and bioluminescence emission

Electroretinographic S(λ) functions obtained from dark-adapted eyes in *Pyrophorus punctatissimus*, *P. divergens*, *Pyrearinus termitilluminans*, and *Fulgeochlizus bruchii* males possess a broad peak in the green with a shoulder in the near-ultraviolet, suggesting the existence of short- and long-wavelength receptors [14]. A spectral correspondence was found in all four species between visual sensitivity in long wavelengths and the BL emission of the ventral lantern that is implicated in the mating ritual. Here is another example of spectral correspondence between green visual spectral mechanisms and bioluminescent optical signals. An example of two of the four species is given in Fig. 6. We have now established the spectral tuning that exists between the species bioluminescent emissions and the visual mechanisms in the compound eyes of above four elaterid species representing three genera (14). These species were selected due to their abundance in the Brazilian *cerrados*. The data reported here, dealing with species of both sympatric and allopatric occurrence offer a basis for better understanding the general patterns of sexual attraction and courtship in bioluminescent beetles.

Similar to nocturnal lampyrids, in the click-beetles, *Pyrophorus punctatissimus* and *P. divergens*, vision is mediated by P540 rhodopsin overlaid

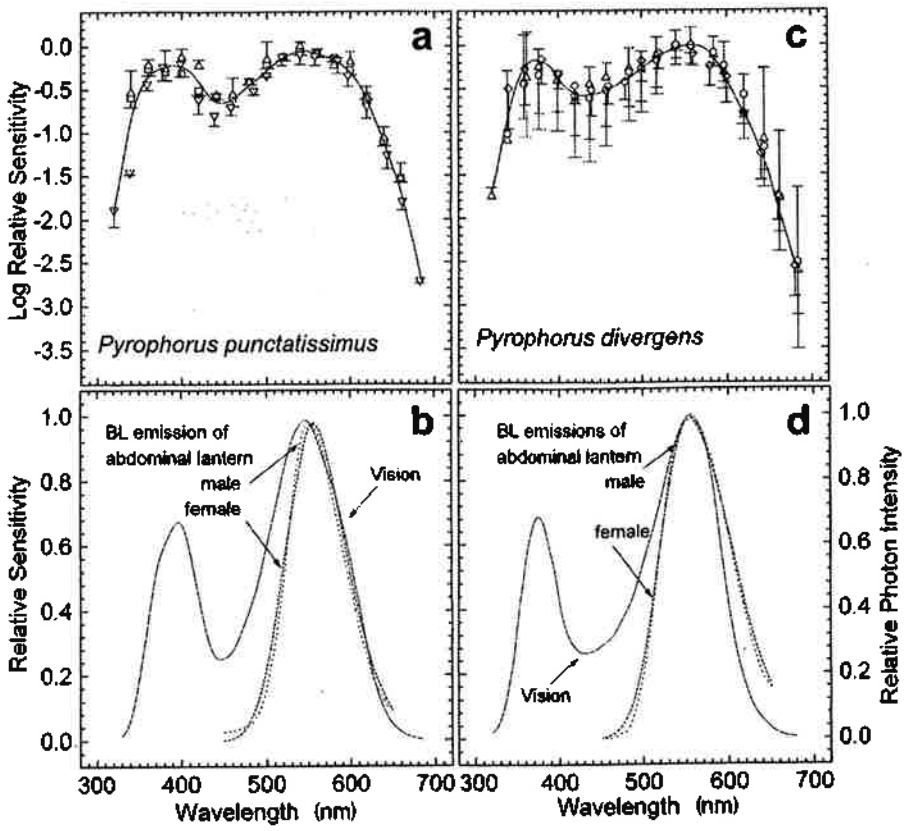


Figure 6. Electroretinographic spectral sensitivity functions in dark-adapted compound eyes in the males of two species of click beetles: *Pyrophorus punctatissimus* (a) and *Pyrophorus divergens* (c) Error bars ±1 S.D. A comparison between spectral sensitivity functions and the species' *in vivo* bioluminescence of the ventral abdominal lantern in the male and the female for each species is given in the lower panels b and c respectively.

with O. D. 1.6 broad species-specific yellow screening pigments (λ_{max} = 448-478 nm and λ_{max} = 459-488 nm respectively). The yellow screening pigment shifts the absorbance of the P540 to longer wavelengths such that the λ_{max} of the calculated relative spectral sensitivity of *P.punctatissimus* and *P. divergens* shifted to 550 nm and 562 nm respectively and the species BL from the abdominal lantern match (Fig. 7). In these two species, then, the broad green visual sensitivity is mediated by a species-specific rhodopsin absorbing maximally in the green in conjunction with a yellow species-specific blue-absorbing screening pigment, which is similar to the situation in nocturnal lampyrids (Fg. 1).

5. Phengodidae: Some ecological considerations

The Phengodidae (railroad worms) include 31 genera and 244 species distributed throughout the New World from extreme southern Canada to Chile,

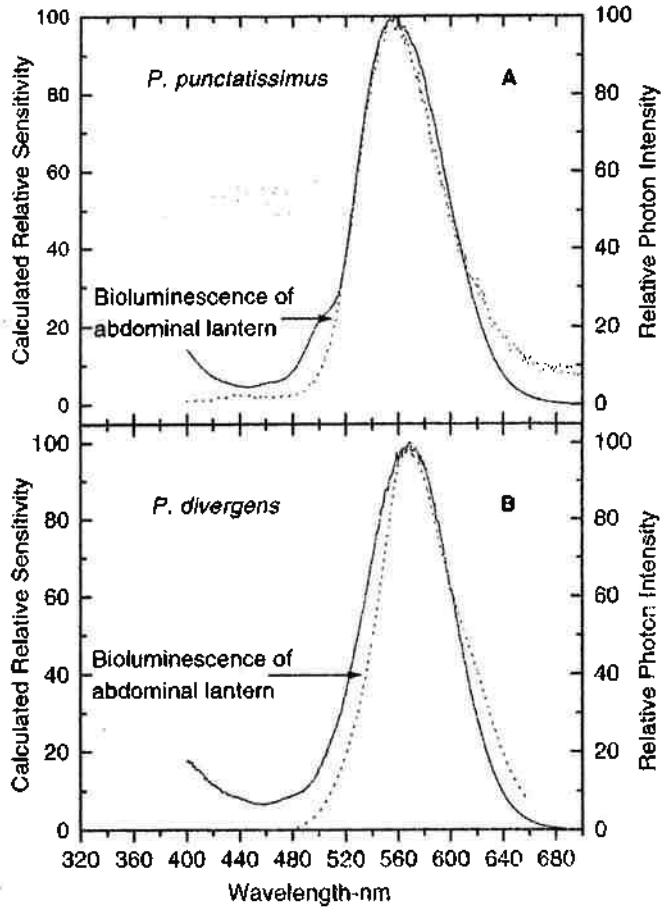


Figure 7. A comparison of calculated absorbance of P540 overlaid with species screening pigment of O. D.1.6 in *Pyrophorus punctatissimus* (A) and *P. divergens* (B) compared with species' *in vivo* bioluminescence emission (dotted line) from the ventral abdominal lantern of the female.

including the Nearctic and Neotropical biogeographical regions [57-60]. Females are neotenic, much larger than males and larviform, retaining many larval features and attaining the highest level of paedomorphosis [50, 60, 66, 67]. The larviform female pupa of *Phrixothrix hirtus* can be easily distinguished from the larva and larviform adult female by the lighter coloration of the integument and non-sulcate triangular-shaped mandible, the presence of an oopore (absent in larvae) and by the annular spiracles, biforous in larvae [60, 67]. The larviform male pupates and metamorphoses within three weeks into a flying adult (an adult survived one week in Viviani's lab). Adults and pupae of *Phrixothrix hirtus* were collected in the *cerrados* of Central Brazil and their complete life cycles and bioluminescence spectra for all stages have been described [67].

The larvae of both sexes possess 11 pairs of lanterns (2-3 on prothorax and 8-9 on abdomen) as well as two (one in some species) large anterior

cephalic lanterns. The variation in the color of the lanterns in phengodids far exceeds that among the lampyrids and the elaterids. *Brasilocerus* spp possess green to yellow-green (λ_{max} = 550-557 nm) dorso-lateral lanterns and yellow (λ_{max} = 565-570 nm) anterior (cephalic) lanterns. *Mastinocerus* spp possess amber (λ_{max} = 578-580 nm) dorso-lateral lanterns and yellow-orange (λ_{max} = 580-597 nm) cephalic lanterns, while *Phrixothrix* spp possess green to yellow (λ_{max} = 540-568 nm) dorso-lateral lanterns and orange-red (λ_{max} = 592-636 nm) cephalic lanterns [66].

The ecological significance of the diversity of bioluminescent colors in phengodids is not well understood. Similarly the role of the two sets of lanterns in the inter- and intra-specific visual guided behavior is not well defined. In the undisturbed state the railroad worms crawl with only their head lanterns emitting steady illumination [66]. When the worm is disturbed, the dorso-lateral lanterns light up. Initially they emit brightly, but then gradually the intensity is lowered. Such a behavior would suggest that the lateral lanterns are utilized to ward off predators [68]. Most predatory insects possess broad green vision, and undoubtedly they would detect green to yellow bioluminescence with ease.

One of the possible functions of the light emitted from lateral lanterns [50, 60, 68] is aposematism associated with distateful properties. It has also been suggested that the cephalic lantern(s) emission is used for illumination during the search for prey (millipedes) [67, 68]. According to Sivinsky [66], because most organisms are insensitive to red light, the production of red light in *Phrixothrix* could have a double advantage during the search for prey: (a) finding the prey without advertising its presence, and (b) avoiding being targeted by predators, a potential risk with continuous light. This assumes that the worms themselves have red-sensitive photoreceptors. As yet there is only weak evidence for their existence. Furthermore, it has been suggested that the cephalic lantern(s) emission is used for illumination when the animal is crawling. A similar function is implicated for bioluminescence in fireflies, where bioluminescence illuminates the area where the firefly is landing [69].

Since nocturnal lampyrids possess broad green visual sensitivity, green to yellow bioluminescence can be used for illuminating the path the firefly is navigating. Similarly if Phengodid larviform females can utilize their steady lemon-yellow to red emissions (565 to 636 nm) for illumination [66], it would be necessary that the insect possess visual receptors maximally absorbing in the yellow-green or red region of the spectrum depending upon the species. The presence of a long-wavelength spectral mechanism in the compound eyes of Phengodidae larviform females as yet has not been documented.

It has also been suggested that sexual communication between the sexes in the railroad worm is mediated via pheromones. In nocturnal and crepuscular lampyrids the sexual communication is predominantly mediated by BL optical signaling at a distance, but pheromone communication has a significant role in close proximity. Hence bioluminescence for distance communication, and pheromone communication for close proximity, both may operate in railroad worms as well.

5.1. Spectral tuning of vision and bioluminescent optical signals

The ERG S(λ) function (Fig. 8) of dark-adapted compound eyes in the railroad worm *Phrixothrix heydeni* was found to possess a maximum in the yellow-green (λ_{max} = 565 nm) with a shoulder in the blue-violet (λ_{max} = 420 nm). The bioluminescence emission of the lateral lanterns (BL λ_{max} = 568 nm) corresponds with the ERG S(λ) function in the yellow-green part of the spectrum. However the red emission (BL λ_{max} = 638 nm) from the cephalic lanterns is out of the expected range for the visual system. The sex of the specimens from which the above data were obtained is not known. There are some preliminary data to suggest the presence of a red receptor in one specimen. However confirmatory data as yet are not available.

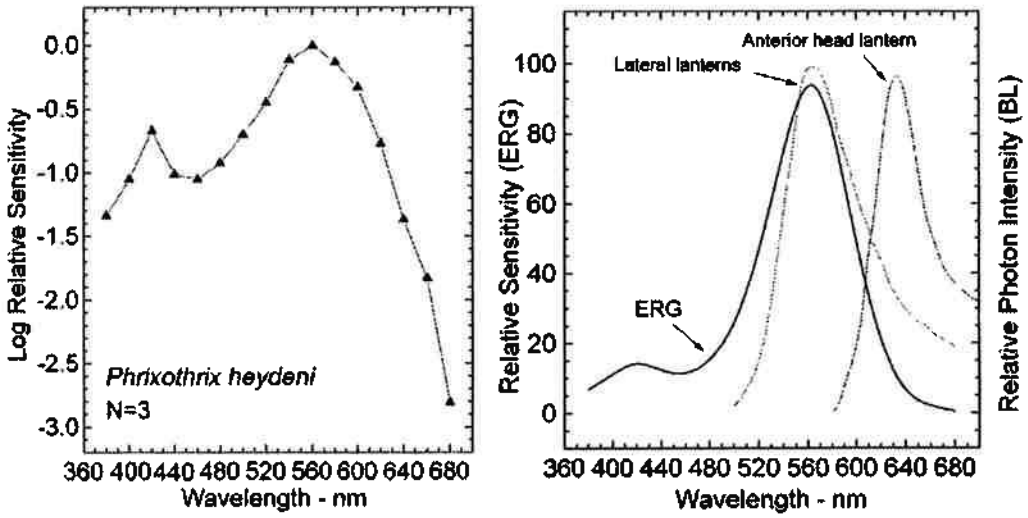


Figure 8. A comparison of visual spectral sensitivity with bioluminscence emission from the lateral and the cephalic lanterns in the railroad worm *Phrixothrix heydeni*. *Left panel*: Spectral sensitivity of ERG response from the dark-adapted compound eyes. *Right panel*: The data points for threshold curve from the upper panel are plotted on a percentage scale and a comparison is made with the species yellow BL emission from the lateral lanterns (λ_{max} = 568 nm) and the red BL from the cephalic lantern (λ_{max} = 638 nm).

6. Discussion

In Table 1 is presented the data from earlier sections can be summarized as follows.

1. The nocturnal species of beetles, both fireflies (Fig. 1) and click beetles (Fig. 6), in general emit green bioluminescence with few exceptions that emit yellow BL. These beetles possess bimodal S(λ) functions with broad green maxima (Figs.1 & 6) and a shoulder in the near-uv. Vision in nocturnal *Photuris versicolor* is mediated by green absorbing rhodopsin (P545) in conjunction with a broad species-specific blue absorbing yellow screening pigment (λ_{max} = 456 nm, Fig. 2 b) (19). A similar situation exists for the twi-night active lampyrids. Similarly vision in click beetles *Pyrophorus punctatissimus* and *P. divergens* is mediated by P540 rhodopsin overlaid with broad species-specific yellow screening pigments. In both families, the yellow screening pigment shifts the absorbance of the green visual pigment to longer wavelengths such that a close match exists between λ_{max} of species BL with calculated S(λ) functions (Fig. 7) [20].
2. The visual S(λ) functions of twilight-active lampyrids are also bimodal with near-uv and narrow long wavelength peaks. Here the shapes of the species BL and species visual S(λ) functions match in the long wavelength region. (Fig. 3) [6, 7, 9, 11]. Vision in these species in the long wavelength region is mediated by P545 or P557 rhodopsin overlaid with species-specific magenta screening pigments (Fig. 4) which result in narrow visual sensitivity peaks in the yellow-green [9, 19].
3. The bimodal S(λ) function in phengodid *Phrixothrix heydeni* has one broad peak in the long wavelength region at 560 nm. The peak of BL from the lateral lanterns match as the S(λ) maximum (Fig. 8). The detector for the BL emission from the head lanterns is yet to be found. Physiological and MSP data from other phengodid species are much needed.
4. The evolutionary selection of different colors of bioluminescence and the tuning of visual spectral mechanisms and BL emission among beetles can be explained with Seliger's mathematical optimization model [16, 17], which was proposed for lampyrids and can now be extended to other Elateroidea families. The model is based upon correlating three sets of information, the BL emission spectrum, visual spectral sensitivity of the eye, and the characteristics of the photic environment during the activity period of the beetle. A summary of this model based upon the optimization of signal-to-noise (noise being environmental photons) ratio for the detection of BL optical signal in North American fireflies is given below.

Table 1. Relationship between species visual spectral sensitivity, rhodopsin, screening pigment and species bioluminescence emission maximum in Coleoptera representing three families.

Species	ERG S(λ_{max}) (nm)	Rhodopsin	Screening Pigment λ (nm)	BL(λ_{max}) (nm)		Reference
Lampyridae						
Twilight-active						
Photinus scintillans	**580⁺**	**P557**	**525⁺**	**579**		**7,9**
Photinus macdermotti	570⁺		510⁺	569		
Photinus marginellus	**565⁺**		**512⁺**	**565**		**9**
Photinus pyralis	565⁺	P545	517⁺	564		6, 7, 9
Photinus collustrans	**560⁺**		**512⁺**	**560**		**9**
Macrolampis omissa	575⁺			572		11
Twi-night active						
Photuris potomaca	555*		461*	560		9
Photuris frontalis	**560***			**572**		**9**
Nocturnal						
Photuris versicolor	**555***	**P545**	**456***	**551**		**6**
Photuris lucicrescens	555*			551		8
Pyractomena barberi	**555***			**563**		
Pyrogaster sp1	550*			556		11
Photinus sp1	**555***			**563**		**11**
Brazil: Elateridae				**Dorsal**	**Ventral**	
Pyrophorus punctatissimus	**545***	**P540**	**463***	**546**	**557**	**14**
Pyrophorus divergens	555*	P540	472*	542	550	13, 14
Pyrearius termitilluminans	**545***			**545**	**552**	**12, 14**
Fulgeochlizus bruchii	545*				543	12, 14
Brazil: Phengodidae				**Head**	**Body**	
Phrixothrix heydeni	565*			635	565	15

* broad maximum ⁺ narrow maximum

6.1. Optimization model for the detection of bioluminescent optical signal

For two symmetrical functions, one the optical signal and other the detector, the efficiency for the detection of the signal by the detector is the product of the two functions, which can be mathematically represented as follows:

$$\int S(\lambda)BL_0(\lambda)d\lambda \qquad \text{Eq. 1}$$

Where $S(\lambda)$ is the spectral sensitivity of the detector (i. e., eye), $BL(\lambda)$ is the emission spectrum of the optical signal, and the function is integrated over the overlap of these two functions. At twilight there is sufficient sunlight such that the visual system (detector) responds to both the optical signal and the ambient illumination (Ai), the environmental photon noise. The efficiency for the detection of the ambient photon noise is given below:

$$\int S(\lambda)Ai(\lambda)d\lambda \qquad \text{Eq. 2}$$

Therefore the detection of optical signal (BL) in the presence of interfering "noise" due to ambient light is the optimization of the ratio (R) between the efficiency for signal detection and that for the 'noise' given by the following equation.

$$R = \frac{\int S(\lambda)BL_0(\lambda)d\lambda}{\int S(\lambda)Ai(\lambda)d\lambda} \qquad \text{Eq. 3}$$

Case a. For nocturnal species, the environmental photon noise (denominator) is almost negligible. Thus the efficiency of the detection of the optical signal is the overlap of the BL emission with the visual spectral sensitivity function of the eye.

Wald [45] had suggested that the selection of the rhodopsin molecule for vision is based upon the fact that the maximal absorption of rhodopsin dovetails with the sunlight reflection from green foliage. Figure 9 presents dovetailing of absorption spectrum of P550 rhodopsin with the reflectance spectrum of two grasses and the irradiance of light reflected from the foliage in the habitat of firefly *Photinus pyralis* during its activity period at twilight. Vision mediated by visual pigments (range P510-550) is ubiquitous among insects [review: 44]. It is presumably an adaptation to green, foliage-rich habitats [16].

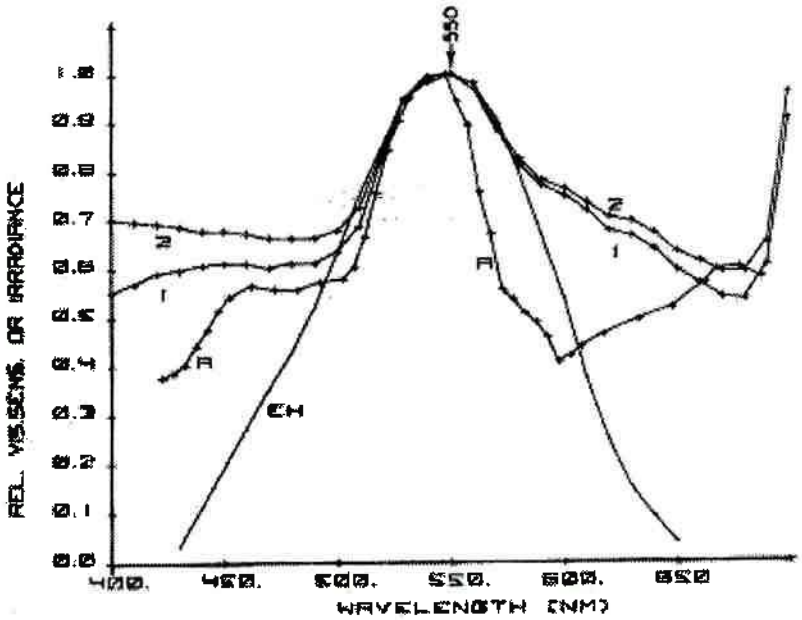


Figure 9. Normalized diffuse spectral reflectance of two grasses (1,2) from *Photinus pyralis* site. A. Spectral radiance during the flashing period. EH represent the Ebrey-Honig nomogram for P550 rhodopsin. Taken from Seliger et al., 1982 Fig. 1. [16]. With kind permission of the publisher Wiley-Blackwell.

In situ MSP measurements of the absorption spectrum of the photopigment in the rhabdomeric segment in the compound eyes of the firefly *Photuris versicolor* and the elaterid *Pyrophorus punctatissimus* show the presence of a green P540-545 nm visual pigment. The selection of green absorbing visual pigment (P540-545 nm) follows the prediction of the sensitivity hypothesis of visual ecology. Bioluminescence arose subsequent to vision [70], hence the pressure will be for the selection of a luciferase enzyme for emitting green BL to optimize signal detection. In general, the nocturnal beetles emit green BL that indeed matches their broad $S(\lambda)$ functions (Figs. 1 and 7) [8, 14, 19].

Case b. We described earlier that the fireflies which restrict their flashing activity to twilight emit yellow BL and that their emission spectra and their $S(\lambda)$ functions (Fig. 3) narrowed by screening pigments (Fig. 4) match. At twilight there is a substantial amount of sunlight reflection from green foliage, the environmental noise of the system. Under these conditions the optimization ratio, R^i for the detection of BL is given:

$$R^i = \frac{\int S^i(\lambda) BL_0(\lambda) d\lambda}{\int S^i(\lambda) Ai(\lambda) d\lambda} \qquad \text{Eq. 4}$$

where $S^i(\lambda)$ represents the narrow spectral sensitivity function, Ai represents the sunlight reflected from green foliage, and BL_0 represents the ancestral green BL emission.

The value of the denominator of Eq. 4 is very important. It can be minimized if the peak of the modified visual spectral sensitivity function $[S^i(\lambda)]$ is offset from the peak of the green foliage-reflected sunlight ('noise'). We observed that the visual $S(\lambda)$ functions in twilight-active fireflies were shifted from the green into the yellow region of the spectrum. There are three ways to shift the $S(\lambda)$ towards longer wavelengths: (1) select a rhodopsin whose peak absorption is shifted from green (540-545 nm) to longer wavelengths; (2) have screening pigments which shift the $S(\lambda)$ maximum to still longer wavelengths with a reduction in absolute sensitivity; (3) combine both (1) and (2). MSP data show that options number 2 and 3 are selected by different N. American lampyrids. The narrow-bandwidth magenta-colored screening pigments (Fig. 4) described earlier for twilight-active fireflies act as environmental noise filters, absorbing the green foliage-reflected sunlight in the species' habitat [16]. The screening pigments (Fig. 4) shift the $S^i(\lambda)$ function to longer wavelengths from their nocturnal ancestral peaks in the green (compare Figs. 1 and 2).

Seliger [16] argued that in the presence of ambient sunlight at twilight screening pigment-mediated narrow $S^i(\lambda)$ functions would be more adaptive than broad $S(\lambda)$ functions mediated by rhodopsin shifted to longer wavelengths. This optimization was qualified as what he called Biological Effective Adaptation (BEA) a measure of the capability of a species for intra-specific BL communication. BEA is a dimensionless ratio between the efficiency of the detection of green ancestral bioluminescence (BL_0) with a modified $S^i(\lambda)$ representing a twilight species (Eq. 4) and the efficiency of detection with a yellow shifted rhodopsin with broad $S(\lambda)$ function. BEA can be calculated by the following equation.

$$BEA = \frac{R^i}{R} = \underbrace{\frac{\int S^i(\lambda)BL_0(\lambda)d\lambda}{\int S^i(\lambda)Ai(\lambda)d\lambda}}_{I} \div \underbrace{\frac{\int S(\lambda)BL_0(\lambda)d\lambda}{\int S(\lambda)Ai(\lambda)d\lambda}}_{II} \qquad \text{Eq. 5}$$

The two parts, I and II, of the above equation, were evaluated independently and their ratio (BEA) presented in Fig 10. The values of BEA tend to diminish beyond 580 nm suggesting that beyond that wavelength there is no selective advantage by having a narrow $S^i(\lambda)$ function for the detection

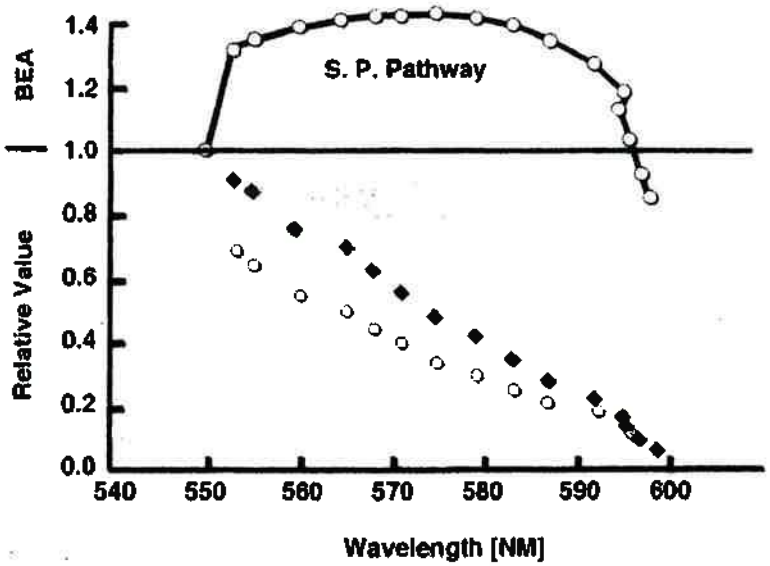


Figure 10. Calculated values for the family of curves of the product terms in Eq. 5 (see text) for the detection of green bioluminescence and of the green ambient light, indicating that the decrease in detection efficiency for ambient light (o) is greater than the decrease in detection efficiency for bioluminescence (■). The values of BEA as shown are maximal (1.4) for λ_{max} values between 565 and 580 nm. Taken from Seliger et al., 1982 Fig. 4A. [16]. With kind permission of the publisher Wiley-Blackwell.

of ancestral green BL. Thus far the longest wavelength of BL emission known in fireflies is at 579 nm for the twilight-active *Photinus scintillans*. The value for BEA at 580 nm is 1.4 in Fig. 10 with green ancestral BL emission and increases to 1.61 when considering *P. scintillans* BL emission at 579 nm [16].

Lythgoe [71] proposed that for enhancing brightness and/or color contrast, it is necessary to have the visual spectral mechanism offset from the peak of the ambient illumination (Contrast Hypothesis of Visual Ecology). The narrow $S^{i}(\lambda)$ function of the twilight-active *Photinus scintillans* (λ_{max} = 580 nm) coincided with the (λ_{min}) of its photic habitat (Fig. 11a) down-welling light, and is offset from the broad green maximum (around 550 nm) (Fig. 11b) of the sunlight reflected from green grass (up-welling light) at twilight in the species habitat [17] such that the BL is detected with the greatest possible contrast. Yellow BL is a contrast signal against green (analogous to a yellow buttercup flower in green grass).

The optimization model quantitatively explains the selective advantage of the magenta-colored screening pigments found in those firefly species which restrict their flashing to a short interval at twilight and predicts the color of bioluminescence emission in nature [16]. The predictions of the model with

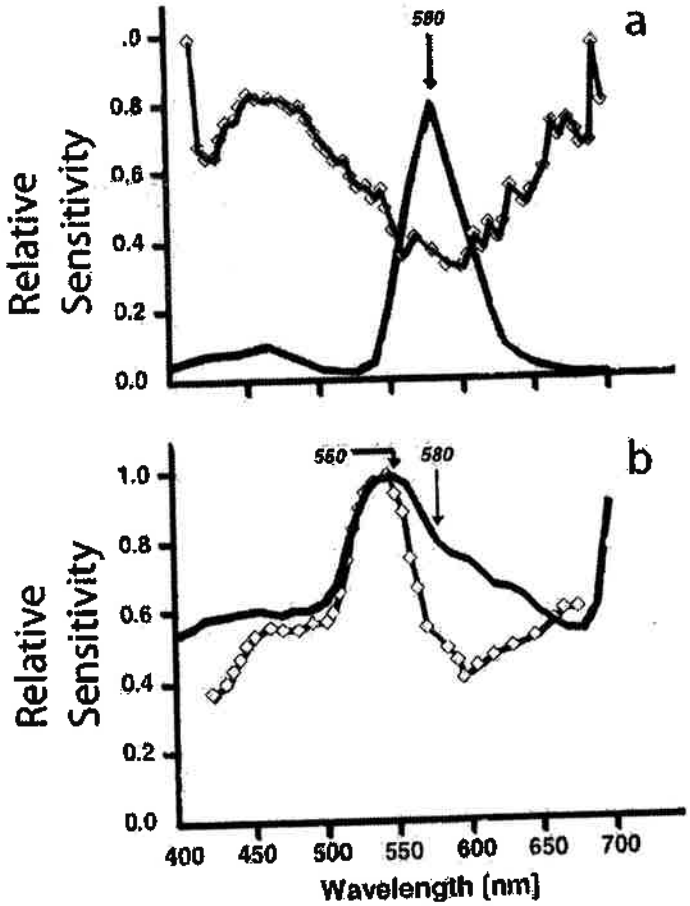


Figure 11. Down-welling (upper panel) and up-welling (lower panel) ambient sunlight in the habitat of *Photinus scintillans* during its flashing activity period. The S(λ) of *P. scintillans* is offset from the λ_{max} of its ambient photic environment and is in the λ_{min} of the photic environment. Taken from Seliger et al., 1982 Fig. 7. [17]. With kind permission of the publisher Wiley-Blackwell.

respect to different combinations of visual pigments and screening pigments resulting in broad or narrow visual $S^{i}(\lambda)$ functions among night- or twilight-active fireflies respectively, have now been verified by MSP measurements [19]. Since the BL signals are spectrally narrow and limited in photon content and appear against a spectrally broad background, the spectral correspondence between the visual S(λ) functions of the visual receptors (i.e., the detectors for the BL optical signals) and the emission spectrum of the BL optical signals optimizes the detection of species-specific BL signals. These signals are utilized for sexual communication [16, 17, 25, 26] in species inhabiting different habitats and photic niches. The pressure exists for the selection of luciferase enzymes that results in yellow-to-orange bioluminescence emissions to match the peak of the screening pigment-modified spectral sensitivity functions $[S^{i}(\lambda)]$.

6.2. Bioluminescence and color vision in beetles

The presence of three spectral mechanisms, near-UV, violet-blue and yellow in *Photinus pyralis* [72] and near-UV, blue and green in *Photuris lucicrescens* [8] was strongly suggested by selective adaptation experiments in which we obtained ERG S(λ) functions from the compound eyes of fireflies. Later a blue receptor (λ_{max} = 435 nm) was identified in the dorsal sector of the compound eyes in *P. frontalis* [9]. It was also shown that the action spectrum of the intracellular response thought to arise from $R_{1\text{-}6}$ retinular cells in *Photinus pyralis* compound eyes is that of a green-yellow receptor with a narrow spectral sensitivity function (λ_{max} = 565 nm) [19]. Kelber et al. [73] have shown that color discrimination is possible even in starlight illumination conditions for the elephant hawkmoth *Deilephila elpenor*. In a superposition compound eye, the superposition aperture can increase the photon count of an image of a dim object by combining rays coming through various facets and thus "increase the eye's sensitivity by a factor of up to 1000" [74]. Recently, behavioral evidence has been presented for the presence of color vision in the European lampyrid *Lampyris noctiluca*. Here the blue chromatic mechanism inhibited the male's response to the female's green BL light [27]. Similar evidence is now available for the *Photinus pyralis* female's response to a simulated species-specific male flash. When a blue adapting light is presented simultaneously with the 565 nm test stimulus, the female's response is inhibited [75]. Thus we see, not that the blue receptor exists in the fireflies, but also that it plays an inhibitory role (– ive) while the green/yellow receptor is excitatory (+ive) in nature. Such an arrangement is common in insect color vision [reviews: 42, 76].

7. Conclusions

Knowledge of the spectral characteristics of vision in fireflies has been pivotal in unraveling the enigma of the presence of different colors of bioluminescence, ranging from green through yellow and amber, among lampyrids and elaterids. Seliger at al [16, 17] hypothesized that an adaptive co-evolution of vision and bioluminescence in lampyrids has occurred, and this concept can now be extended to other families. Our findings also further support and provide mathematical underpinning for von Uexküll's [3] theory that instinctive behavior involves the dovetailing of the sensory receptor and the effector signal for unambiguous communication.

Acknowledgements

Thanks are due to Professor Howard H. Seliger for his earlier collaboration and support of this work. Professor Seliger and William H.

Biggley provided bioluminescence emission spectra for all North American firefly species discussed in this review during the years when the author was a guest in his laboratory. Many of the ideas presented here have come out of laboratory conversations with him and his colleagues Mr. Biggley and Professor James E. Lloyd. The Brazilian co-authors of this review were funded by the Fundação de Amparo á Pesquisa do Estado de São Paulo (FAPESP) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors are deeply indebted to Jean Lall for her expert editorial assistance in the preparation of this manuscript.

References

1. Lloyd, J. E. 1977, In: How Animals Communicate, T. A. Sebeok, (ed.), Indiana University, Bloomington, 164.
2. Seliger, H. H. and McElroy, W. D. 1964, Proc. Natl. Acad. Sci. U.S. 52, 75.
3. McElroy, W. D. and Seliger, H. H. 1966, In: Molecular Architecture in Cell Physiology, T. Hayashi and A. Szent-Gyorgyi (eds.), Prentice-Hall, Englewood Cliffs, New Jersey, 63.
4. Buck, J. B. 1937, Physiol. Zool. 10, 45.
5. von Uexküll J. 1934, Streifzüge durch die Umwelten von Tieren und Menschen, Springer, Berlin (English translation), In: Instinctive Behavior, C. H. Schiller (ed.), 1957, International Universities Press, New York.
6. Lall, A. B., Chapman, R. M., Trouth, C.O. and Holloway, J. A. 1980, J. Comp. Physiol. A 145, 135.
7. Lall, A. B., Seliger, H. H., Biggley, W. H. and Lloyd, J. E. 1980, Science 210, 560.
8. Lall, A. B., Lord, E.T. and Trouth, C.O. 1982, J. Comp. Physiol. 147, 195.
9. Lall, A. B., Strother, G. K., Cronin, T. W. and Seliger, H. H. 1988. J. Comp. Physiol. A 162, 23.
10. Eguchi, E., Nemoto, A., Meyer-Rochow, V. B. and Ohba, N. 1984, J. Insect Physiol. 30, 607.
11. Lall, A. B., Carvalho, A. H., de Souza, J. M., Ventura, D. S. F., Viviani, V. R. and Bechara, E. J. H. Unpublished manuscript.
12. Lall, A. B., Carvalho, A., de Souza, J. M., Bechara, E. J. H. and Cronin, T. W. 2000, In: Bioluminescence and Chemiluminescence, J. F. Case, P. J. Herring, B. H. Robinson, S. H. D. Haddock, L. J. Kricka and P. E. Stanley (eds.), World Publishing Company, Singapore.
13. Lall, A. B., Ventura, D. S. F., de Souza, J. M., Bechara, E. J. H., Colepicolo-Neto, P. and Viviani, V. R. 2000, J. Insect Physiol. 46, 1137.
14. Lall, A. B., Järvilehto, M. V. A., Cronin, T. W., Carvalho, A. H., de Souza, J. M., Barros, M. P., Stevani, C. V., Ventura, D. S. F., Bechara, E. J. H., Viviani, V. R. and Hill, A. A. Unpublished manuscript.
15. Lall, A. B., Carvalho, A. H., Viviani, V. R., Ventura, D. S. F. and Bechara, E. J. H. Unpublished manuscript.
16. Seliger, H. H., Lall, A. B., Lloyd, J. E. and Biggley, W. H. 1982, Photochem. Photobiol. 36, 673.

17. Seliger, H. H., Lall, A. B., Lloyd, J. E. and Biggley, W. H. 1982, Photochem. Photobiol. 36, 681.
18. Horridge, G. A. 1969, Proc. Royal Soc. Lond. Sr. B 171, 445.
19. Cronin, T. W., Järvilehto, M., Weckström, M. and Lall, A. B. 2000, J. Comp. Physiol. A, 186, 1.
20. Järvilehto, M., personal communication.
21. Lloyd, J. E. 1966, Univ. Michigan Mus. Zool. Misc. Pub. No. 130, 1.
22. Lloyd, J. E. 1971, Annu. Rev. Entomol., 16, 97-122.
23. Lloyd, J. E. 1978, In: Bioluminescence in Action, P. J. Herring (ed.), Academic Press, New York.
24. Lloyd, J. E., 1983, Annu. Rev. Entomol. 28, 131.
25. Seliger, H. H., Buck, J. B., Fastie, G. F. and McElroy, W. D. 1964, J. Gen. Physiol. 48, 95.
26. Biggley, W. H., Lloyd, J. E. and Seliger, H. H. 1997, J. Gen. Physiol. 50, 1681.
27. Booth, D. J., Stewart, A. and Osorio, D. 2004, J. Exp. Biol. 207, 2373.
28. Case J. F. 1984. In: Insect Communication, T. Lewis (ed), Royal Entomol. Soc. London, London, 195.
29. Lloyd, J. E. 1984, Florida Entomologist 67, 228-239.
30. Lall, A. B. 1981, J. Insect Physiol. 27, 461.
31. Lall, A. B. and Worthy, K. M. 2000, J. Insect Physiol. 46, 965.
32. McElroy, W. D. and Seliger, H. H. 1966, In: Molecular Architecture in Cell Physiology, T. Hayashi and A. Szent-Gyorgi (eds.), Prentice Hall, Englewood Cliffs, New Jersey.
33. Costa, C. 1975, Arq. Zool. 265, 49.
34. Costa, C. 1976, Papéis Avulsos Zool. 29, 141.
35. Colepicolo–Neto, P. and Bechara, E. J. H. 1984, Arq. Biol. Tecnol. 27, 439.
36. Costa, C., Lawrence, J. F. and Rosa, S. P. (in press). Chapter Elateridae. In: Handbook of Zoology / Handbuch der Zoology. Band IV Arthropoda, Insecta, Teilband, Coleoptera: Evolution and Systematics (Polyphaga Part). Jena, Friedrich-Schiller-Universität Jena.
37. Rosa, S. P. 2007, Análise filogenética e revisão taxonômica da tribo Pyrophorini Candèze, 1863 (Coleoptera, Elateridae, Agrypninae). PhD thesis, Universidade de São Paulo. Brazil.
38. Arnoldi, F. G. A., Ogoh, K., Ohmiya, Y. and Viviani, V. R. 2007, Gene 405, 1.
39. Rosa, S. P. 2004, Rev. Bras. Entomol. 48, 203.
40. Costa, C. 1991, Rev. Bras. Entomol. 35, 567.
41. Costa, C. 1982, Rev. Bras. Entomol. 1, 23.
42. Bechara, E. J. H. 1988, In: Advances in Oxygenated Processes, L. Baumstark (ed.), 1. JAI Press, London, 123.
43. Bechara, E. J. H., Colepicolo-Neto, P., Viviani, V. R., Barros, M. P. and Costa, C. 1999, An. Acad. Bras. Cienc. 71, 169.
44. Briscoe, A. D. and Chittka, L. 2001, Ann. Rev. Entomol. 46, 471.
45. Wald, G. 1960, In: Comparative Biochemistry, M. Florkin and H. S. Mason (eds.) Vol. 1 Academic Press. New York.
46. Whittaker, R. J. and Jones, S. H. 1994, J. Biogeography 21, 245.
47. Brossard, M., Lopez-Hernandez, D., Lepage, M. and Leprun, J. 2007, Bio. Fert. Soils. 43, 437.

48. Yarranton G. A. and Morrison, R. G. 1974, J. Ecology 62, 417.
49. Reis, A., Bechara, F. C., Espindola, M. B., Vieira, N. K. and Lopes, L. 2003, Braz. J. Natur. Conserv. 1, 85.
50. Crowson, R. A. 1981, The Biology of Coleoptera. Academic Press, New York.
51. Halliwell, B. and Gutteridge, J. M. C. 2006, Free Radicals in Biology and Medicine, 4th ed., Oxford University Press, London.
52. Colepicolo-Neto, P., Bechara, E. J. H. and Costa, C. 1986, Insect Biochem. 16, 381.
53. Barros, M. P. and Bechara, E. J. H. 1998, Free Radic. Biol. Med., 24, 767.
54. Bechara, E. J. H., Costa, C., Colepicolo, P., Viviani, V. R., Barros, M. P., Timmins, G. S., Lall, A. B., Terra, W. R., Ferreira, C., Stevani, C. V. and Torres, M. A. 2007, Arch. Org. Chem. viii, 311.
55. Viviani, V. R. and Bechara, E. J. H. 1995, Photochem. Photobiol. 62, 490.
56. Colepicolo-Neto, P., Costa, C. and Bechara, E. J. H. 1986a, Insect Biochem. 16, 803.
57. Lawrence, J. F. 1982, In: Synopsis and Classification of Living Organisms, S. P. Parker (ed.), Vol. 2., McGraw–Hill, New York.
58. Zaragoza-Caballero, S. 1984, An. Inst. Biol. Univ. Nal. Autón. México, Ser. Zoología 55, 203.
59. Costa, C., Vanin, S. and Casari–Chen, A. 1988, Larvas de Coleoptera do Brasil, Museu de Zoologia, Universidade de São Paulo.
60. Costa, C. and Zaragoza, S. (in press). Chapter Phengodidae. Handbook of Zoology / Handbuch der Zoology. Band IV Arthropoda, Insecta, Teilband, Coleoptera: Evolution and Systematics (Polyphaga Part). Jena, Friedrich-Schiller-Universität Jena.
61. Buck, J. B. 1950, Ant. Rec. 108, 121.
62. Tiemann, D. 1967, Proc. Calif. Acad. Sci. 35, 235
63. Wittmer, W. 1976, Entomol. Arb. Mus. Frey 27, 41559.
64. LeSage, L. 1991, In: Immature Insects, F. W. Stehr, (ed.), Vol. 2., Kendall/Hunt Publishing Co., Dubuque, Iowa, 424.
65. Viviani, V. R. and Bechara, E. J. H. 1993, Photochem. Photobiol. 58, 615.
66. Viviani, V. R. and Bechara, E. J. H. 1997, Entomol. Soc. Am. 90, 389.
67. Costa, C., Vanin, S. A., Casari, S. A. and Viviani, V. R. 1999, Iheringia, Ser. Zool. 86, 6.
68. Sivinsky, J. 1981, Coleopt. Bull. 35, 167.
69. Lloyd, J. E. 1968, Entomol. News 10, 265.
70. Seliger, H. H. 1975, Photochem. Photobiol. 21, 355.
71. Lythgoe, J. N. 1966, In: Light as an Ecological Factor, R. Bainbridge, G. C. Evans and O. Rackham (eds.), Bracewell, Oxford, 375.
72. Lall, A. B. and Jensen, T. 1973, Biol. Bull. 145, 444.
73. Kelber, A., Balkenius, A. and Warrant, E. J. 2002, Nature 419, 922.
74. Kelber, A. and Roth, L. S. N. 2006, J. Exp. Biol. 209, 781.
75. Lall, A. B. 2006. Twenty-ninth Euro. Conf. Visual Percept. Abs., 140.
76. Menzel, R. and Backhaus, W. 1991, In: The Perception of Colour, P. Gouras (ed.), Macmillan, London.

Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 229-242
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

12

The synchronous flashing signal of *Pteroptyx effulgens* in Papua New Guinea is used by *P. tarsalis* to form aggregations

Nobuyoshi Ohba[1] and Ayu Shimoyama[2]
[1]"The Ohba Firefly Institute", 4-1-12-204 Maborikaigan, Yokosuka City 239-0801, Japan; [2]c/o Meyer-Rochow Research Group, Eläinmuseo University of Oulu, SF-90014 Oulu, Finland

Abstract

In the Papua New Guinean firefly Pteroptyx effulgens *more than several thousand individuals gather together in specific trees to emit their flashes in synchrony. The pronotum in this species is orange in colour, the elytra are black and body length is approx. 7 mm. Synchronous flashing is only seen in males and flash intervals amount to approximately 0.9 sec. It is an unusual flashing pattern. The females*

Correspondence/Reprint request: Dr. Nobuyoshi Ohba, "The Ohba Firefly Institute", 4-1-12-204 Maborikaigan, Yokosuka City, 239-0801, Japan. E-mail: qgb00523@nifty.ne.jp

fly about and emit a weak green continuous light for 3-4 seconds to attract a male. The females of another species, P. tarsalis., *do not fly and only perch on a leaf. When males of* P. effulgens *locate a female, they orientate towards her and approach her. An emission of rapid flashing signals precedes copulation. Often there are two species of fireflies in one tree, one of them being the yellow* P. tarsalis *and the other* P. effulgens P. tarsalis *resembles* P. effulgens *in much of its external morphology, but the two species can be distinguished by the colour of their elytra. In* P. tarsalis *the pronotum is yellow and the apices of the elytra are dark. The ratio of* P. effulgens *to* P. tarsalis *in a tree is approximately 5:1, with* P. effulgens *always being the more abundant species. Regarding luminescence behaviour,* P. tarsalis *males begin flashing at midnight, but do not synchronize their flashes; futhermore the luminescent calling signal of the female has a different colour from that of* P. effulgens. *Yet, based on field and laboratory observations,* P. tarsalis *appears to make use of the other species' communication system. It is thought that the small population of* P. tarsalis *achieves greater efficiency through the use of the luminescent signals employed by the larger population of* P. effulgens *males.*

1. Introduction

Fireflies, which flash together at the same time on a particular tree are well known in South East Asia [1-5]. In *Pteroptyx effulgens* only the males possess the flashing characteristic; the females produce their light continuously. This phenomenon has been described repeatedly, but the mechanism behind it is still not fully understood.

According to my research on *Pteroptyx effulgens* in Papua New Guinea and also *P. tener* in Singapore and Malaysia, I confirmed many of the observations made by Case [6] and was able to show that the flash pattern represents a very important component of the synchronous flashing signal and also demonstrated its ethological meaning [7-12]. During this research, it was found that the Malaysian *P. valida* shares its habitat with *P. tener*. However, the two species occupy different layers of the tree. Furthermore, *P. valida* does not join in the synchronous flashing of the other species [10].

A somewhat similar phenomenon of co-existence can be observed in Papua New Guinea with *P. tarsalis* and *P. effulgens*. In this article, We are going to present comparative data on the morphology of *P. tarsalis* and *P. effulgens*, and shall then explain the function of their luminescence behaviours in the wild.

2. Method

The targets of this research have been *Pteroptyx effulgens* and *P. tarsalis,* which occur together in the same habitat and which were observed in the field and in the laboratory (Figs.1, 2). *P. effulgens* males constitute the larger group resident in a particular tree, and their lighting patterns rapidly become synchronous once luminescent activity starts. This synchronous flashing behaviour can be observed throughout the whole year. The precise location of the research site can be revealed on request.

Figure 1. Tree with large numbers of *P. effulgens* and *P. tarsalis* and the environment.

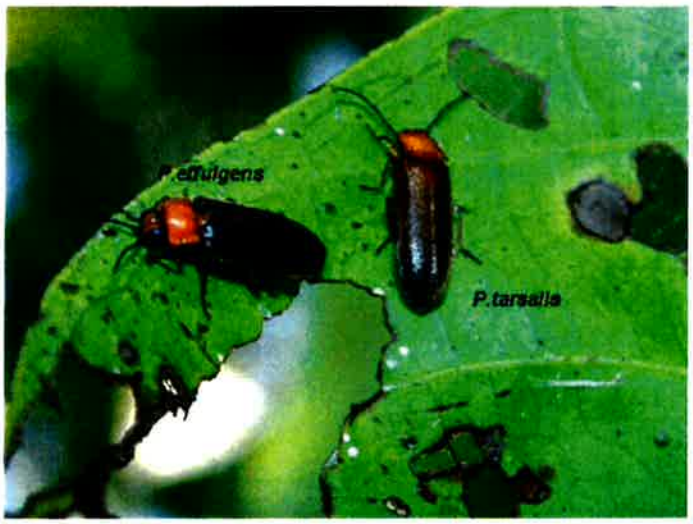
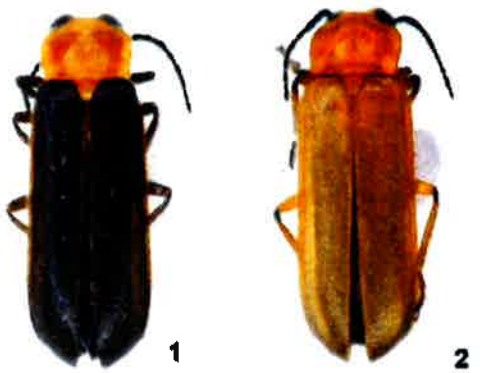


Figure 2. *P. effulgens* (left) and *P. tarsalis* (right) gather on the same tree.

3. Morphological and ecological observations

3.1. Comparisons of the external morphologies of *P. effulgens* and *P. tarsalis* adults

The external morphology of the two species is very similar, but the elytra of *P. effulgens* are of a black colour and the pronotum is reddish orange (Fig. 3). On the other hand *P. tarsalis* possesses some yellow colour on both of these body parts. Given this slight difference only in coloration, one might think that they could face some difficulty in distinguishing each other by appearance. Yet, because the compound eyes of these two firefly species are



1. *P. effulgens* 2. *P. tarsalis*

Figure 3. Dried specimens of male *P. effulgens and P. tarsalis.*

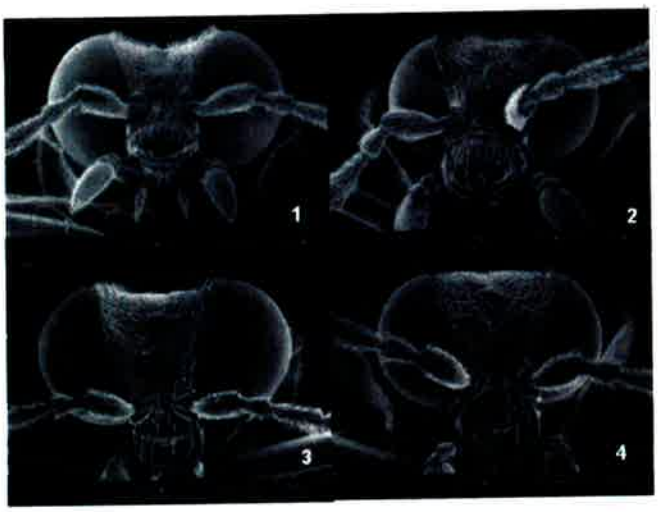


Figure 4. Head of male *P. effulgens* and *P. tarsalis.* 1. *P. effulgens* (male), 2. *P. effulgens* (female), 3. *P. tarsalis* (male), 4. *P. tarsalis* (female).

highly developed, it is possible that at least over short distances they might be able to perceive the distinct colours of their respective elytra.

3.2. Observations by scanning electron microscopy: SEM

Head shapes and dimensions of *P. tarsalis* are very similar to those of *P. effulgens* (Figs. 4, 5). In both species the compound eyes are well developed and the ommatidia are of uniform and hexagonal shapes (Fig. 6) The mandibles are small, curved, and their apices are sharply pointed (Fig. 5). The

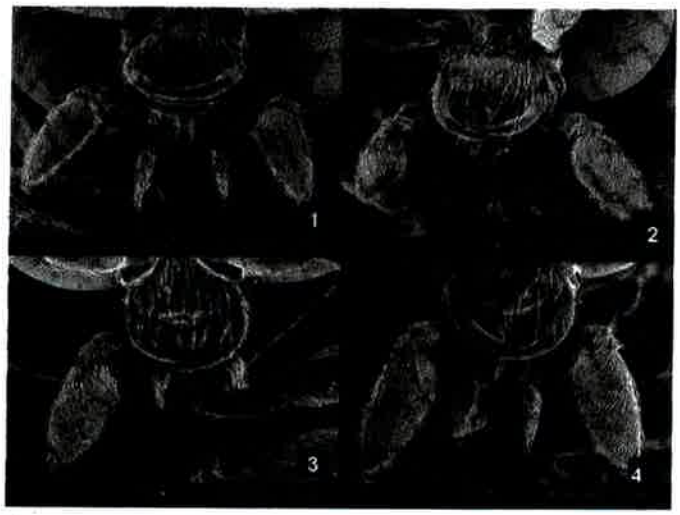


Figure 5. Mouth parts of male *P. effulgens* and *P. tarsalis* 1. *P. effulgens* (male), 2. *P. effulgens* (female), 3. *P. tarsalis* (male), 4. *P. tarsalis* (female).

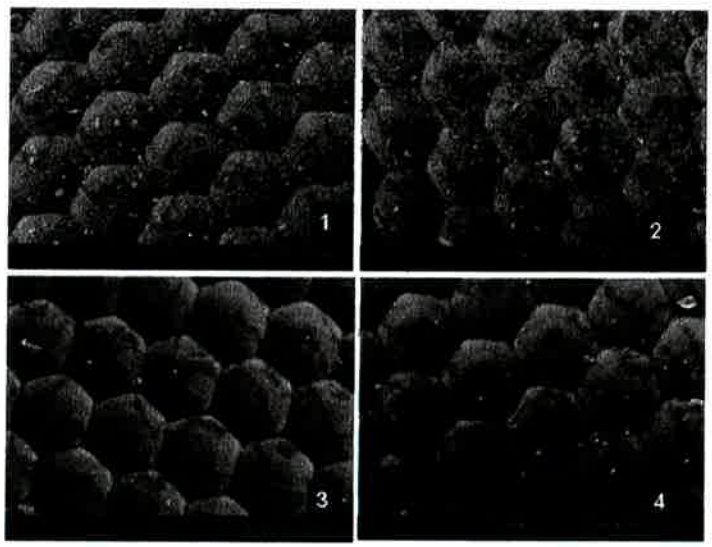


Figure 6. Compound eyes of male and female *P. effulgens* and *P. tarsalis.* 1, 2. *P. effulgens;* 3, 4. *P. tarsalis.*

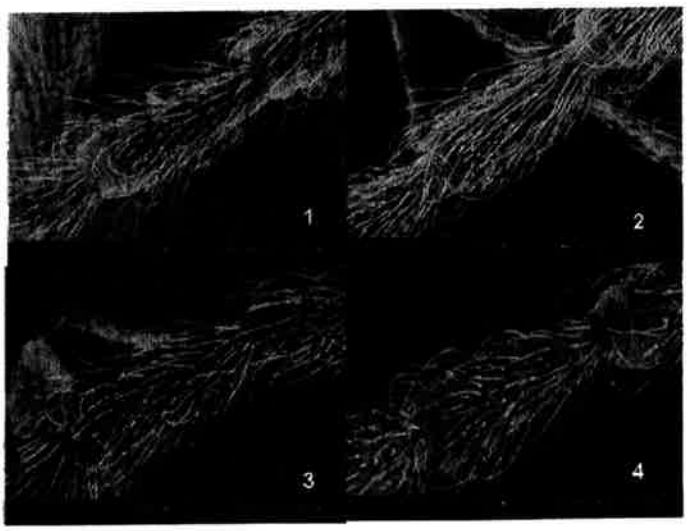


Figure 7. Antennae of male *P. effulgens* and *P. tarsalis*. 1, 2. *P. effulgens* 3, 4. *P. tarsalis*.

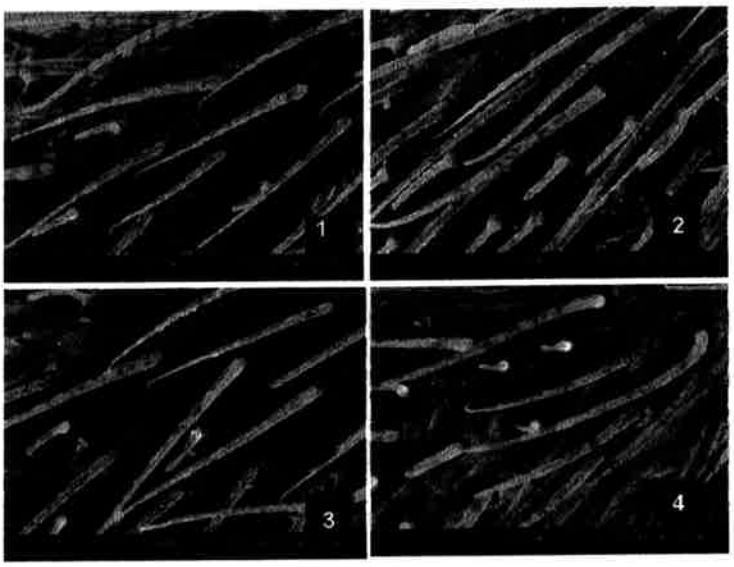


Figure 8. Antennae of female *P. effulgens* and *P. tarsalis*. 1, 2. *P. effulgens* 3, 4. *P. tarsalis*.

antennae are slim and filiform, covered luxuriantly by sensory hairs (Figs. 7, 8). There is, thus, very little difference between the two species.

3.3. Ratios of *P. effulgens* and *P. tarsalis*

The results of a single random midday sweeping-catch at the observation site was: 376 specimens of *P. effulgens* and 75 of *P. tarsalis*. This overwhelming dominance of *P. effulgens* (on average representing 83.4%)

over *P. tarsalis* (16.6%) is characteristic even at night and was present also at other collection sites. At all times and under all conditions *P. effulgens* is always the dominant species. Yet, *P. tarsalis* can certainly maintain its own population, as its flashing pattern differs from that of *P. effulgens*.

4. Observations on luminescence

4.1. Records of flashing patterns

We used an image intensifier tube, connected to a small videotape recorder for recording the flashing patterns. The image was then played on a monitor in the laboratory and the flashing signals were changed into electronic signals by a sensor. The signal needed to be amplified by an amplifier. The signal was then passed through an AD board and analyses of wave forms, flash durations and power spectra were carried out with the aid of a specific flashing pattern analysis programme [13].

4.2. Flashing activity of *P. tarsalis* in the field

4.2.1. Natural conditions

The fireflies seen flashing in the field were definitely almost always the males of *P. effulgens*. Those of *P. tarsalis* hardly ever emitted any flashes. Therefore, in order to compare the role of the flashing pattern in the two species and to demonstrate that species-specific matings do occur, females of each species were put into separate plastic bags, with the latter being placed 2 m away from the tree. Females of *P. effulgens* started emitting light signals at 19:30h, while those of *P. tarsalis* started emitting light signals at 0:00h and although to the human eye identical, the males of the two species were apparently able to distinguish the signals as they sat with their corresponding females.

Although it is difficult to distinguish the differences of the flashing patterns of male and female *P. tarsalis* in a large group of *P. effulgens* individuals, it is nevertheless possible, because the colour of the lights of each species is slightly different and only just perceptible to the human eye.

According to [7], the spectral emission peek of the light of *P. effulgens* is 565 nm for the male and 558 nm for the female, a difference of only 7 nm. Even though the human eye can barely see the difference, the firefly's compound eyes almost certainly can distinguish the differences of these flashing colours, because of the well developed sensitivity that is characteristic of firefly eyes generally [14-16]. It is further possible that the males of both species can distinguish each other's light signals from those of their own females, but this has not yet been able to be verified. However, when very close to the females, the males can possibly identify their own female's elytra.

It is poorly known whether and how male individuals belonging to different species communicate with each other when present in a group of different firefly species in the wild [11]. According to our research on coincident luminescent signalling in *P. tarsalis* and *P. effulgens* that of the former would not be recognizable, because it would be 'drowned out' by the signals of the dominant *P. effulgens*. Moreover, the females of *P. effulgens*, unlike those of *P. tarsalis*, produce a continuous light during peak activity and fly around the trees in the company of luminescing males to attract the latter.

4.2.2. Calling signals of female *P. effulgens* and *P. tarsalis*

According to the flash pattern analysis of the luminescent calling signals of *P. effulgens* females, the wave shape is complicated (Fig. 9.5-8), but to the human observer simply seems to be a green and continuous light. The flash pattern of *P. tarsalis* females is also like that (Fig. 12) and therefore difficult

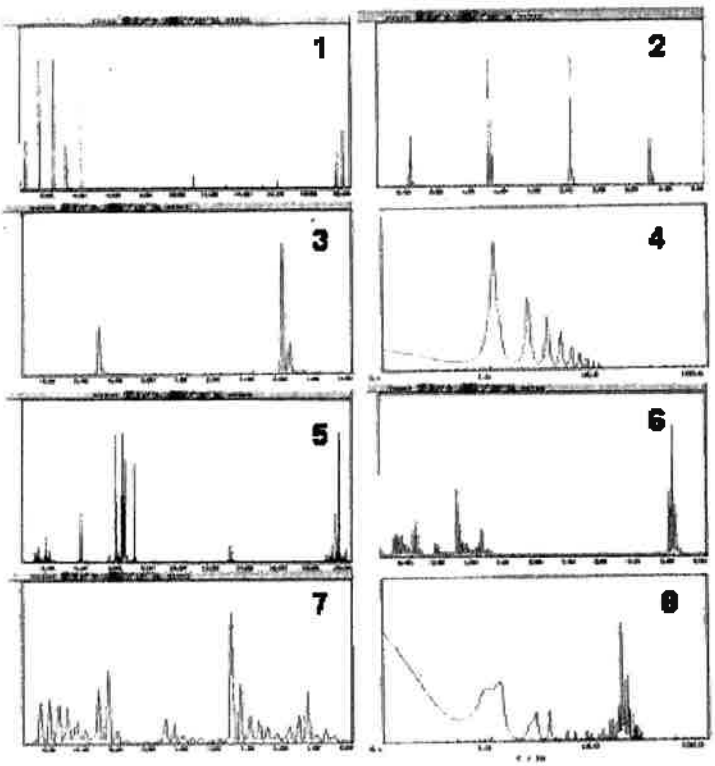


Figure 9. Flashing patterns of *P. effulgens*. Beginning of flash recordings at 21:00h; atmospheric temperature: 22°C. 1. Male (recorded 20 sec), 2. Male (recorded 4 sec), 3. Male (recorded 2 sec), 4. Power spectrum of male light. 5. Female (recorded 20 sec), 6. Female (recorded 4 sec), 7. Female (recorded 2 sec), 8. Power spectrum of female light. In recordings 1-3 and 5-7 the x-axis is time (sec) and y-axis is relative light intensity; in recordings 4 and 8 the x-axis is Hz and y-axis is relative intensity.

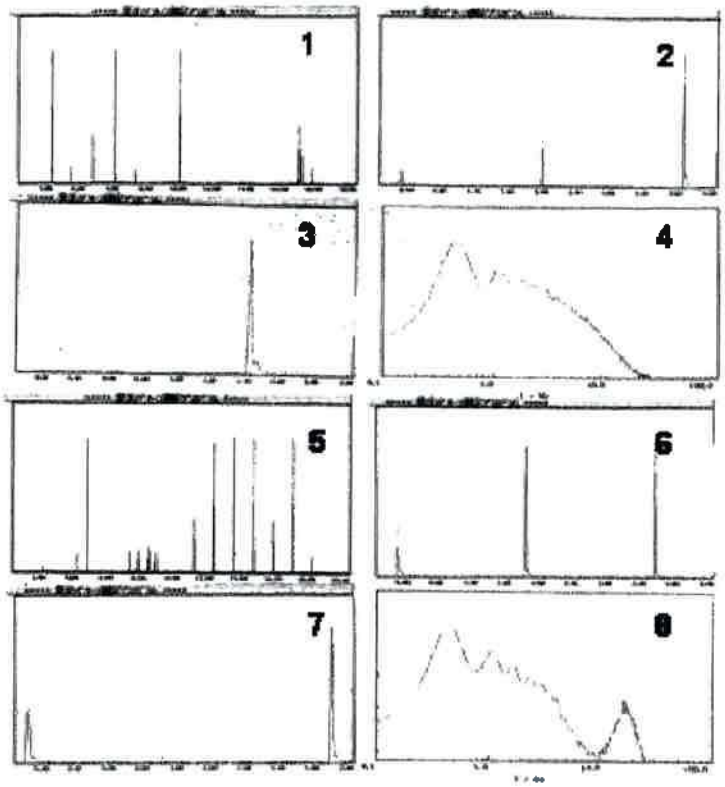


Figure 10. Flashing patterns of male *P. tarsalis*. Beginning of flash recordings at 0:00h. 1. Beginning flashes (recorded 20 sec), 2. Beginning flashes (recorded 4 sec), 3. Beginning flashes (recorded 2 sec), 4. Beginning flashes, power spectrum, 5. Flashing pattern including short cycle flashing (recorded 20 sec), 6. Flashing pattern, including short cycle flashing (recorded 4 sec), 7. Flashing pattern including short cycle flashing (recorded 2 sec), 8. Flashing pattern including short cycle flashing, power spectrum. In recordings 1-3 and 5-7 the x-axis is time (sec) and y-axis is relative light intensity; in recordings 4 and 8 the x-axis is Hz and y-axis is relative intensity.

to distinguish from that of *P. effulgens* females by human eye (Fig. 12.1-8). Although the spectral distribution of the females' light signals has not been analysed, that of the males of the two species is nearly identical and indistinguishable to the human eye. That the males can distinguish the signals of their corresponding females was shown with the experiment of the light-emitting females in a plastic bag (see above).

4.3. Flashing activity under indoor conditions

General luminescence activity in *P. effulgens* started at 19:30h, while that of *P tarsalis* did not commence until after 0:00. In *P. effulgens* it was the males that began to emit light at 19:30 h and to synchronize their signals soon after that. The males of *P. tarsalis* started their light production after 0:00 and

did not synchronize their flashes. The flashing patterns of both species are totally different (Figs. 9-12.). In *P. effulgens* regular flash intervals of around 0.9 seconds occur (Figs. 9. 1-3), the power spectrum has its main peak at around 1 Hz (Fig. 9.4) trailed by 8 shoulder peaks at around 2 to 10 Hz. The luminescence in *P. tarsalis* exhibits much greater changes over the time course of one flash (Figs. 10-11).

In males of *P. tarsalis* the wave of each flash cycle ranges from 0.3 to over 20 Hz (Figs. 10-11), and the flash periodicity is irregular. The irregularities are present in all observations and remarkably short intervals between flashes are recordable. The power spectrum of this short-interval light lies around 17 Hz (Fig. 11.8). When the flashing activity quickens, short

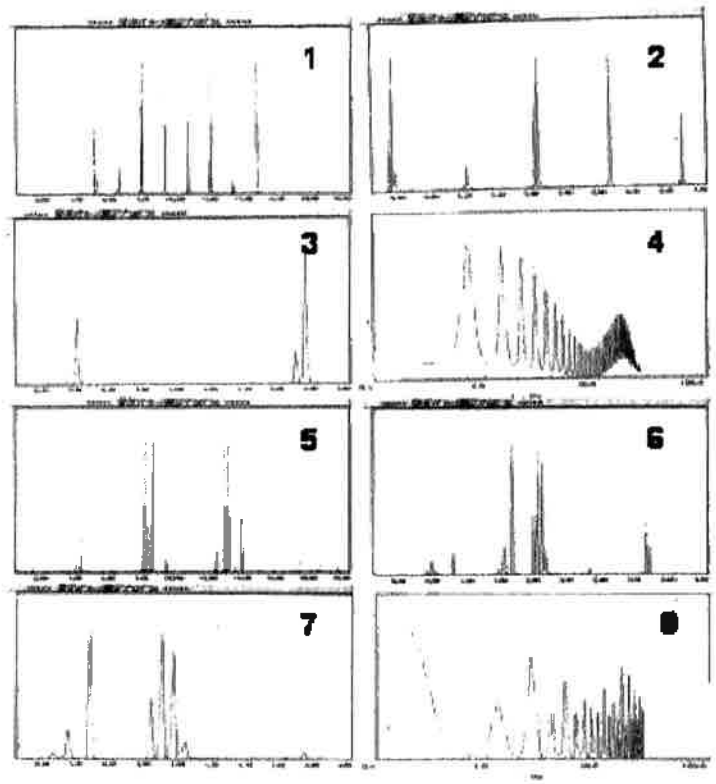


Figure 11. Flashing patterns of *P. tarsalis* (most active time 02 : 00h). 1. Male flashing pattern with increased short cycle (recorded 20 sec), 2. Male flashing pattern with increased short cycle (recorded 4 sec), 3. Male flashing pattern with increased short cycle (recorded 2 sec), 4. Male flashing pattern with increased short cycle, power spectrum, 5. Male flashing pattern with increased short cycle (recorded 20 sec), 6. Male flashing pattern with increased short cycle (recorded 4 sec), 7. Male flashing pattern with increased short cycle (recorded 2 sec), 8. Male flashing pattern with increased short cycle, power spectrum. In recordings 1-3 and 5-7 the x-axis is time (sec) and y-axis is relative light intensity; in recordings 4 and 8 the x-axis is Hz and y-axis is relative intensity.

interval light dominates (Fig. 11) and specific peaks of 18 Hz can be seen in the power spectrum. The shoulder peaks are most obvious in Fig. 11.5-7, with one flashing cycle containing 3 to 4 smaller shoulders. In the power spectrum, cycles with ranges from 11 Hz to 20 Hz can be recorded (Fig. 11.8).

Flashing patterns of female specimens: In both species the flashing patterns are very similar (Figs. 9.1,2). The flashing intervals in *P. effulgens* are irregular, but with regard to the smaller shoulder peaks the intervals are almost regular.

Intervals between shoulder peaks amount to 0.05 seconds, which is why humans can see the signal only as a continuous light. The power spectrum shows long and short cycles (Fig. 9.8). A 10 Hz cycle has not been observed, but numerous 17 Hz cycles were present. On the other hand, females of *P. tarsalis*

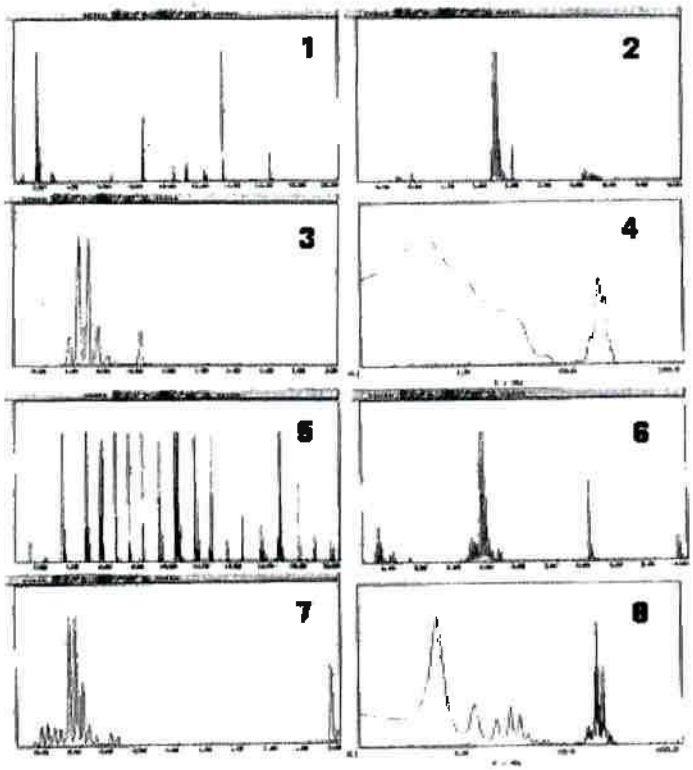


Figure 12. Flashing patterns of female *P. tarsalis*. 1. Beginning flashes (recorded 20 sec), 2. Beginning flashes (recorded 4 sec), 3. Beginning flashes (recorded 2 sec), 4. Beginning flashes, power spectrum, 5. Flashing pattern with increased short cycle (recorded 20 sec), 6. Flashing pattern with increased short cycle (recorded 4 sec), 7. Flashing pattern with increased short cycle (recorded 2 sec), 8. Flashing pattern with increased short cycle, power spectrum. In recordings 1-3 and 5-7 the x-axis is time (sec) and y-axis is relative light intensity; in recordings 4 and 8 the x-axis is Hz and y-axis is relative intensity.

possess a variety of shorter and longer cycles with strikingly short cycles of ca. 17 Hz. Flash pattern analyses show 0.4 Hz and 17 Hz cycles to represent the main components (Fig. 12.4, 8).

P. tarsalis does not start flashing until midnight, and, as mentioned above, has no synchronous flashing behaviour to attract the sexes to each other. After midnight it produces short flashing signals, with specific cycles as shown in Fig. 10.1-8.

These flashing signals are likely to increase following the detection of a female's calling signal. The female then communicates with the male via her own flashing signal. This was observed by putting males and females into two separate plastic bags.

Based on indoor, laboratory observations, *P. tarsalis,* with regard to communication by luminescent signals, uses different activity times from those seen in *P. effulgens*.

4.4. Differences of the flashing patterns of both species under indoor conditions

Figs. 10-11 show the male *P. tarsalis* signal, which the female individual can recognize. The males of *P. effulgens* employ synchronous flashing and of around 1 Hz repetition rates at peak activity. Following the peak, there are 8 shoulders in a 10 Hz cycle. On the other hand, *P. tarsalis* males do not employ synchronous flashing and their flashes cover a range of 0.2 to 20 Hz cycles with irregular intervals.

Furthermore, flashing patterns may change with flashing activity. Especially lights of around an 18 Hz cycle increase, while longer cycles decrease. As mentioned above, each of the two species has its own different characteristic flashing patterns, so that the females of both species can find their own males by their own characteristic flashing patterns.

Although, the flashing patterns of the females of both species are very similar to each other, the males can apparently distinguish them and may make use of the colour differences in the females' signals.

5. Mechanism for group formation of *P. tarsalis* on the same tree with *P. effulgens*

While the vast majority of *P. effulgens* get together as a species aided by their specific flashing signal [7], *P. tarsalis* does not employ synchronous flashing and may find it more difficult to gather together as a species. It is conceivable that olfaction may help in bringing individuals together.

Furthermore, as *P. tarsalis* starts flashing only at midnight, it is unlikely it could then still call other con-specific males from afar to the same tree, in

which large numbers of synchronously flashing *P. effulgens* males are already emitting their lights, conceivably drowning out the lights of the small numbers of *P. tarsalis* males, when they start to emit their lights.

Based on this scenario, *P. tarsalis* males might well be first attracted by *P. effulgens'* synchronous flashing to a tree on which a population of *P. effulgens* is already resident. After midnight when there are also sufficient females of *P. tarsalis* around, *P. tarsalis* males may also begin to start flashing. There is thus the possibility of inter-specific communication between *P. tarsalis* and *P. effulgens*.

In detail, *P. tarsalis* uses *P. effulgens'* signal as the first step to get together on a tree that is already occupied by *P. effulgens* males. *P. tarsalis'* own flashing activity then evolved as a consequence of using *P. effulgens'* synchronous flashing signals to get together. This has not been reported before and represents a newly-discovered phenomenon.

As mentioned above, it seems an exceptional situation that similar congeneric species live in the same place and at the same time, but remain segregated in mating. We regard this as an important discovery in view of species differentiation and the role of activity and behaviour in such speciation.

One of us (N. Ohba) intends to examine the male genitalia of the two firefly species to determine, whether there are clear morphological differences that preclude bastardisations between the species. N. Ohba also plans to carry out similar research and a similarly detailed analysis of the seemingly related phenomenon of *P. calida* and *P. tener* co-occurrences in Singapore and Malaysia.

Acknowledgment

N. Ohba very much appreciates the following people's cooperation in the research: Mr. Michiaki Hirowatari from NHK, Mr. Sonny Wong from the Malaysia Nature Society, Mr. Sim Sian former Curator of "Night Safari" in Singapore. Both authors also express their sincere thanks to Prof. V.B. Meyer-Rochow, who read and corrected the draught and provided them with useful hints and comments.

References

1. Bassot, J.M., and Polunin, I.V. 1967, Sci. Rept. Yokosuka City Mus., 13,18-23.
2. Buck, J., and Buck, E.1966, Nature, 211, 562-564.
3. Haneda, Y. 1966, Sci. Rept. Yokosuka City Mus., 12, 1-8.
4. Hanson, F. E., Case, J. F., Buck, J., and Buck, E.1971, Science, 174, 161-164.
5. Lloyd, J. E. 1973, Nature, 245, 268-270.
6. Case, J. 1980, Biol. Bull., 159, 613-625.

7. Ohba, N. 1999, Sci. Rept. Yokosuka City Mus., 46, 33-40.
8. Ohba, N.2004, Mystery of Fireflies (in Japanese). Yokosuka City Museum, Yokosuka.
9. Ohba, N. 2006, Sci. Rept. Yokosuka City Mus., 53, 1-8.
10. Ohba, N. 2006, Sci. Rept. Yokosuka City Mus., 53, 9-17.
11. Ohba, N., and Sim, S.H. 1994, Sci. Rept. Yokosuka City Mus., 42, 1-11.
12. Ohba, N., and Wong, C.H. 2004, Sci. Rept. Yokosuka City Mus., 51, 1-33.
13. Makino, T., Suzuki H., and Ohba, N. 1994, Sci. Rept. Yokosuka City Mus., 42, 27-57.
14. Eguchi, E,, Nemoto, A., Meyer-Rochow, V.B., and Ohba, N. 1984, J. Insect Physiol., 30, 607-612.
15. Lall, A.B., and Worthy, K.M. 2000, J. Insect Physiol., 46, 965-968.
16. Lall, A.B. 1993, J. Exp. Zool., 265, 609-612.

Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 243-253
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

13

Larval respiration system and evolution in aquatic fireflies (Coleoptera: Lampyridae: Luciolinae)

Xinhua Fu[1] and Lesley Ballantyne[2]
[1]Department of Plant Science and Technology, Hua Zhong Agricultural University, Wuhan, 430070, China; [2]School of Agricultural and Wine Sciences Charles Sturt University, PO Box 588, Wagga Wagga 2678, Australia

Abstract

Aquatic larvae of the firefly Luciola substriata Gorham are of two different morphological types. First and second instar larvae have bristle-like structures on the lateral margins of the abdomen, which are confirmed as gills. First to second instar larvae use both gills and tracheae to respire. Third to sixth instar larvae lack lateral abdominal gills and depend on a well-developed metapneustic tracheal

Correspondence/Reprint request: Dr. Xinhua Fu, Department of Plant Science and Technology, Hua Zhong Agricultural University, Wuhan, 430070, China. E-mail: fuxinhua2001@yahoo.com

respiration system. Four other species with very similar aquatic larvae, viz. Luciola leii Fu et Ballantyne, L. ficta Olivier, L. lateralis Motsch., and L. cruciata Motsch., have gill-spiracle compound structures along the sides of the abdomen. The possibility that aquatic fireflies evolved from terrestrial lampyrids, and that Luciola substriata may have secondarily returned to water, is explored.

1. Introduction

Aquatic fireflies are rare and most of them belong to the genus *Luciola* [1-3]. Jeng *et al.* [2] reviewed aquatic fireflies in China, confirmed the aquatic status in five *Luciola* species from Taiwan and Japan (*Luciola substriata*, *L. ficta*, *L. lateralis*, *L. cruciata* and *L. owadai*), and described a new Taiwanese species *Luciola hydrophila.* Three species, *L. lateralis, L ficta*, and *L substriata,* are more widespread and have ranges that include parts of mainland China [4, 5]. Recently, two species of aquatic firefly, *Luciola leii* [3] and *Luciola aquatilis* Thancharoen [6] were discovered from mainland China and Thailand, respectively.

Among these aquatic fireflies, *L. substriata* and *L. aquatilis* share morphologically similar larvae. Both species, which are backswimmers, possess two types of larvae with very different external morphological structures [2, 5, 7]. The first and second instar larvae possess lateral bristles determined here to function as gills in *L. substriata*, while third to sixth instar larvae have lost abdominal gills and are metapneustic [5].

Other aquatic firefly larvae, viz. *L. lateralis*, *L. cruciata*, *L. owadai*, *L. ficta*, *L. hydrophila* and *L. leii* were reported to respire via tracheal gills [1-4, 8-10] but detailed observations on the structure and the function of the tracheal gills is lacking. Here we describe the transformation of the larval respiration system in the backswimming firefly *L. substriata* and compare this with other aquatic firefly larvae, which possess only tracheal gills, and explore a possible scenario for the evolution of the larval respiration system of aquatic fireflies.

2. Materials and methods

Adults of both sexes of *L. substriata* were collected in Lake Tangxun and several nearby fishponds on the campus of Huazhong Agricultural University, Wuhan City, Hubei Province (30.5° North, 114.3° East) during evenings in the month of July by Fu X H. Adult fireflies were kept in an aqua-terrarium measuring 30×20×6 cm, with water and soil exposed to air, and the giant duckweed *Spirodela polyrhiza* Schleiden (Monocotyledoneae: Lemnaceae). When the eggs hatched, newly-hatched aquatic snails were

offered as food for the first-instar larvae. Mid-instar and last-instar larvae were collected from Lake Tangxun in the evening, using their light impulses to locate them. The collected larvae were reared in an aquarium for further study.

To observe the larval surface structures and spiracles, scanning electron microscopy (SEM) was used. The terminal two abdominal segments of older and newly hatched larvae were cut through the light organ and fixed for 24 h in 4°C cold, phosphate-buffered 2.5% glutaraldehyde, pH 7.4. The material was then rinsed twice in phosphate buffer at 10-min intervals and post-fixed for 3- 4 hrs in 1% osmium tetroxide at room temperature. Two rinses in phosphate buffer and dehydration in a graded series of ethanol with 12 h stays at each concentration (30, 50, 70, 80, 90, and 100%) followed. The specimens were then placed in acetone for two 12 h periods before finally being subjected to critical point drying. Each specimen was attached with double-sided sticky tape to an aluminium stub and sputter-coated with gold to a thickness of about 200 nm. The specimens were observed under a Hitachi S450 scanning electron microscope at an accelerating voltage of 25 kV [11, 12].

For ultrastructural observations of the bristle-like structure on the lateral abdominal region in 1st instar larvae, the newly hatched larvae were fixed in 2% cold glutaraldehyde at pH 7.3 in a 50 M sodium cacodylate buffer (with 150 M sucrose added). After post-fixation in 2% cold osmium tetroxide in the same buffer, the 1st instar larvae were dehydrated through a graded series of acetone and embedded in araldite. The sections were made along the dashed vertical to the stem of the bristle-like structure (Fig. 3 D). Semi-thin sections for light microscopy were stained with Delafield's haematoxylin and eosin; ultrathin sections for transmission electron microscopy were double-stained with uranyl acetate and lead citrate for a few minutes each (Meyer-Rochow and Liddle, 1988), and were examined in a Hitachi H-600 TEM microscope at 75 KV accelerating voltage. The 1st instar and final instar larvae were cross-sectioned to examine the respiration system.

The respiration systems of two types of larvae were dissected and observed under the microscope and photographed (Olympus Bx51, SZ51, coupled with Dp20 CCD).

3. Results

3.1. Respiratory system in *Luciola substriata* larva

In first and second instar larvae, SEM revealed that bristle-like structures are located on the sides of the abdominal segments (Figs. 1 A; 2 A, B; 3 A - E). One pair of posterior spiracles occurs at the tip of the 8th abdominal segment (Figs. 3 E, F). Light microscope observation revealed that the primary

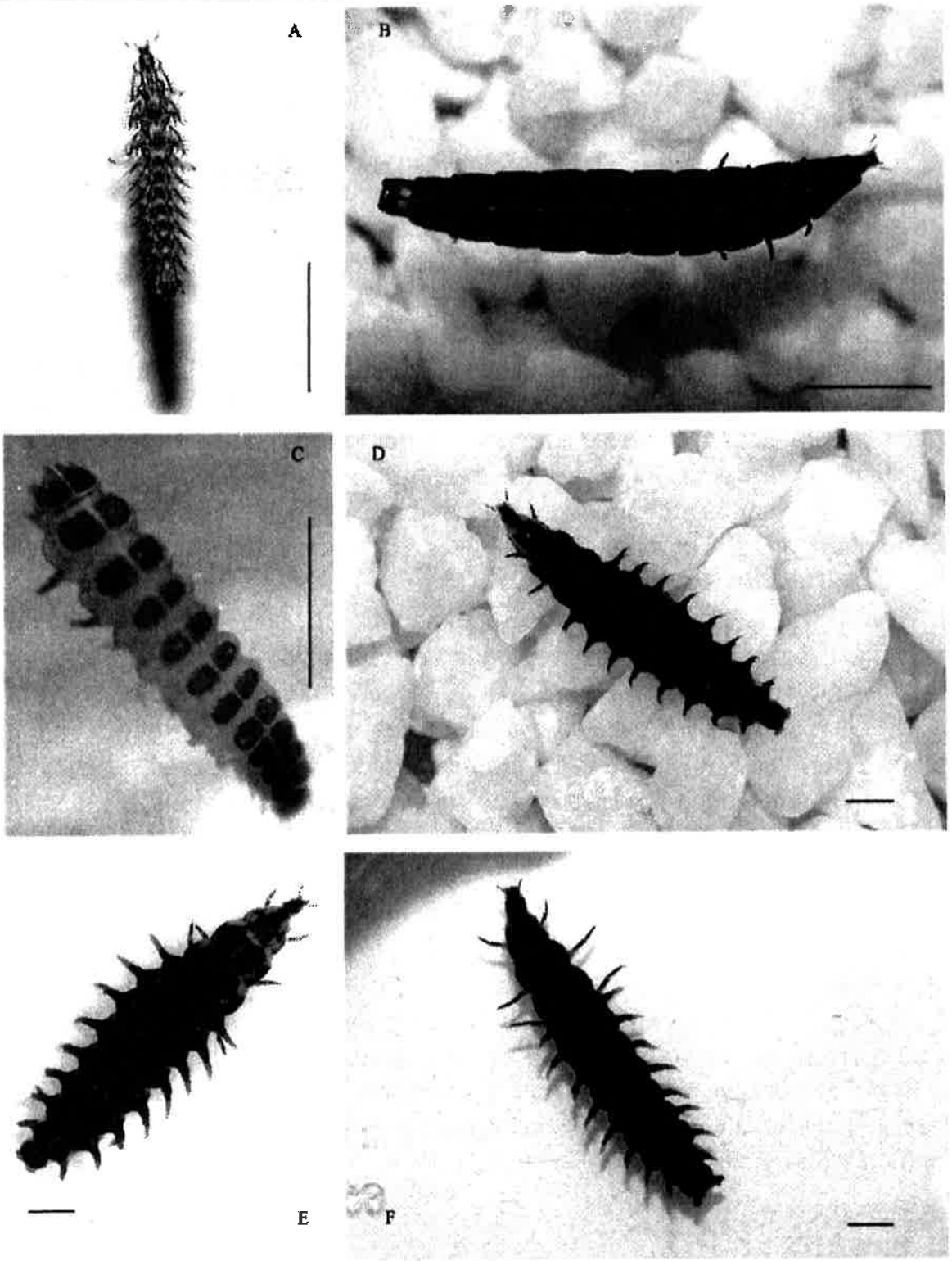


Figure 1. Larvae of aquatic fireflies. A. *Luciola substriata*: first instar larva, back-swimming. Scale bar: 1mm. B. *L. substriata*: final instar larva, back-swimming. Scale bar: 5mm. C. *L. leii*: first instar larva. Scale bar: 1mm. D. *L. leii*: final instar larva. Scale bar: 5 mm. E. *L. ficta*: final instar larva. Scale bar: 5 mm. F. *L. cruciata*: final instar larva. Scale bar: 5 mm.

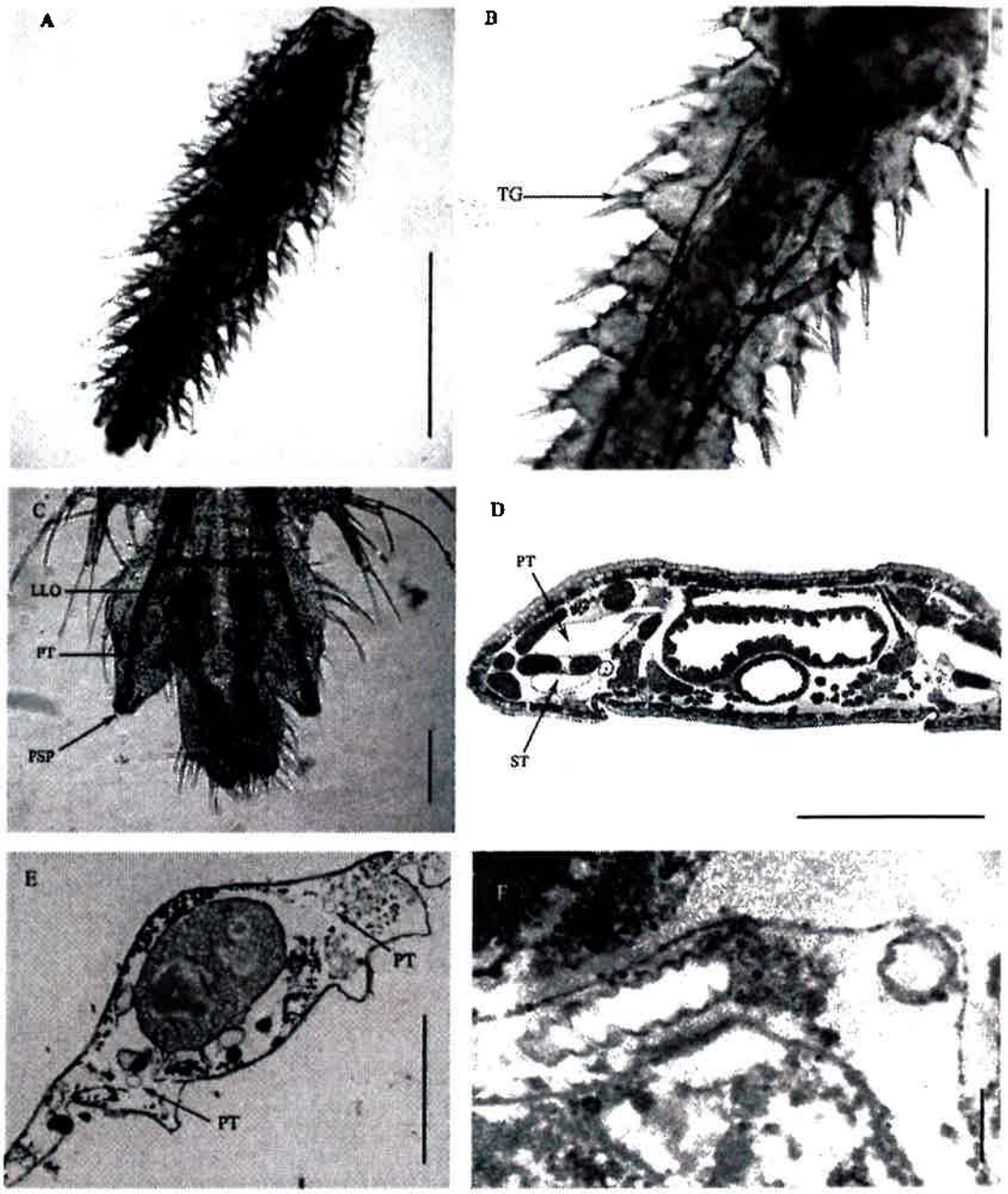


Figure 2. Morphology of larval respiratory system of the aquatic firefly *Luciola substriata.* A. First instar larva: dorsal view (light micrograph). Scale bar: 1 mm. B. First instar larva: dorsal view, showing details of abdominal segments (light micrograph). Scale bar: 1 mm. C. First instar larva, 8th and 9th abdominal segments, in dorsal view (light micrograph). Scale bar: 50µm. D. Final instar larva, transverse section of abdominal segments (dorsal side uppermost). Scale bar: 1 mm. E. First instar larva, transverse section of abdominal segments (dorsal surface uppermost). Scale bar: 0.5 mm. F. First instar larva, transverse section, showing lateral abdominal bristles. Scale bar: 0.5µm.

Abbreviations used: TG, Tracheal gill; PT, Primary trachea; VCOM, Ventral tracheal commissura; ST, Second Trachea; VNC, Ventral nerve cord; G, Ganglion; TRA, Tracheole; LLO, Larval light organ; PSP, Posterior spiracle; SP, spiracle.

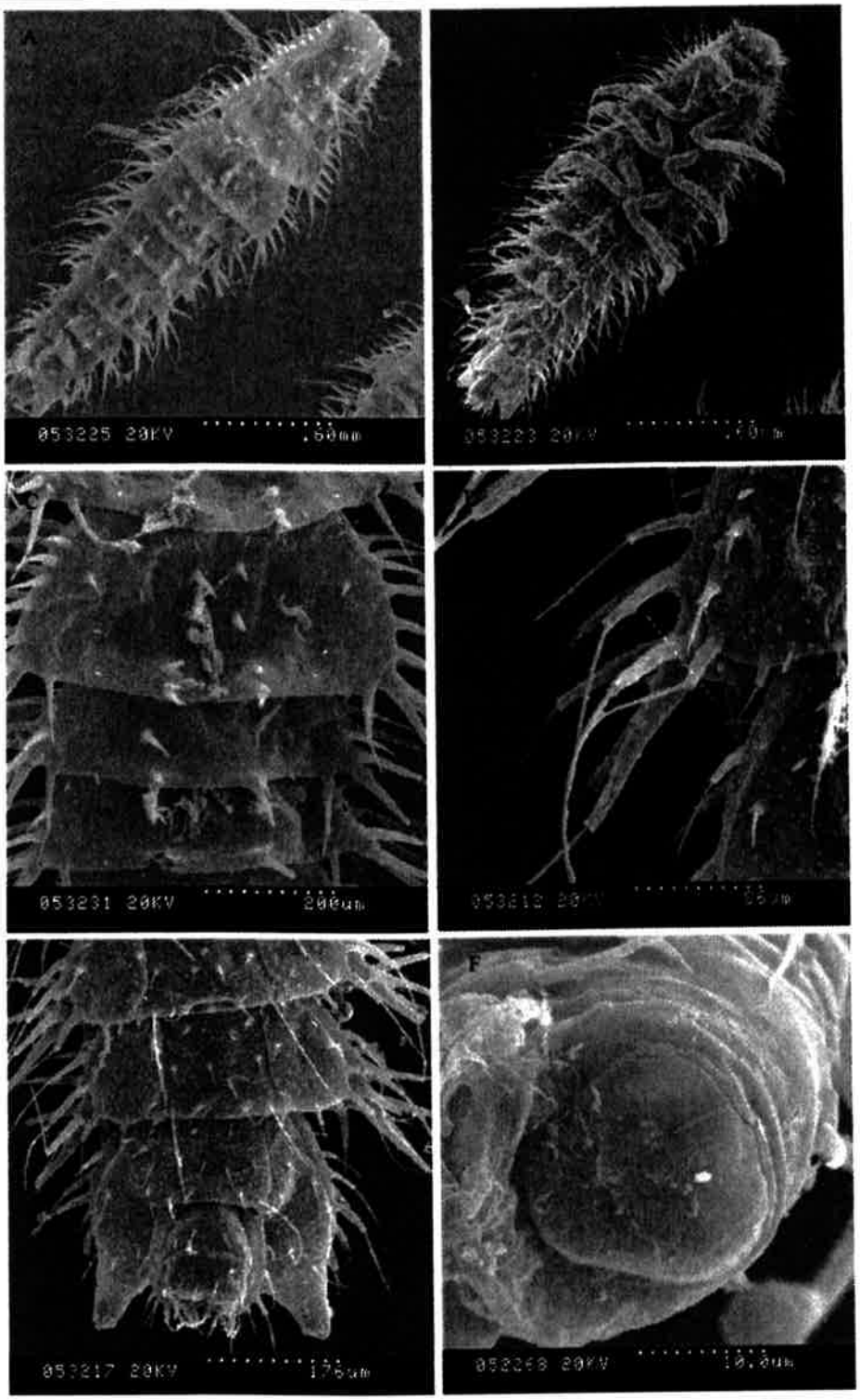


Figure 3. External morphology of respiratory system in first instar larvae of aquatic firefly *Luciola substriata*. Scales: as indicated on the figures. A. Dorsal view (scanning electron micrograph). B. Ventral view (scanning electron micrograph). C and D. Details of the bristle-like structures on the lateral margins of the abdominal segments. E and F. Detailed view of the posterior spiracles at the tip of the 8th abdominal segment.

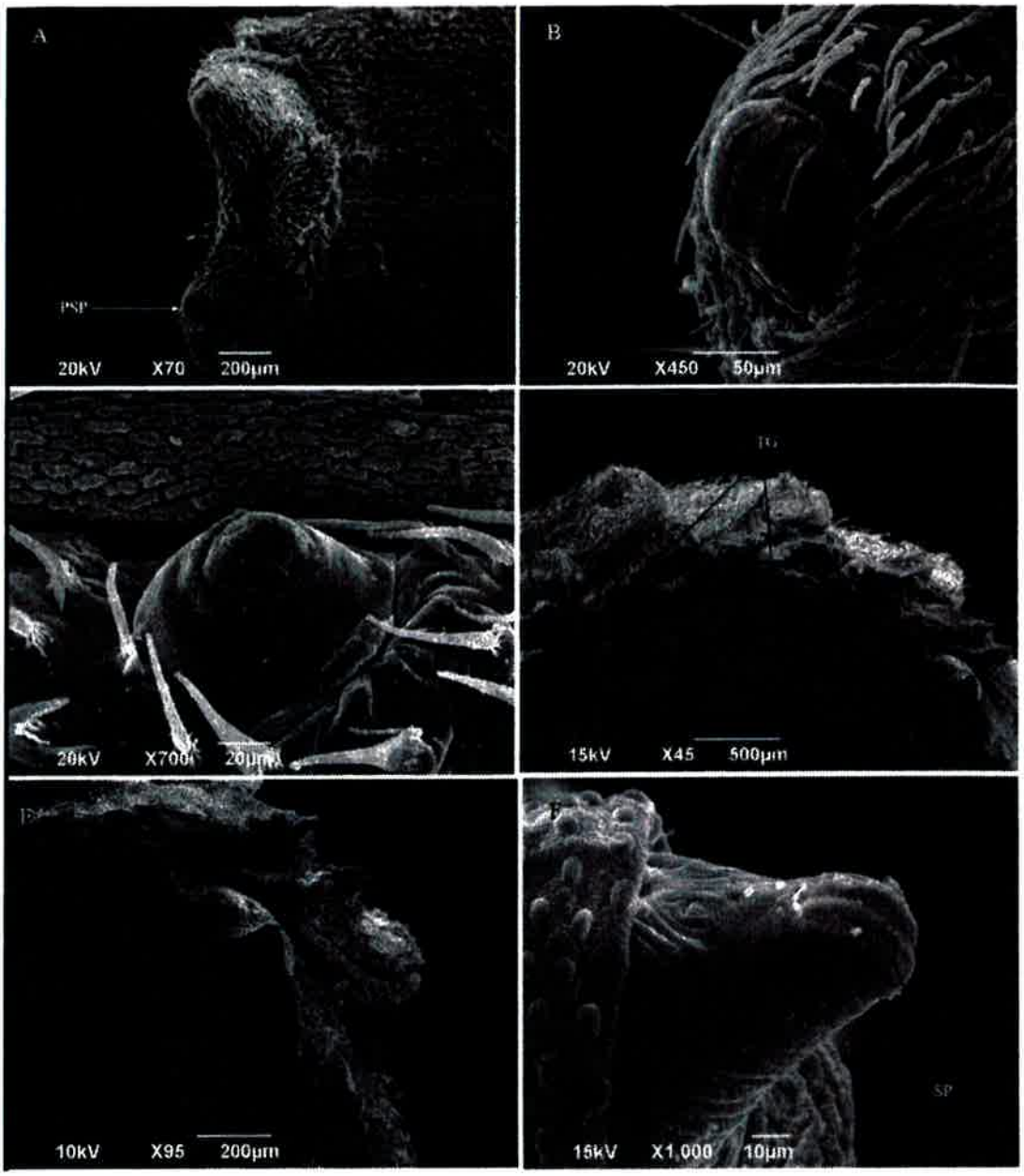


Figure 4. Spiracles and gills in larval aquatic fireflies. Scales: as on indicated on the figures. A and B. *Luciola substriata* final instar larva, spiracles at the tip of 8th abdominal segment (scanning electron micrograph). C. *L. substriata* final instar larva, showing abdominal spiracle (scanning electron micrograph). D and E. *L. leii* final instar larva, showing gill-spiracle compound structures along the sides of the abdomen (scanning electron micrograph). F. *L. leii* final instar larva with spiracle in the gill-spiracle compound (scanning electron micrograph).

tracheae connect with the posterior spiracles and run along the sides of the abdomen to the head (Fig. 2 A). Two parallel symmetrical longitudinal tracheal trunks exist running down each side of the body (Figs. 2 B, E; 5). Secondary tracheae, arising from the primary tracheae, connect with the lateral

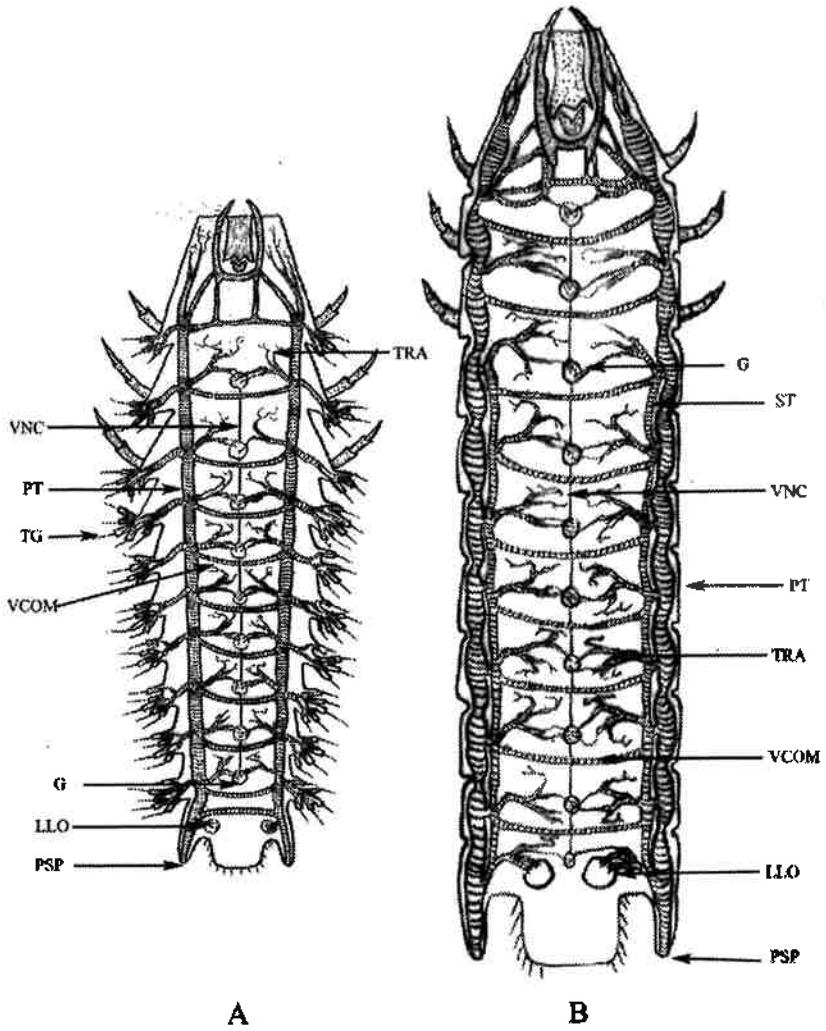


Figure 5. Respiratory system in first and final instar larvae of aquatic firefly *Luciola substriata.* A. First instar larva. B. Final instar larva.

abdominal bristle structures (Fig. 2 B). TEM revealed tiny tracheae in the bristle structures (Fig. 2 F). It is reasonable to conclude that the abdominal bristle like structures in first and second instar larvae are functioning as gills.

In third to sixth instar larvae, lateral abdominal gills of any form are missing (Fig 1 B). These later instar larvae develop a holopneustic respiratory system with 10 pairs of spiracles. The first pair of spiracles is located on the lateral margin of the meso-thorax, the second pair on the lateral margin of the meta-thorax. Abdominal spiracles of segments 1-7 are located on the epipleural plates (Fig. 4 C) and the largest pair, the posterior spiracles on the end of epipleurite 8 (Figs. 4 A, B). There are two longitudinal tracheal trunks running down each side of the body with the outer trunk of wider diameter. There is no indication of connections with the spiracles along the side of the body (with the exception of the terminal spiracles on segment 8) and it is not

yet clear if any other spiracles apart from the terminal ones actually function in these later instars.

3.2. Gill-spiracle compound structures in other aquatic firefly larvae

SEM observation revealed that the tracheal gills in *Luciola leii* are fork-shaped compound structures arising from the lateral abdominal segments (Figs. 4 D, E). A spiracle is located at the tip of the shorter branch of each tracheal gill (Fig. 4 F). First instar larvae only have tracheal gills, spiracles are not evident in this stage.

3.3. Larval locomotion behaviour (crawl vs swimming)

Most aquatic firefly larvae, except for *L. substriata*, crawl on the bottom of a body of water aided by the terminal pygypodia and the lateral gills (Figs. 1 D - F). Larvae of *L. leii*, *L. ficta*, *L. lateralis* and *L. cruciata* use gills to respire in water and do not usually need to obtain oxygen above water unless the dissolved oxygen content is low [9]. Larvae of *L. leii* were observed to crawl above water and remain there overnight if the water was polluted by food. Mature larvae of *L. leii* gathered at the water and land junction for 10 days or more and then moved onto land to construct pupal cells [9].

The first and second instar larvae of *L. substriata* could 'back swim' on the surface of the water, but spent most of their time crawling on the substrate. The third to sixth instar larvae are competent backswimmers and swim with their ventral side up, even when searching for prey. When the larvae were swimming, their thoracic legs sculled backwards continuously, while the abdomen curved upward and downward [5, 14]. The pygopodia enable the larvae to grasp and adhere to floating objects. Swimming could either start or cease. When back-swimming larvae changed direction, the larval abdomen curved rapidly clockwise or counterclockwise and thereafter remained unbent. Larvae of *L. substriata* could also crawl slowly on the substrate aided by the pygypodia to search for prey.

4. Discussion

Aquatic firefly larvae of *L. leii*, *L. ficta*, *L. lateralis* and *L. cruciata* possess fork-shaped tracheal gill plus spiracle structures in all instars except for the first instar. Final instar larvae of *L. leii* and *L. ficta* could survive more than 10 days without water in the laboratory. The larvae could apparently respire through the spiracles if without water, or if on land to construct pupal cells, and were able to absorb dissolved oxygen through tracheal gills when beneath the water and when they presumably close the spiracles.

Among the known species of aquatic fireflies, *L. substriata* is distinctive ecologically and morphologically [5]. The first and second instar larvae can live under water and swim on the surface of water. They respire through tracheal gills when in water, but probably also through the pair of posterior spiracles when back-swimming. The third to sixth instars transform their external morphologies and lose the tracheal gills; instead, additional 9 pairs of spiracles appear. They may be able to store huge amounts of oxygen in the large primary tracheae and thus remain submerged for a long time when they dive in search of prey. Both *L. substriata* and *L. aquatilis* larvae possess a pair of posterior spiracles on the end of segment 8 [5 6]. Is it possible that aquatic fireflies could have had a terrestrial origin? It is suggested that all aquatic firefly may have evolved from terrestrial ones, with the possibility that *L. substriata* moved from water to land and back again to water.

The aquatic fireflies *L. cruciata*, *L. lateralis*, *L. owadai*, *L. ficta* and *L. hydrophila* all share a similar habitat and have similar tracheal gills located on the lateral portions of their abdominal segments. Their habitats are shallow streams or ditches, which provide enough solute oxygen [2]. As it is not necessary for the larvae to breathe on the surface of the water, these larvae only crawl in and on the substrate of their habitats and possess no ability to swim.

The habitats favoured by *L. substriata* are very different from those of the other five species of aquatic fireflies mentioned above. *L. substriata* is widely distributed and inhabits large fresh water lakes and ponds [5]. In such habitats the larvae that are bottom feeders would be unable to live at the bottom, because of decreased oxygen and unfavourable water pressure. The characters of this habitat, including the poor oxygen supply might suggest that the larvae evolved a long, flat body shape with little exposed membranous area, and a unique back swimming behaviour to adapt to the environment. Lack of exposed membrane and a hard exoskeleton could give the surface swimming larva extra protection against surface predators. Back swimming with the capacity to respire at the water surface allows such larvae to live in oxygen-deficient waters.

References

1. Okada, Y.K. 1928, Trans. Entomol. Soc. Lond. (Pt.), 101-107.
2. Jeng, M.L., Lai, J., and Yang, P.S. 2003, Water Beetles of China (III), J. Jäch and J. Ji (Eds), 539–562.
3. Fu, X. H., and Ballantyne, L.A. 2006, Can. Entomologist, 138, 339–347.
4. Yeh, S. 1999, Master's Thesis. Grad. Inst. Plant and Entomology, Natl. Taiwan University, Taipei.
5. Fu, X.H., Ohba, N., Vencl, F.V., and Lei, C.L., 2005 Can. Entomologist, 137, 83–90.

6. Thancharoen, L. A., Ballantyne, M. A., Branham, M. L., and Jeng, J. 2007 Zootaxa, 1611, 55-62.
7. Ho, J.Z., Ju, J. S., and Ju, J. C. 1998, Nature Conservation Quart. 22, 47-51.
8. Ohba, N., Azuma, S., Nishiyama, K., and Goto, Y. 1994, Sci. Rep. Yokosuka City Mus., 42, 13-26.
9. Fu, X.H., Ohba, N., Vencl, F.V., and Lei, C.L. 2006, Can. Entomologist, 138, 860-870.
10. Fu, X.H. , Ohba, N., Zhang, Y., and Lei, C.L. 2006, Can. Entomologist, 138, 399-406.
11. Fu, X.H., Vencl, F.V., Ohba, N., Meyer-Rochow, V.B., Lei, C.L. and Zhang, Z.N. 2007, Chemoecology, 17, 117-124.
12. Mora, R., Retana, A., and Espinoza, A.M. 2001, Ann. Entomol. Soc. Am., 94, 438-448.
13. Meyer-Rochow, V.B., and Liddle, A.R. 1988, Proc. Roy. Soc. London B, 233, 293-319.
14. Fu, X.H., Wang, Y.Y., Ohba, N., and Lei, C.L. 2005, Coleopterists Bull., 59, 501-505.

Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 255-276
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

14 Beetle bioluminescence: A genetic and enzymatic research review

John Day
Centre for Ecology and Hydrology (CEH) Oxford, Mansfield Road
Oxford, OX1 3SR, U.K.

Abstract

Bioluminescence is the emission of visible light by living organisms and is most evident terrestrially in beetles i.e fireflies, glow-worms, railroad worms and some click beetles. A summary of the research conducted during the past 30 years on the bioluminescence mechanisms of beetles is presented. The review concentrates on luciferase, the principle enzymes involved in bioluminescence, and the luciferin regenerating enzyme (LRE) speculated to be necessary for maintaining luminescence, as well as providing an overview of current opinions on the genetics of bioluminescence in beetles.

Correspondence/Reprint request: Dr. John Day, Centre for Ecology and Hydrology (CEH) Oxford, Mansfield Road, Oxford, OX1 3SR, UK. E-mail: jcda@ceh.ac.uk

1. Introduction

From the steady green radiance of the solitary glow-worm to the synchronized flash display of hundreds of fireflies the light emission of bioluminescent beetles has appealed to man for thousands of years. Such luminary figures as Pliny the Elder, Robert Boyle and Charles Darwin have recorded their own fascination with these remarkable creatures. However, it has only been in the last century that the mechanism for beetle bioluminescence has begun to be resolved. Over the years the study of luminescence in fireflies and other beetles has attracted many disciplines; taxonomy, physiology, biochemistry, physical chemistry and genetics to name but a few. In this review it is not possible to provide a complete overview of beetle bioluminescence research. I have therefore concentrated on the studies that have examined the genes and their products that provide the principle components for bioluminescence.

2. Bioluminescent beetles

Luminescence is evident in many terrestrial organisms such as fungi, worms, millipedes, centipedes and flies, but the most resolved are the bioluminescent beetles. The firefly that emits light in the form of pulses and flashes from specialized organs, known as the lantern, located in the last abdominal segments are the most well know around the world. This light emission forms the basis of what has been established for a long time as the basis of sexual communication between adults. Bioluminescence is also evident in other life stages of luminescent beetles from the egg through to the pupae, although not all species exhibit bioluminescence in all life stages. As far as is known all firefly larvae are bioluminescent but the function of this luminescence is less obvious. A number of theories have been proposed to account for this mechanism [1], the most plausible being defense from predation [2].

Three main coleopteran families contain luminescent beetles; the Lampyridae (fireflies & glow-worms), the Phengodidae (railroad worms) and the Elateridae (click beetles) [3]. The former two families belong to the superfamily Cantharoidea with the latter being found in the more distant Elateroidea. With nearly 2000 species the Lampyridae constitute the largest family of bioluminescent beetles. It is commonly regarded that the larvae of all lampyrids are luminescent, including those that have non-bioluminescent adults [4, 5]. As a result it has been suggested that not only were the ancestral lampyrids luminescent [5] but, potentially, early cantharoids exhibited bioluminescence in the larval stage [4]. However, Crowson felt that this ancestral state did not extend to the Elateridae despite certain affinities. Such

affinities, as will be evident, extend to the genes and enzymes involved in bioluminescence.

3. The bioluminescent reaction

The first modern study of bioluminescence began with a luminous click beetle. In 1885 Dubois demonstrated the first example of a luciferin-luciferase reaction from a West Indies *Pyrophorus* species by preparing two extracts from the light organs that, when combined, produced light [6]. Dubois concluded that one extract contained a heat labile enzyme necessary for the light emission and called this 'luciferase' whilst the other extract contained a heat stable substance he designated "luciférine". The requirement of adenosine tri-phosphate (ATP) and Mg^{2+} for the bioluminescent reaction were identified in the 1940's [7, 8] and in 1978 McElroy & DeLuca [9] proposed a two step scheme for the overall reaction of firefly bioluminescence.

$$E + LH_2 + ATP + Mg^{2+} \rightarrow E{\bullet}LH_2\text{-}AMP + PPi + Mg^{2+} \quad (1)$$

$$E{\bullet}LH_2\text{-}AMP + O_2 \rightarrow E{\bullet}L + CO_2 + AMP + Light \quad (2)$$

Both steps are catalysed by the enzyme luciferase (E). In the first stage luciferin (LH_2) is converted into a luciferyl adenylate (LH_2-AMP) by ATP in the presence of Mg^{2+}. In the second step, luciferyl adenylate is oxidized by molecular oxygen resulting in the emission of light and the production of oxyluciferin (L).

Luciferin is a general term defined as an organic compound that exists in a luminous organism and provides the energy for light emission by being oxidized, normally in the presence of a specific luciferase [10]. Firefly luciferin was first purified and crystallized in 1957 [11] ultimately leading to the determination of its structure in 1961 [12] (shown in figure 1). The product of the luminescent oxidation of luciferin is oxyluciferin, a compound which is extremely unstable (shown in figure 1). A number of studies focused on the intervening steps between luciferin and oxyluciferin resulting in the postulation of the bioluminescent reaction shown in figure 1 [12-16]. Luciferase-bound luciferin is converted into an adenylate in the presence of ATP and Mg^{2+} with the release of pyrophosphate (PPi). The adenylate in the presence of oxygen forms a peroxide intermediate (A) which then forms a dioxetanone intermediate (B) by splitting off AMP. Dioxetanes are heterocyclic compounds which consist of a four-membered ring that contains two oxygen atoms and two carbon atoms. The subsequent decomposition of the dioxetane intermediate produces an excited state of oxyluciferin in the form of either a monoanion (C1) or a dianion (C2). When the energy levels of

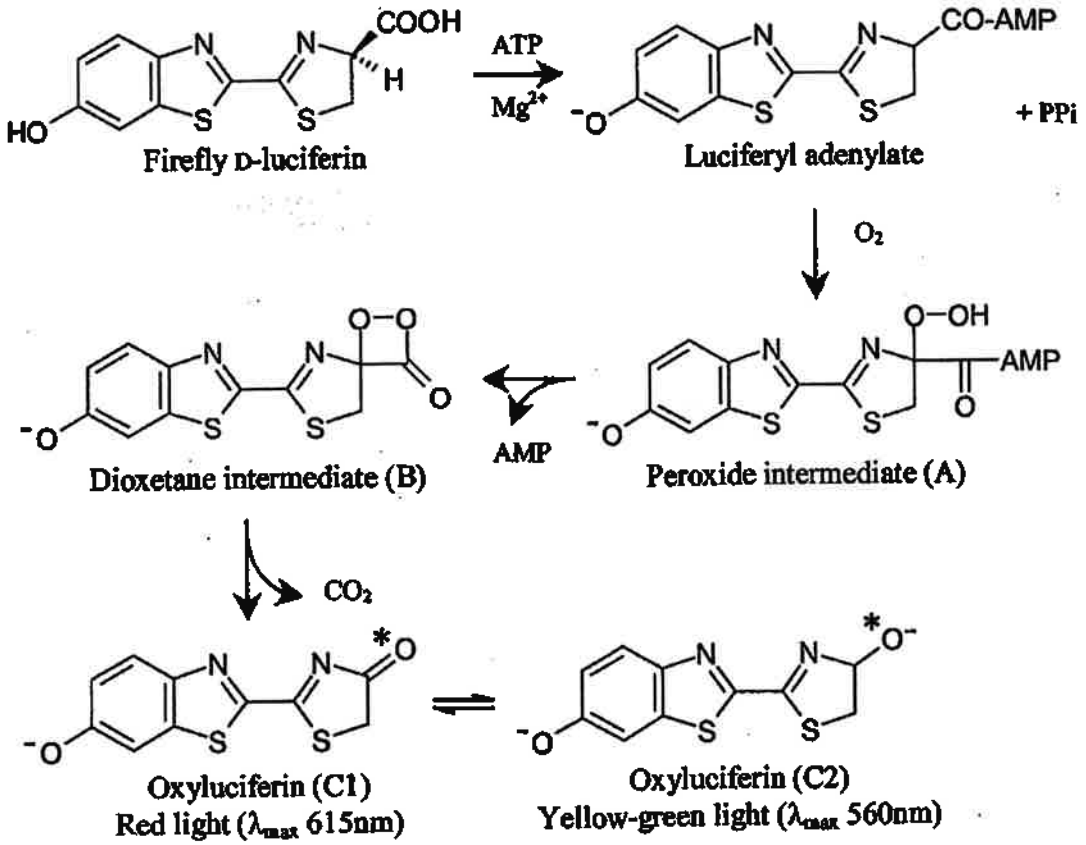


Figure 1. Mechanism of the bioluminescent reaction of firefly luciferin catalyzed by firefly luciferase.

the excited states fall to the ground states, C1 emits red light (λ_{max} 615nm) and C2 emits yellow-green light (λ_{max} 560nm).

More recently, alternative candidates have been proposed as the light emitters in the firefly luminescent system including the adenylate of D-5,5-dimethylluciferin which emits light in two different colours [17]. Although numerous emitters have been proposed, the initial substrate for all three principle bioluminescent beetle families is identical and the difference in bioluminescent colour is achieved by variation in the luciferase amino acid sequence coded for by the luciferase gene.

4. Luciferase

Beetle luciferase has been intensively studied for over 50 years and is probably the most well characterized of all bioluminescent enzymes. As the firefly luminescent reaction is dependent upon ATP as a luciferin activator it has been extremely versatile in determining enzymes and metabolites involved in ATP-dependent reactions. Consequently firefly luciferase has been used in a range of applications in both medical and biotechnological

research where sensitive photocounters and luminometers can detect ATP down to pico or even femtomole concentrations. In addition, the use of luciferase as a reporter gene has meant it has played a significant role in modern gene expression studies.

4.1. Firefly luciferase

The luciferase from the firefly *Photinus pyralis* was first purified, crystallized and partially characterized in 1956 [18]. The molecular weight was estimated as 100,000 and the isoelectric point at pH 6.2-6.3. Purification based upon high-performance liquid chromatography (HPLC) was later reported for luciferase from *Photinus pyralis* and *Photinus macdermotti* [19]. In 1984 Wood and co-workers cloned *P. pyralis* luciferase by *in vitro* translation and determined the molecular weight to be 62,000 as opposed to the previously reported 100,000 [20]. Wienhausen and DeLuca identified luciferases from other bioluminescent beetle species, including the click beetle *Pyrophorus plagiophthalamus*. These migrated at a similar rate, although not identically, and exhibited extensive cross-reactivity with antibodies raised against *P. pyralis* luciferase [21]. Thus it was anticipated that luciferases from other bioluminescent beetles would have similar molecular weights.

The firefly *P. pyralis* was again used to provide the material for the first cloning of luciferase into a bacterial system. De Wet and coworkers in 1985 expressed the cDNA of *P. pyralis* luciferase in *Escherichia coli* providing the basis for mass production of luciferase *in vitro* and the further characterization of the enzyme through mutagenesis studies in the coming years. To date the luciferase cDNA has been characterized from over twenty bioluminescent beetle taxa and extensive information has been collated about these enzymes (Table 1). In fireflies the luciferase enzyme is composed of one polypeptide chain ranging in size from 545–552 highly conserved residues. Over half are non-polar or ambivalent amino acids and the number of charged residues is virtually the same for all lampyrid species.

It was not until 1996 that Conti *et al.* resolved the crystal structure of the *P. pyralis* luciferase at a resolution of 2.0 Å [22]. The protein was found to be folded into two compact domains connected by a short flexible hinge (Figure 2A). The large N-terminal domain being composed of a β-barrel and two β-sheets flanked by α-helices to form an αβαβα five-layered structure. The C-terminal portion of the molecule formed a distinct domain separated from the N-terminal domain by a wide cleft. Conti *et al.* proposed that the cleft was far too big to accommodate the substrate and the domains will close in the course of the reaction to sandwich the substrates.

Table 1. Cloned beetle luciferases and their characteristics.

	Residues	Sequence identity (%)†	pI	λ_{max} (nm) *	Reference
LAMPYRIDAE (fireflies & glow-worms)					
Lampyrinae					
Cratomorphus distinctus	547	83	5.85	550	[23]
Diaphanes pectinealis	547	83	6.09	-	[24]
Lampyris noctiluca	547	84	6.08	550	[25]
Nyctophila cf. caucasica [a]	547	84	6.19	-	[26]
Photinus pyralis	550	100	6.43	562	[27]
Pyrocoelia miyako	548	82	6.11	550	[28]
Pyrocoelia pygidialis	548	83	6.03	-	[29]
Luciolinae					
Hotaria parvula	548	67	6.27	568	[28]
Hotaria unmunsana	548	67	6.10	-	[30]
Lampyroidea maculata	548	64	5.99	-	[31]
Luciola cruciata	548	67	7.17	562	[32]
Luciola italica	548	65	5.99	566	[33]
Luciola lateralis	548	67	6.52	552	[34]
Luciola mingrelica	548	67	6.24	570	[35]
Luciola terminalis	548	65	6.47	-	*unpublished*
Photurinae					
Photuris pennsylvanica					
Ppe1	552	69	7.23	560	[36]
Ppe2	545	59	8.29	538	[36]
Phengodidae (railroad worms)					
Phrixothrix vivianii	545	55	6.39	548	[37]
Phrixothrix hirtus	546	48	7.00	623	[37]
Ragophthalmus ohbai	543	53	7.93	555	[38]
Elateridae (click beetles)					
Pyrophorus mellifluus					
Green (dorsal)	543	47	6.92	549	[39]
Green (ventral)	543	47	7.63	554	[39]
Pyrophorus plagiophthalamus					
Green	543	47	6.71	546	[40]
Yellow Green	543	47	6.71	560	[40]
Yellow	543	47	6.39	578	[40]
Orange	543	47	6.71	593	[40]
Pyrearinus termitilluminans	543	46		538	[41]

† Amino acid sequence identity to *Photinus pyralis* luciferase.

[a] Reported as *Lampyris turkistanicus* see [42].

* For comparative purposes the *in vitro* emission is reported. *In vivo* measurement of bioluminescence can be affected by a number of factors and does not necessarily depict the true light emission of the enzyme.

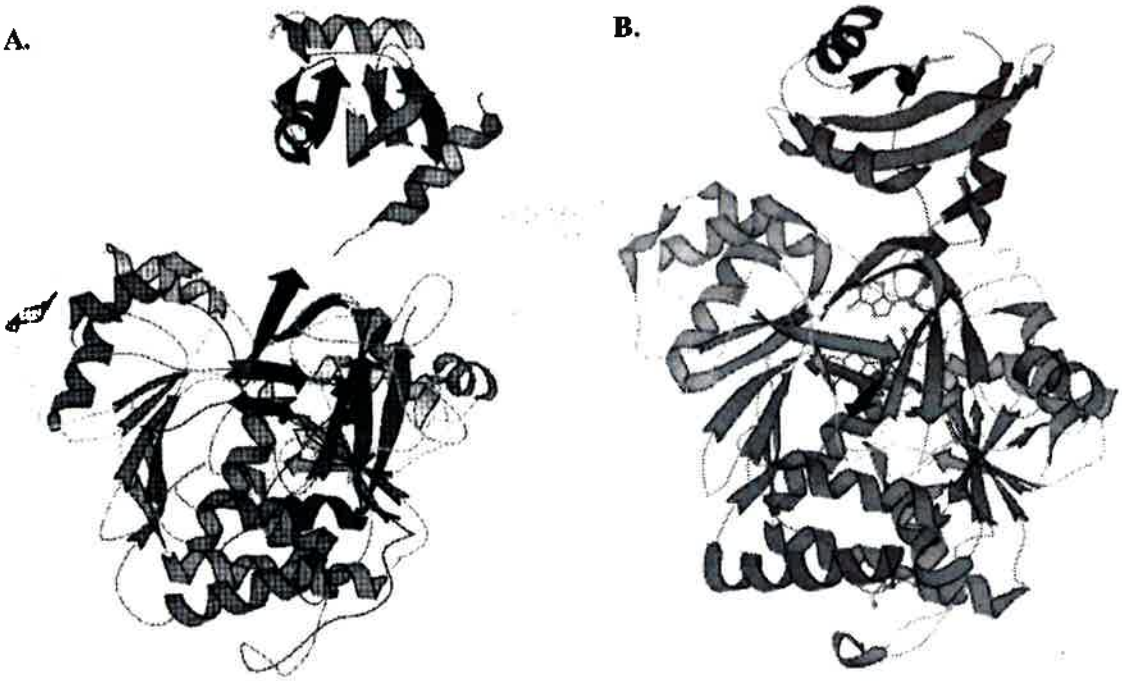


Figure 2. Ribbon representations of the firefly luciferase protein. (A) *Photinus pyralis* luciferase structure. (B) *Luciola cruciata* luciferase complexed with DLSA. (Generated using the program KiNG vers. 2.12).

In 2006 the crystal structure of the wild-type luciferase from *Luciola cruciata* complexed with a high-energy intermediate analogue of luciferin, 5'-O-[N-(dehydroluciferyl)-sulfamoyl] adenosine (DLSA) was determined at 1.3 Å resolution (Figure 2B) [43]. It is apparent from the comparative structures that indeed the domains are closer when bound to a substrate (Figure 2).

Two models have been proposed for the substrate binding active site of the enzyme [44, 45]. According to both models the luciferin binding site should include residues R^{218}, H^{245}–F^{247}, A^{313}–G^{320}, G^{339}–I^{351} and K^{529} with a hydrophobic region composed of F^{247}, A^{313}, A^{348} and I^{351*}. Whilst similar, the two models differ in the proposed residue interaction with the luciferin phenol group; one argues in favour of R^{337} whilst the other presents R^{218} [46].

4.2. Railroad worm and click beetle luciferases

The bioluminescent mechanism in the Phengodidae and luminescent members of the Elateridae is considered to be the same as that found in the fireflies (Lampyridae). Each mechanism is dependent upon ATP, luciferin, Mg^{2+} and the enzyme luciferase to create light. Beetle luciferin is regarded to

* Specified residues refer to positions in the *Photinus pyralis* luciferase [26].

be the same structure in the Phengodidae and Elateridae as the Lampyridae [47, 48]. Despite these similarities the difference in colours of light produced in these families is quite dramatic. In lampyrids the light is limited in range from green to yellow (λ_{max} 538 – 584 nm). However, bioluminescent click beetles have three light organs; a pair of dorsal oval light organs on the pronotum which emit a green light (λ_{max} 536 – 559 nm) and a ventral organ located on the first abdominal segment which ranges in colour from green through to orange (λ_{max} 549 – 594 nm). In railroad worms the number of lanterns increases with eleven pairs of luminous organs located dorso-laterally along the abdominal and thoracic segments. These emit green through to orange light (λ_{max} 535 – 592 nm) and are present in both adults and larvae. In addition, some species such as the railroad worm *Phrixothrix* have a luminous organ on the head which emits red light (λ_{max} 600 – 620 nm). These colour differences are a result of amino acid changes in the luciferase protein.

Shortly after the publication of the first firefly luciferase sequence Wood *et al.* in 1989 characterized four different luciferase sequences from a single click beetle species *P. plagiophthalamus* [40]. Sixty beetles were used to construct a cDNA library from which the luciferases where characterized. It was not evident at the time whether these different enzymes could be found in a single beetle or whether the dorsal and ventral lanterns were under different genetic control. However, in 2003 Stolz *et al.* conducted a large study on the same species in Jamaica and found by comparing genomic clones with cDNA sequences there were two different genes controlling bioluminescence independently in the dorsal and ventral lanterns [39]. Luciferase from one further species of elaterid was carried out in 1999. One luciferase clone was characterized from *Pyrearinus termitilluminans* which produced a blue shifted bioluminescence which, *in vitro*, is the same as that found in *Photuris pennsylvanica Ppe2*. Although one clone was evaluated four other clones were bioluminescent but unfortunately were not characterized. Additional luciferase genes may exist in the genome of *P. termitilluminans* which have yet to be identified. In 1998 the luciferase from *Rhagophthalmus ohbai* was characterized [38]. Although *R. ohbai* is currently classified in its own family the Rhagophthalmidae, opinion is still divided as to its placement. In the past it has been contained in the Phengodidae and the Lampyridae. The *R. obhai* luciferase shares greatest sequence identity with the Phengodidae luciferase sequences but this comparison is limited to one species.

4.3. Beetle luciferases and bioluminescence spectra

As previously mentioned beetle luciferases use the same luciferin substrate to naturally display light ranging in color from green (λ_{max} ~ 530nm)

to red (λ_{max} ~ 635nm). A specific property that differentiates firefly luciferase from those of the click beetles and railroad worms is pH sensitivity. The optimum pH for luminescence is around 7.8 but in 1964 Seliger and McElroy reported a strong pH dependence of the colour of the emitted light [47]. In acidic (pH < 6.5) buffer solutions the intensity of the normal yellow-green emission of *P. pyralis* (λ_{max} 562nm) decreases markedly and a low intensity red emission was observed (λ_{max} 616nm). In addition, Seliger and McElroy found divalent heavy metal cations Cu^{+2} and Zn^{+2}, denaturants such as urea and an increase in temperature could illicit the same shift in the spectrum to the red [47].

Conversely, subsequent studies of click beetle and railroad worm luciferases exhibited no red shift in acidic conditions [40, 41, 49]. As a result firefly luciferases have been described as 'pH sensitive' and both elaterid and phengodid luciferases as 'pH insensitive'. It is interesting to note that the original work carried by Seliger and McElroy evaluated the effect of pH and metal cations on the click beetle *Pyrophorus plagiophthalamus* and although they found no red shift in the ventral organ luciferase they did observe a small shift in spectra in the dorsal lantern luciferase suggesting some pH effect. Interestingly they found a blue shift in the dorsal organ luciferase in basic conditions and with the addition of metal cations [47].

To date, four main hypotheses have been presented to account for the range of colour emitted in the beetle bioluminescent reaction. The first explanation was presented in 1971 by White *et al.* who proposed that the excited state of the keto-form of the oxyluciferin anion can relax by emitting red light, whilst the excited state of the enol-form emits yellow-green light (figure 1) [50].

Later, McCapra proposed an alternative model, that colour variation is associated with conformations of the keto form of excited-state oxyluciferin [51]. McCapra proposed that all of the luminescent colours ranging from green to red are generated from twisted intramolecular charge transfer (TICT) excited states of the keto form. The colour of the light emission should depend on the rotation around the C–C bond of the –N C–C N– moiety. Branchini and co-workers presented partial experimental support for McCapra's mechanism [17].

The third hypothesis assumes that the colour of the bioluminescence is dependent upon the polarization of the oxyluciferin in the microenvironment of the luciferase–oxyluciferin complex: the higher the polarization, the larger the red shift of bioluminescence [52-54].

The fourth hypothesis, published in 2006 by Nakatsu and co-workers proposed an energy loss control mechanism which is dependent upon the size of the cavity between the luciferase domains [43]. A non-relaxed form of the

keto oxyluciferin should emit yellow-green light. Conversely after geometrical relaxation it should emit red light. The geometrical relaxation is determined by the size of the luciferase cavity.

There has been much discussion about the mechanism that can explain the effect of pH on bioluminescence and the precise nature of these emitters. However, irrespective of the molecular structure of the emitting forms, the organization of the protein environment of the emitter and the flexibility of key amino acid residues contribute significantly to the spectral parameters of beetle bioluminescence.

4.4. Adenylate-forming protein family

Beetle luciferase belongs to a large family of adenylate-forming enzymes (PFAM00501). The adenylate-forming proteins catalyze a two-step reaction converting an organic acid to a CoA thioester [55, 56]. This mode of substrate activation is commonly used by adenylate-forming enzymes such as acyl-CoA ligases [57], acetyl-CoA synthetases [58], non-ribosomal peptide synthetases (NRPSs) [59] and aminoacyl-tRNA synthetases [60], as well as luciferase. These enzymes are relatively large, ranging in size from 500 to 700 residues. Structurally they are composed of two domains, an N-terminal domain of 400-550 residues and a smaller C-terminal domain of 100-140 residues. An active site is situated at their interface. Members share limited sequence homology of 20-30%, however, several well-conserved sequence motifs have been identified between members and three principle motifs have been attributed with an adenylation function [57, 61-63]. Of particular note is the invariant residue K^{529} which was shown to be important in the adenylation step [64].

These enzymes activate a variety of different substrates, including aromatic acids, acetic acid and long-chain fatty acids, to the corresponding enzyme-bound acyl-adenylates, which are then transferred to the thiol group of CoA. The two half-reactions occur in a ping-pong mechanism. A domain alternation mechanism has been proposed for these enzymes. Upon completion of the initial adenylation reaction, the C-terminal domain of these enzymes undergoes a 140° rotation to perform the second thioester-forming half-reaction.

It has recently been speculated that beetle luciferase may have evolved from an ancestral fatty acyl-CoA synthetase as firefly luciferase retains this activity *in vitro* [65, 66]. As such beetle luciferin may not itself have originally been the substrate for the ancestral luciferase, but rather a 'luciferin-like' molecule, with beetle luciferin appearing as a substrate later in evolution In support of this, dehydroluciferin, differing from luciferin by only two hydrogen atoms and inactive for chemiluminescence, can be

efficiently ligated to CoA by firefly luciferase [67]. Luciferase may still function as a fatty acyl-CoA synthetase involved in the oxidation of fatty acids in the peroxisome of beetles. Interestingly, it was shown that firefly luciferase had a marked preference for fatty acids such as arachidonic acid [68]. This may be unsurprising as arachidonic acid, although typically occurring in very small amounts in the phospholipids of terrestrial insects, has been found in very high levels in the tissue lipids of adult fireflies [69].

4.5. Luciferase genes and beetle genomes

The first genomic luciferase sequence was characterized from *P. pyralis* and found to be composed of seven exons divided by six introns ranging in size from 48–58 bp [27]. Subsequent studies have shown this arrangement is conserved in lampyrids [42, 70, 71]. To date no genomic luciferase sequences have been characterized from members of the Elateridae or Phengodidae.

Recently paralogous luciferase-like sequences have been identified from the Japanese firefly *L. cruciata* [66] suggesting gene duplication of luciferase-like sequences in bioluminescent beetle genomes. Despite extensive sequence identity of the *L. cruciata* luciferase-like genes to the *bona fide* luciferase, the two paralogous enzymes revealed no bioluminescence activity. Furthermore, only one gene product exhibited long-chain fatty acyl-CoA synthetic activity. It was subsequently proposed that luciferase has arisen from a gene duplication event of an ancestral acyl-CoA synthetase and functionally diverged to acquire a novel bioluminescent function [66]. Six luciferase-like sequences have been found to be present in the genome of the red flour beetle *Tribolium castaneum* illustrating the degree of gene duplication of luciferase-like genes within beetle genomes [72]. These luciferase-like sequences showed extensive sequence identity to orthologues fround in *Tenebrio molitor* which exhibited no bioluminescent activity and were reported to have acyl CoA synthetase activity [73]. It is therefore likely the *T. castaneum* enzymes will exhibit similar proterties. These genes were found on four different chromosomes suggesting potential mobilization of luciferase-like sequences in beetle genomes. A putative retrotransposase sequence was identified upstream of the luciferase gene in the glow-worm *L. noctiluca* suggesting a putative translocation event [71]. With multiple luciferase-like genes identified in members of the Lampyridae a similar scenario may be evident in lampyrids as is found in *T. castaneum*.

Recent studies have not only identified luciferase-like sequences but multiple luciferase sequences within lampyrid genomes [72]. In particular three partial gene sequences were identified for the firefly *Photuris congener* (Figure 3). Two luciferase cDNA clones have previously been characterized

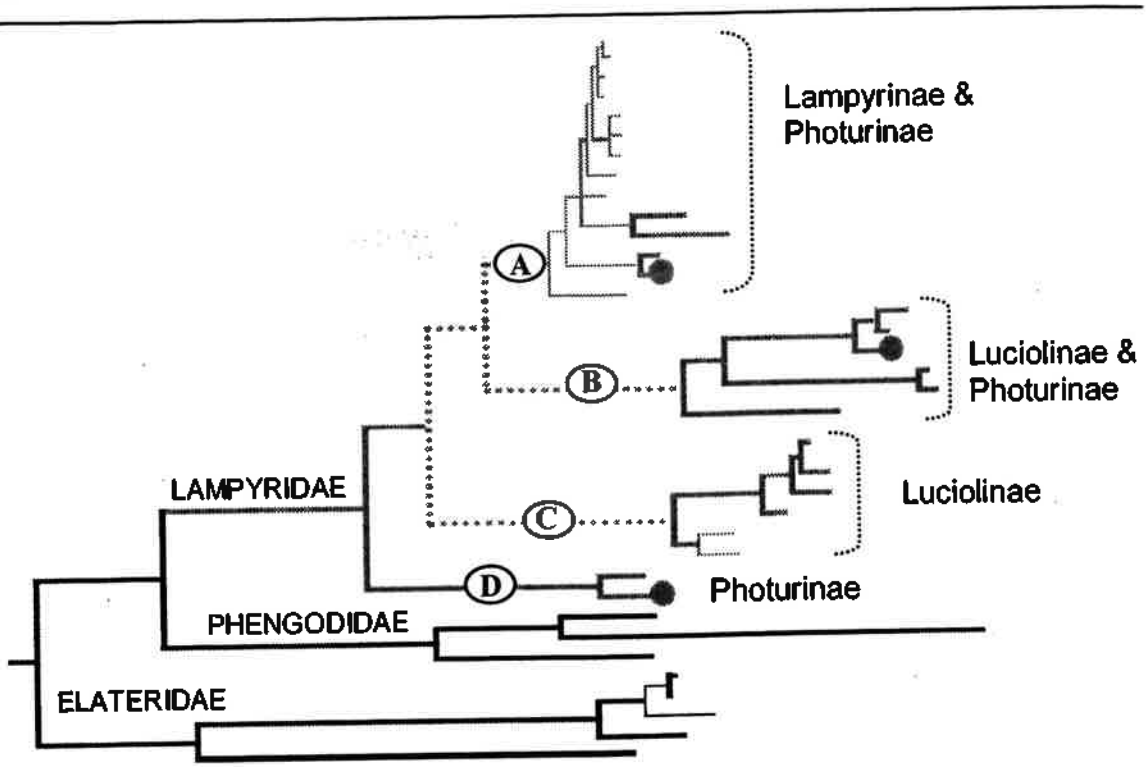


Figure 3. Luciferase gene paralogue phylogeny based upon Bayesian analysis. Clades A-D represent lampyrid luciferase sequence groups. Three *Photuris congener* paralogues are highlighted with filled circles at terminal branches. Dashed and thick branches represent 91-95% and >95% Bayesian posterior probability respectively. Summarized from [72].

from *Photuris* and shown to exhibit different spectra [36]. It has yet to be established whether a third functional luciferase is present in fireflies.

Further evidence of paralogous luciferase genes were found in Luciolinae species, *Luciola italica* and *Lampyroidea maculata*. Interestingly, paralogues *LuiLUC2* and *LdmLUC2* formed a subclade with *Ppe2*. Cho *et al.* [70] identified the possibility of three luciferase alleles in *Luciola lateralis* indicating the presence of at least two luciferase genes within this species supporting the findings of Day *et al.* for two or more luciferase genes within the members of the Luciolinae.

5. Luciferin

Luciferin, 2-(6-hydroxybenzothiazol-2-yl)-2-thiazoline-4-carboxylic acid, was first purified and crystalized from the North American firefly *Photinus pyralis* in 1957 by Bitler and McElroy [11]. This process required 15,000 firefly lanterns to produce 9 mg of crystalline luciferin. Proof of its structure came from the successful chemical synthesis of enzymatically active luciferin in 1961 [12] and the structure confirmed by X-ray crystallography [74].

It is commonly regarded that all lampyrids emit light as larvae and many seem to produce light throughout all their life stages. Interestingly the non-luminescent adults of *Ellychnia corrusca* can be induced to elicit a flash of light indicating the expression of the enzyme and the synthesis of the substrate in the adult beetle (pers. comm. J. Copeland). Luciferin appears to be conserved in structure between bioluminescent beetle species and even families irrespective of metamorphic stage or lantern location [75-77].

5.1. Luciferin chirality

In chemistry the term chiral is used to describe a compound that cannot be superimposed on its mirror image. Biologically active natural products are usually homochiral e.g. proteins are predominantly composed of mainly L-amino acids. In general, most bioluminescent reactions employ a single chiral luciferin and are biosynthesized from peptides or L-amino acids as is postulated for the luminescent ostracod *Cypridina* [78]. The firefly bioluminescent reaction is no exception. Only firefly D-luciferin contributes to bioluminescence and several researchers noted that no light is produced from L-luciferin [79, 80]. However, Lembert reported that L-luciferin produced weak light but extremely slowly [81]. As a result Lembert proposed that L-luciferin was racemized to give D-luciferin [81]. It has been recently presented that luciferase could be responsible for the stereoisomeric inversion of L-luciferin to D-luciferin which would explain the weak bioluminescence observed by Lembert [82]. To further support this Niwa *et al.* 2006 measured levels of both D- and L-luciferins in the firefly *Luciola lateralis*; both forms were detectable in all firefly life stages, including the egg [83]. The enantiomeric excess of D-luciferin was highest at the adult stage, while it was lower during larval and pupal stages suggesting L-luciferin is converted to D-luciferin as the beetle matures.

5.2. Luciferin biosynthesis

Luminescence was originally thought to be produced by symbiotic bioluminescent bacteria in the firefly [84, 85]. This notion was dispelled when Harvey in 1929 demonstrated that the adult firefly develops new lanterns after the larval lanterns have been excised [86]. Seliger predicted that the adult lampyrids emerge with sufficient luciferin for luminescence during their lifetime[87]. Strause *et al.* studied the levels of luciferase and luciferin in larvae, pupae and adults of *Photuris pennsylvanica* [88]. They calculated there would be sufficient luciferin (15 μmole) and luciferase in the newly emerged adult to provide 10,000 flashes, adequate for the lifespan of an average firefly. They concluded there would be no need for the adult to synthesize luciferin thereby supporting Seliger's hypothesis.

It has been suggested that, based upon the structure of beetle luciferin and its chemical synthesis, that the origin of the thiazoline ring is likely to be a cysteine [89]. Indeed studies using radiolabelled cysteine apparently confirm this. However, the origin of the benzothiazole portion is less clear but it has been suggested that cysteine is also a building block of this part of the luciferin molecule. Early attempts to chemically synthesise luciferin were based upon the possibility that *in vivo* quinones, e.g. p-benzoquinone, known to be present in coleopterans, often as part of defense secretions, may react with cysteine to produce the 6-hydroxybenzothiazole moiety of beetle luciferin. The addition of a subsequent cysteine gives beetle luciferin. This proven chemical synthesis was seen as a model for the biosynthesis *in vivo* [90]. However, the ability to chemically synthesis luciferin from p-benzoquinone and cysteine is not proof of how luciferin is made *in vivo*. An alternative mechanism for the formation of beetle luciferin comes from hypotheses related to the recycling of the product of the light reaction, oxyluciferin, back to luciferin.

6. The luciferin regenerating enzyme (LRE)

In 1974 Okada and co-workers injected ^{14}C oxyluciferin and ^{14}C-2-cyano-6-hydroxybenzothiazole (2C6HB) into living fireflies and detected ^{14}C luciferin after a number of hours [91]. They concluded that the luminescent product, oxyluciferin, is recycled to the substrate luciferin for subsequent light emission [92]. Okada *et al.* also identified that the addition of cysteine improved the yield of luciferin. As a result in *Pyrophorus pellucens* it was found that radiolabelled cysteine was incorporated into newly synthesized luciferin [90] These results have been explained in the following two-step reaction: (1) transformation of oxyluciferin to 2-cyano-6-hydroxybenzothiazole and (2) condensation of 2C6HB with D-cysteine to yield luciferin. Derivatives of 2C6HB are now used for the large-scale chemical synthesis of beetle luciferin [93, 94]. However, it should be noted that it has been established that the reaction with cysteine and 2C6HB occurs non-enzymatically [91, 95].

Along with luciferase a second enzyme has been implicated in the process of beetle bioluminescence. A protein fraction of three firefly extracts (*Photinus pyralis*, *Luciola lateralis* and *L. cruciata*) were originally found to exhibit an activity that enhanced bioluminescence *in vitro* [95]. This enzyme, the luciferin regenerating enzyme (LRE) was further characterised by obtaining the cDNA for all three fireflies which, when expressed and assayed, exhibited the same properties of enzymatically regenerating oxyluciferin back into luciferin in the presence of D-cysteine [95, 96] (Figure 4). The three LRE cDNA sequences coded for between 307–309 amino acids

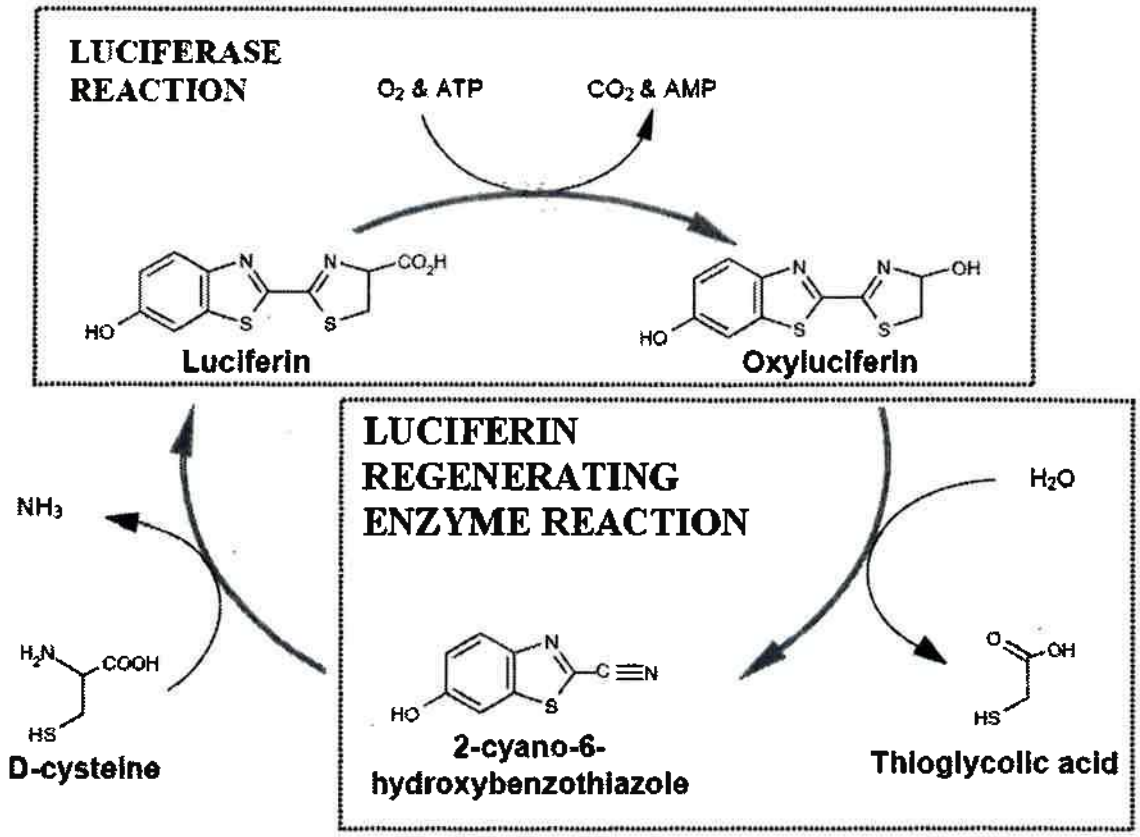


Figure 4. Theoretical pathway for the regeneration of luciferin from oxyluciferin.

with predicted molecular masses of 33.6–34.3 kDa. The LRE gene for *P. pyralis* is composed of five exons divided by four introns ranging in size from 53 – 904 bp [97]. It is not yet known whether LREs are found within the genome of click beetles and railroad worms. Sequence comparisons revealed that the three LRE's shared significant identity with a group of proteins known as senescence marker protein-30 (SMP30) [96].

6.1. Senescence marker protein 30 (SMP30)

To understand age-associated modifications at the genetic level Fujita *et al.* in 1992 surveyed differential levels of proteins produced by in different aged rats. A novel hepatic protein was identified and found to be produced in reduced amounts (60-70% less) in older rats [98]. Due to the relationship with aging and the molecular mass of 30 kDa the protein exhibited, the protein was named senescence marker protein 30 (SMP30) [98].

Molecular analysis of the rat SMP30 cDNA revealed a transcript coding for an enzyme composed of 299 amino acids with an estimated molecular weight of 33,387 [98]. Independently a Ca^{2+}-binding protein called regucalcin had been characterised and subsequently found to be the identical to SMP30

[99-102]. Fujita *et al.* went on to characterise the cDNA encoding SMP30 from human RNA and the cDNA, gene and putative promoter sites in the mouse [103, 104]. In the mouse the gene is composed of seven exons and spans approximately 17.5 kb [105]. A number of promoter regions have been predicted including a cluster of motifs (Sp1) that, in aged rats, decrease in binding efficiency [104]. Although first identified in the liver, SMP30 transcripts have been found in other tissues such as the kidney, lung, testes and cerebrum [106]. In mice the SMP30 has been shown to protect the liver, and potentially other organs, from apoptosis [107]. SMP30 also potentially facilitates detoxification from harmful compounds classed as diisopropyl phosphorofluoridates (DFP) such as the chemical warfare nerve agents sarine, soman and tabun [107, 108]. This evidence along with other studies proposing antioxidant properties, thereby protecting cells from oxidative stress, has led SMP30 to be regarded as an anti-aging molecule [109].

6.2. SMP30 and ascorbic acid synthesis

Recently Kondo *et al.* (2006) showed that SMP30 is involved in the synthetic pathway of L-ascorbic acid or vitamin C [110]. Ascorbic acid is a reducing agent and antioxidant and in mammals is synthesised in the liver whereas reptiles and fish produce it in the kidney [111, 112]. Gluconolactonase (GNL) is known to catalyse the penultimate reaction [113-115] and Kondo *et al.* (2006) showed that rat GNL and SMP30 are one and the same with regard to their catalytic activity [110]. SMP30 exhibited catalytic acitvity with a range of aldonolactone substrates including L-gluconic acid, but the most activity was exhibited with D-glucono-δ-lactone. Furthermore, SMP30 knockout mice were prone to scurvy when subjected to a vitamin C deficient diet. Ascorbic acid is synthesized by the majority of vertebrates and the presence of a biosynthesis pathway in the sea lamprey suggests this appeared early in the evolutionary history of fishes prior to the emergence of terrestrial vertebrates [116, 117]. This biosynthetic capability has been lost in a number of diverse organisms including teleost fishes [118], passeriform birds [119], bats [120], guinea pigs [121] and primates including humans [122]. Enzymological studies in the late 1950s revealed that the inability to synthesize ascorbic acid was caused by the lack of L-gulonolactone oxidase (GLO) activity [123]. Disrupted GLO genes have been characterized in humans [124], primates [125], guinea pigs [126] and some breeds of pig [127].

6.3. The role of SMP30 homologues in insects

SMP30 homologues have been identified from four different insect families and a number of putative roles ascribed. In *Drosophila* a SMP30 orthologue, *Dca*, exhibited increased expression levels as a result of cold

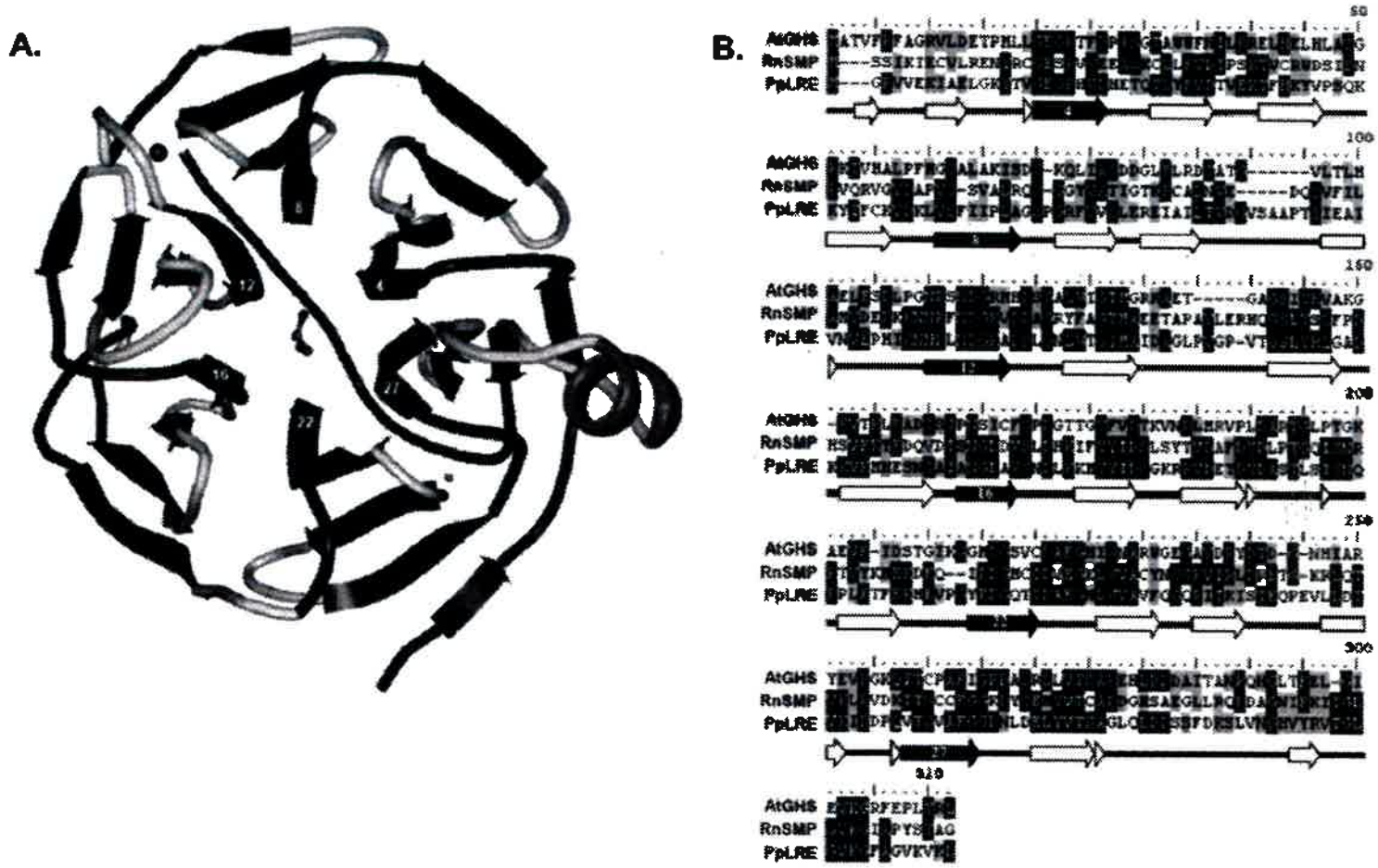


Figure 5. (A) Crystal structure of SGL protein from *Agrobacterium tumefaciens*. The six beta sheets found at the centre of the protein are numerically highlighted. (B) SGL amino acid sequence alignment from *A. tumefaciens* (AtGHS), rat (SMP30) and the firefly *P. pyralis* (PpLRE). The six sheets found at the centre of the protein are highlighted in black.

acclimatisation [128]. Dipteran SMP30 orthologues have also been identified from the flesh fly *Sarcophaga peregrina* [129] which was found to be restricted almost exclusively to the anterior fat body [129] and was hence referred to as the anterior fat body protein (AFP). In late larvae of the blowfly, *Calliphora vicina*, this AFP was found to bind to the hexamerin receptor arylphorin-binding protein (ABP) and predicted to play a role in the regulation of hexamerin uptake by fat body cells along the anterior–posterior axis [130]. It has yet been established whether insect SMP30 homologues have a GNL function.

6.4. SGL protein family

Based upon sequence conservation and the recent evidence for conserved function the three classes of enzyme GNL, SMP30 and LRE have been designated as the SGL (SMP-30/Gluconolaconase/LRE-like) protein family (PFAM08450). However, despite the sequence identity, SMP30 in mice has been shown to lack any luciferin regenerating activity; conversely both LRE and SMP30 have been shown to hydrolyze diisopropyl phosphorofluoridate (DFP) [108]. Firefly LRE therefore stands apart as a enzyme with a unique catalytic activity, the regeneration of oxyluciferin into luciferin, an activity not exhibited by mammalian SMP30 [108]. LRE therefore may have arisen from an ancestral GNL in beetles which may have originally played a part in the synthesis of ascorbic acid.

A number of SGL gene copies have been identified in lampyrids along with other beetles suggesting extensive SGL gene duplication events in the history of the Coleoptera [131]. In the genome of *Tribolium castaneum* alone seven paralogues were identified in a tandem array but it is not yet known what function these enzymes serve [131]. Amino acid alignments show, like the adenylate-forming enzymes, highly conserved core motifs (figure 5). The tertiary structure of SGL proteins is known only from one organism, the bacteria *Agrobacterium tumefaciens* (RCSB protein databank accession 2GHS). The structure revolves around a putative active site composed of six beta sheets and it is in most of these sheets and adjacent regions that the core conserved motifs are found (figure 5). It is likely that the tertiary structure of firefly LRE will be similar to that of *A. tumefaciens* and that through gene duplication has evolved a novel function in bioluminescent beetles to regenerate luciferin.

References

1. Sivinski, J. 1981, Coleopterists Bulletin, 35, 167-179.
2. De Cock, R. and Matthysen, E. 2003, Behavioral Ecology, 14, 103-108.
3. Harvey, E.N. 1952, Bioluminescence. Academic Press, New York, (date).

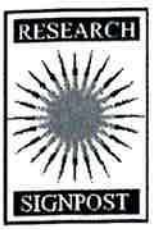
4. Crowson, R.A. 1972, Rev. Uni. Madrid, 21, 35-77.
5. McDermott, F.A. 1964, Trans. Amer. Ent. Soc., 90, 1-72.
6. Dubois, R. 1885, Compt. Rend. Soc. Biol., 37, 559-562.
7. McElroy, W.D. 1947, Proc. Natl Acad. Sci. USA, 33, 342-345.
8. McElroy, W.D., and Strehler, B.L. 1949, Arch. Biochem., 22, 420-433.
9. McElroy, W.D., and DeLuca, M. 1978, Bioluminescence in Action, P. J. Herring, (Ed). Academic, London, pp. 109-127.
10. Shimomura, O. Bioluminescence: Chemical principles and methods. World Scientific, Singapore, (date).
11. Bitler, B., and McElroy,W.D. 1957, Arch. Biochem. Biophys., 72, 358-368.
12. White, E.H., McCapra, F.M., Field, G.C., and McElroy, W.D. 1961, J. Am. Chem. Soc., 83, 2402-2403.
13. Hopkins, T.A., Seliger, H.H., White, E.H., and Cass, M.W. 1967, J. Am. Chem. Soc., 89, 7148-7150.
14. McCapra, F., Chang, Y.C., and Francois,V.P. 1968, Chem. Commun.,22-23.
15. Shimomura, O., Goto, T. , and. Johnson, F.H 1977, Proc. Natl Acad. Sci. USA, 74, 2799-2802.
16. White, E.H., Rapaport, E., Seliger, H.H., and Hopkins, T.A. 1971, Bioorg. Chem., 1, 92-122.
17. Branchini, B.R. *et al.*, 2002, J. Am. Chem. Soc., 124, 2112-2113.
18. Green, A.A., and McElroy, W.D. 1956, Biochim. Biophys. Acta, 20, 170-176.
19. Branchini,B.R., and Rollins, C.B. 1989, Photochem. Photobiol., 50, 679-684.
20. Wood, K.V., deWet, J.R., Dewji, N., and DeLuca, M. 1984, Biochem. Biophys. Res. Commun., 124, 592-596.
21. Wienhausen, G., and DeLuca, M. 1985, Photochem. Photobiol., 42, 609-661.
22. Conti, E., Franks, N.P., and Brick, P. 1996, Structure, 4, 287-298.
23. Viviani, V.R. *et al.*, 1999, Photochem. Photobiol., 70, 254-260.
24. Li, X., Yang, S., and Liang, X. 2006, Zool. Res., 27, 367-374.
25. Sala-Newby, G.B., Thomson, C.M., and Campbell, A.K. 1996, Biochem. J., 313, 761-767.
26. Said Alipour, B. *et al.* 2004, Biochem. Biophys. Res. Commun., 325, 215-222.
27. de Wet, J.R., Wood, K.V., Helinski, D.R., and DeLuca, M. 1985, Proc. Natl Acad. Sci. USA, 82, 7870-7873.
28. Ohmiya, Y., Ohba, N., Toh, H., and Tsuji, F. 1995, Photochem. Photobiol., 62, 309-313.
29. Dong, P., Hou, Q.I., Li, X., and Liang, X. 2008, Zool. Res., 29, 477-484.
30. Choi, Y.S. *et al.*2002, Comp. Biochem. Physiol. Biochem. Mol. Biol., 132, 661-670.
31. Emamzadeh, A.R. *et al.* 2006, J. Biochem. Mol. Biol., 39, 578-585.
32. Masuda,T., Tatsumi, H., and Nakano, E. 1989, Gene, 77, 265-270.
33. Branchini, B.R., Southworth, T.L., DeAngelis, J.P., Roda, A., and Michelini, E. 2006, Comp. Biochem. Physiol. B, Biochem. Mol. Biol., 145, 159-167.
34. Tatsumi, H., Kajiyama, N., and Nakano,E. 1992, Biochim. Biophys. Acta, 1131, 161-165.
35. Devine, J.H., Kutuzova, G.D., Green, V.A., Ugarova, N.N., and Baldwin, T.O. 1993, Biochim. Biophys. Acta, 1173, 121-132 (1993).

36. Ye, L., Buck, L.M., Schaeffer, H.J., and Leach, F.R. 1997, Biochim. Biophys. Acta, 1339, 39-52.
37. Viviani, V.R., Bechara, E.J., and Ohmiya, Y. 1999, Biochemistry, 38, 8271-8279.
38. Sumiya, M., Viviani, V.R., Ohba, N., and Ohmiya, Y. 1998, Bioluminescence and Chemiluminescence. Proc. 10th Int. Symp., A. Roda, M. Pazzagli, L.J. Kricka, P. E. Stanley, (Eds.), Bologna, Italy, pp. 433-436.
39. Stolz, U., Velez,S., Wood, K.V., Wood, M., and Feder, J.L. 2003, Proc. Natl Acad. Sci. USA, 100, 14955-14959.
40. Wood, K.V., Lam,Y.A., Seliger, H.H., and McElroy, W.D. 1989, Science, 244, 700-702.
41. Viviani, V.R. *et al.* 1999, Photochem. Photobiol., 70, 254-260.
42. Day, J.C., Chaichi, M.J., Najafil, I., and Whiteley, A.S. 2006, J. Insect Sci., 6, 1-8.
43. Nakatsu, T. *et al.* 2006, Nature, 440, 372-376.
44. Branchini, B.R., Magyar, R.A. Murtiashaw, M.H., Anderson, S.M., and Zimmer,M. 1998, Biochemistry, 37, 15311-15319.
45. Sandalova, T.P., and Ugarova, N.N. 1999, Biochemistry (Mosc.), 64, 962-967.
46. Branchini, B.R., Magyar, R.A., Murtiashaw, M.H., and Portier, N.C. 2001, Biochemistry, 40, 2410-2418.
47. Seliger, H.H., and McElroy, W.D. 1964, Proc. Natl Acad. Sci. USA, 52, 75-81.
48. Viviani, V.R., and Bechara, E.J.H. 1993, Photochem. Photobiol., 58, 615-622.
49. Viviani, V.R., and Bechara, E.J.H. 1995, Photochem. Photobiol., 62, 490-495.
50. White, E.H., Rapaport, E., Seliger, H.H., and Hopkins, T.A. 1971, Bioorg. Chem., 1, 92-122.
51. McCapra, F. 2000, Methods Enzymol., 305, 3-47.
52. DeLuca, M. 1969, Biochemistry, 8, 160-166.
53. Gandelman, O.A., Brovko, L.Y., Ugarova, N.N., Chikishev, A.Y., and Shkurimov, A.P. 1993, J. Photochem. Photobiol. B, 19, 187-191.
54. Ugarova, N.N., and Brovko, L.Y. 2001, Russ. Chem. Bull., 50, 1752-1761.
55. Gulick, A.M., Starai, V.J., Horswill, A.R., Homick, K.M., and Escalante-Semerena, J.C. 2003, Biochemistry, 42, 2866-2873.
56. Reger, A.S., Carney, J.M., and Gulick, A.M. 2007, Biochemistry, 6536-46, 6546.
57. Chang, K.H., Xiang, H., and Dunaway-Mariano, D. 1997, Biochemistry 36, 15650-15659.
58. Gulick, A.M., Starai, V.J., Horswill, A.R., Homick, K.M., and Escalante-Semerena, J.C. 2003, Biochemistry, 42, 2866-2873.
59. Kleinkauf, H., and Von Dohren, H. 1996, Eur. J. Biochem., 236, 335-351.
60. Delarue, M. 1995, Curr. Opin. Struct. Biol., 5, 48-55.
61. Morozov, V.M. 1997, Bioluminescence and Chemiluminescence: Molecular Reporting with Photons. Proc. 9th Int. Symp., J. W. Hastings, L. J. Kricka, and P. E. Stanley (Eds.), Wiley, Chichester, pp. 236-239.
62. Stuible, H.-P., Büttner, D, Ehlting, J., Hahlbrock, K., and Kombrink, E. 2000, FEBS Letters, 467, 117-122.
63. Thompson, J.F. *et al.* 1997, J. Biol. Chem., 272, 18766-18771.
64. Branchini, B.R., Murtiashaw, M.H., Magyar, R.A., and Anderson, S.M. 2000, Biochemistry, 39, 5433-5440.

65. Oba, Y., Ojika, M., and Inouye, S. 2003, FEBS Lett., 540, 251-254.
66. Oba, Y., Sato, M., Ohta, Y., and Inouye, S. 2006, Gene, 368, 53-60.
67. Fontes, R., Dukhovich, A., Sillero, A., and Sillero, M.A.G. 1997, Biochemi. Biophys. Res. Comm., 237, 445-450.
68. Oba, Y., Ojika, M., and Inouye, S. 2003, FEBS Lett., 540, 251-254.
69. Nor Aliza, A.R. *et al.* 2001, Comp. Biochem. Physiol. A Mol. Integr. Physiol., 128, 251-257.
70. Cho, K.H. , Lee, J.S., Yang, D.C., and Boo, K.S. 1999, Insect Mol. Biol., 8, 193-200.
71. Day, J.C. 2005, Eur. J. Entomol., 102, 787-791.
72. Day, J.C., Goodall, T.I., and Bailey, M.J. 2008, Mol. Phylogenet. Evol., in press.
73. Oba, Y., Sato, M., and Inouye, S. 2006, Insect Mol. Biol., 15, 293-299.
74. Blank, G.E., Pletcher, J., and Sax, M. 1971, Biochem. Biophys. Res. Commun., 42, 583-588.
75. Colepicolo, R., Pagni, D., and Bechara, E.J.H. 1988, Comp. Biochem. Physiol. B, Biochem. Mol. Biol., 91, 143-147.
76. Hadj-Mohammadi, M.R., and Chaichi, M.J. 1996, Photochem. Photobiol., 64, 821-822.
77. Seliger, H.H., and McELroy, W.D. 1965, Light - Physical and Biological Action. Academic Press, New York.
78. Kato, S.-I., Oba, Y., Ojika, M., and Inouye, S. 2004, Tetrahedron,60, 11427-11434.
79. Branchini, B.R., Hayard, M.M., Bamford, S., Brennan, P.M., and Lajiness, E.J. 1989, Photochem. Photobiol., 49, 689-695.
80. McElroy, W.D. and Seliger, H.H. 1962, Fed. Proc. Fed. Am. Soc. Exp. Biol., 1006–1012.
81. Lembert, N. 1996, Biochem. J., 317, 273-277.
82. Nakamura, M. *et al.* 2006, Tetrahedron Lett., 47, 1197-1200.
83. Niwa, K., Nakamura, M., and Ohmiya, Y. 2006, FEBS Lett., 580, 5283-5287.
84. Kuhnt, P. 1907, Entomol. Wochenblatt, 24, 3-4.
85. Pierantoni, U. 1914, R. C. Accad. Napoli, 20, 15-21.
86. Harvey, E.N., and Hall, R.T. 1929, Science, 69, 253-254.
87. Seliger, H.H. 1973, Chemiluminescence and Bioluminescence, M. J. Cormier, D. M. Hercules, and J. Lee (Eds.), Plenum, New York, pp. 335.
88. Strause, L.G., DeLuca, M., and Case, J.F. 1979, J. Insect Physiol., 25, 339-348.
89. McCapra, F.M., and Perring, K.D. 1985, Chemiluminescence and Bioluminescence, J. G. Burr (Ed.), Marcel Dekker, New York, 359-386.
90. McCapra, M., and Razavi, Z. 1975, J.C.S. Chem. Comm., 2,42-43.
91. Okada, K., Iio, H., Kubota, I, and Goto, T. 1974, Tetrahedron Lett., 15, 2771-2774.
92. Gates, B.J., and DeLuca, M. 1975, Arch. Biochem. Biophys., 169, 616-621.
93. Bowie, L.J. 1978, Meth. Enzymol., 57, 15-28.
94. Branchini, B.R. 2000, Meth. Enzymol., 305, 188-195.
95. Gomi, K., and Kajiyama, N. 2001, J. Biol. Chem., 276, 36508-36513.
96. Gomi, K., Hirokawa, K., and Kajiyama, N. 2002, Gene, 294, 157-166.
97. Day, J.C., and Bailey, M.J. 2003, Insect Mol. Biol., 12, 365-372.
98. Fujita, T., Uchida, K., and Maruyama, N. 1992, Biochim Biophys Acta, 1116, 122-128.

99. Shimokawa, N., and Yamaguchi, M. 1992, FEBS letters, 305, 151-154.
100. Shimokawa, N., and Yamaguchi, M. 1993 FEBS Letters, 327, 251-255.
101. Yamaguchi, M., and Sugai, K. 1981, Chem. Pharm. Bull., 29, 567-570.
102. Yamaguchi, M., and Yamamoto, T. 1978, Chem. Pharm. Bull., 26, 1915-1918.
103. Fujita, T. *et al.* 1995, Biochim Biophys Acta., 1263, 249-252.
104. Fujita, T, Shirasawa, T., Uchida, K., and Maruyama, N. 1996, Mech. Age. Dev., 87, 219-229.
105. Fujita, T., Shirasawa, T., and Maruyama, N. 1996, Biochim Biophys Acta, 1308, 49-57.
106. Mori, T. *et al.*2004, Pathol. Int., 54, 167-173.
107. Ishigami, A. *et al.* 2002, Am. J. Pathol., 161, 1273-1281.
108. Kondo, Y. *et al.* 2004, FEBS Letters, 570, 57-62.
109. Feng, D. *et al.* 2004, Ann. N.Y. Acad. Sci., 1019, 360-364.
110. Kondo, Y. *et al.* 2006, Proc. Natl. Acad. Sci. USA, 103, 5723-5728.
111. Chatterjee, I.B. 1973, Science, 182, 1271-1272.
112. Moreau, R.., and Dabrowski, K. 1998, PNAS, 95, 10279-10282.
113. Burns, J.J. 1960, Metabolic Pathways, D. M. Greenberg, (Ed.), Academic, New York, vol. 1, 341-356.
114. Nishikimi, M., Okamura, M., and Ohta, Y. 2003, Recent Res. Dev. Biophys. Biochem., S. G. Pandalai (Ed.), Research Signpost, Kerala, India, vol. 3, pp. 531-545.
115. Nishikimi, M., and Yagi, K. 1996, Subcellular Biochemistry - Ascorbic Acid: Biochemistry and Biomedical Cell Biology., J. R. Harris (Ed.), Plenum Press, New York, vol. 25, pp. 17-39.
116. Moreau, R., and Dabrowski, K. 1998, Free Radic. Biol. Med., 25, 989-990.
117. Moreau, R., and. Dabrowski, K. 1998, Proc. Natl. Acad. Sci. USA, 95, 10279-10282.
118. Dabrowski, K. 1990, Biol. Chem. Hoppe-Seyler, 371, 207-214.
119. Chaudhuri, C.R., and Chatterjee, I.B. 1969, Science,164, 435-436.
120. Birney, E.C., Jenness, R., and Ayaz, K.M. 1976, Nature, 260, 626-628.
121. Burns, J.J., Moltz, A., and Peyser, P. 1956, Science, 124, 1148-1149.
122. Stone, I. 1965, Am. J. Physic. Anthropol., 23, 83-85.
123. Burns, J.J. 1957, Nature, 180, 553-553.
124. Nishikimi, M., Fukuyama, R., Minoshima, S., Shimizu, N., and Yagi, K. 1994, J. Biol. Chem., 269, 13685-13688.
125. Ohta, Y., and Nishikimi, M. 1999, Biochim. Biophys. Acta, 1472, 408-411.
126. Nishikimi, M., Kawai, T., and Yagi, K. 1992, J. Biol. Chem., 267, 21967-21972.
127. Hasan, L. *et al.* 1992, J Biol Chem., 267, 21967-21972.
128. Goto, S.G. 2000, J. Insect Physiol., 46, 1111-1120.
129. Nakajima, Y., and Natori, S. 2000, J. Biochem, 127, 901-908.
130. Hansen, I.A., Meyer, S.R., Schafer, I., and Scheller, K. 2002, Eur. J. Biochem., 269, 954-960.
131. Day, J.C. The Evolution of Beetle Bioluminescence. Ph.D. Thesis Oxford Brookes Univeristy, (2009).

Research signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India



Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 277-290
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

15 On the origin of beetle luminescence

Y. Oba
Graduate School of Bioagricultural Sciences, Nagoya University, 464-8601 Nagoya, Japan

Abstract

Beetles (the order Coleoptera) include numerous luminous species, such as fireflies (the family Lampyridae), railroad worms (Phengodidae) and firebeetles (Elateridae). Their bioluminescence mechanism, the luciferin-luciferase reaction, has been intensively studied, but its evolutionary origins remain obscure. Recent findings from molecular phylogenetics and homologue hunting have cast new light on the origins of beetle bioluminescence.

Correspondence/Reprint request: Dr. Yuichi Oba, Graduate School of Bioagricultural Sciences, Nagoya University, 464-8601 Nagoya, Japan. E-mail: oba@agr.nagoya-u.ac.jp

1. Introduction

"Can't you go to take a walk like other folks, without a light?"
"It is immemorial custom with my family," answered the firefly.
-from "The Adventures of Grillo" by Ernest Candèze [1]

An orthodox Darwinian will almost necessarily postulate that luminosity in Cantharoidea would first have arisen as an accidental by-product of a biochemical reaction serving some other function.
-Roy Crowson [2]

Bioluminescence, the production of visible light by a living organism, is known to have emerged independently on several occasions, yet the evolutionary origins of most bioluminescence systems remain obscure [3-6]. The firefly (Lampyridae, Coleoptera) is one of the most intensively studied luminous organisms in the fields of chemistry, biology, and biotechnology. Accordingly, the evolutionary aspect of beetle bioluminescence has been discussed extensively in the literature [7-10]. This chapter provides an update of recent findings on the molecular phylogeny of Coleoptera, the molecular evolution of beetle luciferase, and the systematic distribution of beetle luciferin, and reexamines the origins of beetle bioluminescence.

2. Luminous beetles

2.1. Luminescence in beetles

Luminous beetles have only been identified in small groups of Coleoptera, except for some uncertain records. Before initiating a discussion of the origins of beetle luminescence, it would first be important to review luminous beetles in the world and their present taxonomic positions.

2.2. Cantharoids

The cantharoid beetles, formerly the superfamily Cantharoidea [2], consist of Lampyridae (firefly, ~2,000 species in the world), Phengodidae (railroad worm, ~200 species), Lycidae (net-winged beetle, ~3,500 species), Cantharidae (soldier beetle, ~5,000 species), and six other small families (~120 species in total) [11]. All known species of Lampyridae and Phengodidae are luminous, at least at the larval stage [12], but the members of the other families are non-luminous [2]. The luminosity of larval *Omalisus fontisbellaquaei* (Omalisidae, Cantharoidea) was described in an old report [13], but has recently been questioned ([14], M.A. Branham, personal communication).

2.3. Elateridae

Click beetles (Elateridae, ~9,000 species in the world) are widespread throughout the world, but the luminous taxa (~200 species) are only found in tropical and subtropical America and Melanesia [11,15]. All luminous elaterids (or firebeetles) belong to the tribe Pyrophorini (in the subfamily Pyrophorinae), with the single exception of *Campyloxenus pyrothorax* (Campyloxenini in Pyrophorinae [15]). In 1979, Stibick [15] suggested a clear relationship between Pyrophorini and Campyloxenini. The luminosity of *Melanactes* larva (Melanactinae, Elateridae) remains unknown [7].

2.4. Other beetles

Costa [16] reported luminescence in *Balgus schnusei,* a species that has now been assigned to the Thylacosterninae of the Elateridae [17], but its taxonomic status remains unclear [18]. One report of a bioluminescent staphylinid [19] needs to be confirmed [20].

As a consequence, to date, the only true instances of self-luminescence in beetles are observed in Lampyridae, Phengodidae and Elateridae. Hence, the following discussion focuses on the luminous species of these three families.

3. Bioluminescence of beetles

Lampyrids, phengodids, and luminous elaterids utilize the same luciferin compound, (4*S*)-4,5-dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazolecarboxylic acid, in their luciferin-luciferase reaction [21]. The luciferase cDNAs have been isolated from several species of lampyrids, phengodids, and luminous elaterids, and their primary sequences show high similarity (> 48% amino acid identity with few gaps). Thus, the bioluminescence mechanisms of luminous beetles are essentially the same: the initial reaction catalyzed by beetle luciferase is a formation of luciferyl adenylate in the presence of Mg^{2+} (Eq. 1); then, the luciferase-bound luciferyl adenylate reacts rapidly with molecular oxygen to yield light, CO_2, AMP, and oxyluciferin (Eq. 2).

$$\text{Luciferin} + \text{ATP} \leftrightarrows \text{Luciferyl-AMP} + \text{PPi} \qquad \text{Eq. 1}$$

$$\text{Luciferyl-AMP} + O_2 \rightarrow \text{Oxyluciferin} + \text{AMP} + CO_2 + \text{Light} \qquad \text{Eq. 2}$$

When excess amounts of luciferin and ATP are added to the reaction *in vitro*, light emission decreases rapidly. This inhibitory phenomenon has been explained primarily by the by-production of dehydroluciferyl-AMP, which binds tightly with luciferase [22,23]. However, the addition of coenzyme A (CoA) prevents this rapid inhibition, because dehydroluciferyl-AMP is converted to dehydroluciferyl-CoA and released from luciferase.

McElroy *et al.* [24] suggested that the luciferin-luciferase reaction in the firefly is similar in terms of the catalytic mechanism to that of acyl-CoA synthetase and aminoacyl-tRNA synthetase. After two decades, the primary structure of the firefly *Photinus pyralis* luciferase was determined [25]. Interestingly, it has significant homology with plant 4-coumarate:CoA ligases [26] and rat fatty acyl-CoA synthetase [27], but not with any of the luciferases of other luminous organisms.

4. Phylogeny of luminous beetles

4.1. Phylogeny of Lampyridae and Phengodidae

Phylogenetic analyses of lampyrid species have been conducted based on the morphological characteristics of the adult male [12,28] and the mitochondrial 16S ribosomal DNA [29-31]. However, the resultant trees did not support the monophyly of the Lampyridae. Recent molecular analyses, primarily based on nuclear 18S ribosomal DNA, of cantharoids have shown that the Lampyridae are monophyletic [18,32], suggesting that lampyrid species share a common origin of bioluminescence (Fig. 1).

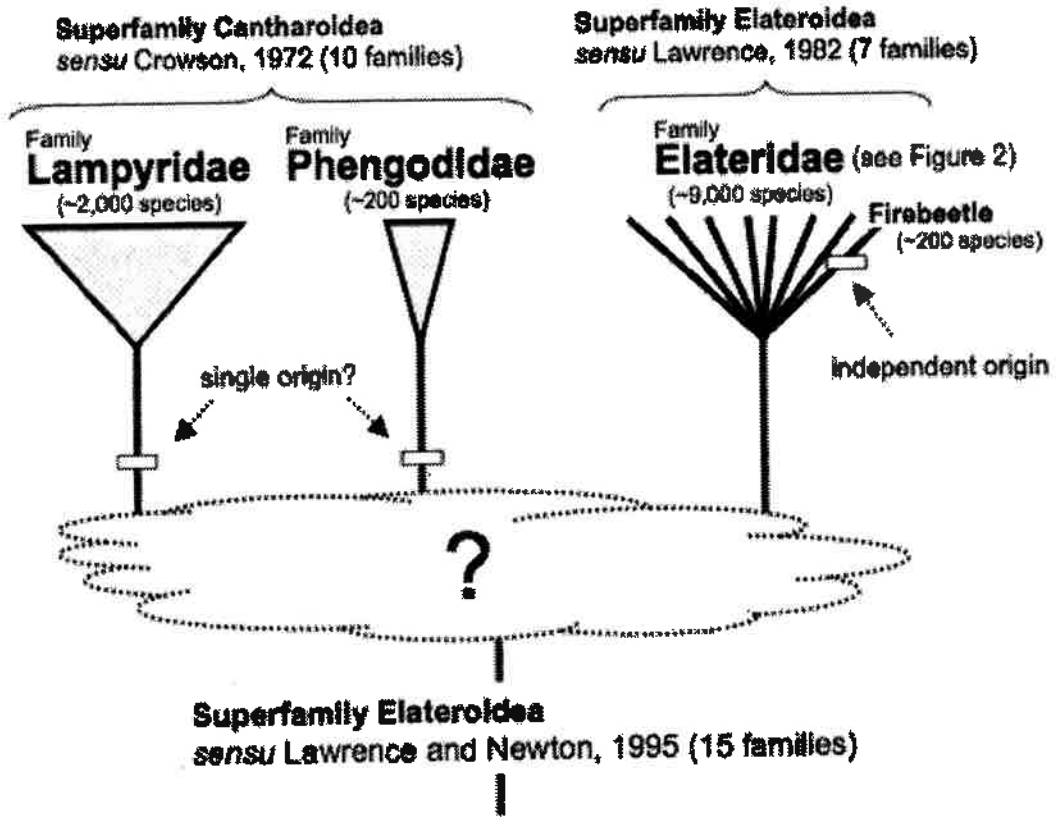


Figure 1. A proposed scheme for evolution of beetle luminescence. Only three families which contain luminous species are shown. Open rectangles indicate a gain of luminescence, thus a simultaneous gain of luciferin and luciferase.

Evolutionary relationships within and around the cantharoid families have been proposed based on their morphological characteristics [2,12,28,33,34]; however, the results have thus far been inconsistent. Crowson [2] and Beutel [33] suggested a sister relationship between Lampyridae and Phengodidae and hypothesized that their luminescence has a common origin. Pototskaja [34] placed the Lampyridae as a sister clade of Lycidae and separated from Phengodidae. Branham and Wenzel [12] considered the subfamily Rhagophthalminae (Phengodidae, [17]) as a sister clade to Lampyridae and as separate from the Phengodinae (Phengodidae). Previous molecular analyses have indicated that Rhagophthalminae is included in the clade of Lampyridae [29-31]. On the other hand, recent molecular analyses demonstrated that Phengodinae and Rhagophthalminae are monophyletic, and that Lampyridae and Phengodidae are not sister groups [18,32]. The former result suggests that the bioluminescence in Phengodinae and Rhagophthalminae is of a common origin (Fig. 1).

Considering the latter results, Bocakova *et al.* [18] proposed that the evolutionary origins of Lampyridae and Phengodidae were independent. Arnoldi *et al.* [35] conducted a mitochondrial genome analysis of the Brazilian firebeetle, *Pyrophorus divergens*, that suggested that the Phengodidae is more closely related to the Elateridae than to Lampyridae, and that the origins of the bioluminescence of the Lampyridae, Phengodidae, and Elateridae were independent. Thus, the phylogenetic relationship between Lampyridae and Phengodidae has yet to be clearly resolved, and the independence of the origins of their bioluminescence has not yet been fully elucidated [32] (Fig. 1). An alternative approach, taken by Kobayashi *et al.* [36] based on studies of early embryonic development, suggested an association between lampyrids and *Rhagophthalmus* (Rhagophthalminae).

4.2. Phylogeny of Elateridae

Although the taxonomy of click beetles has extensively been studied [15,37,38], inconsistencies remain. Recently, we examined the relationships between species of Elateridae based on the partial sequences of nuclear 28S ribosomal DNA [39]. The result indicated that luminous *Pyrophorus* species aggregate at a derived position in the phylogeny of the Elateridae. Therefore, the most parsimonious reconstruction of the "luminous" and "non-luminous" states indicates that the ancestral state of Elateridae was non-luminous (Fig. 2). This suggests that bioluminescence in click beetles evolved independently from that of other luminous beetles, i.e., the Lampyridae and Phengodidae (Fig. 1). This conclusion is consistent with the traditional consensus; indeed, the eminent coleopterist Roy Crowson [2] noted that "there are many indications

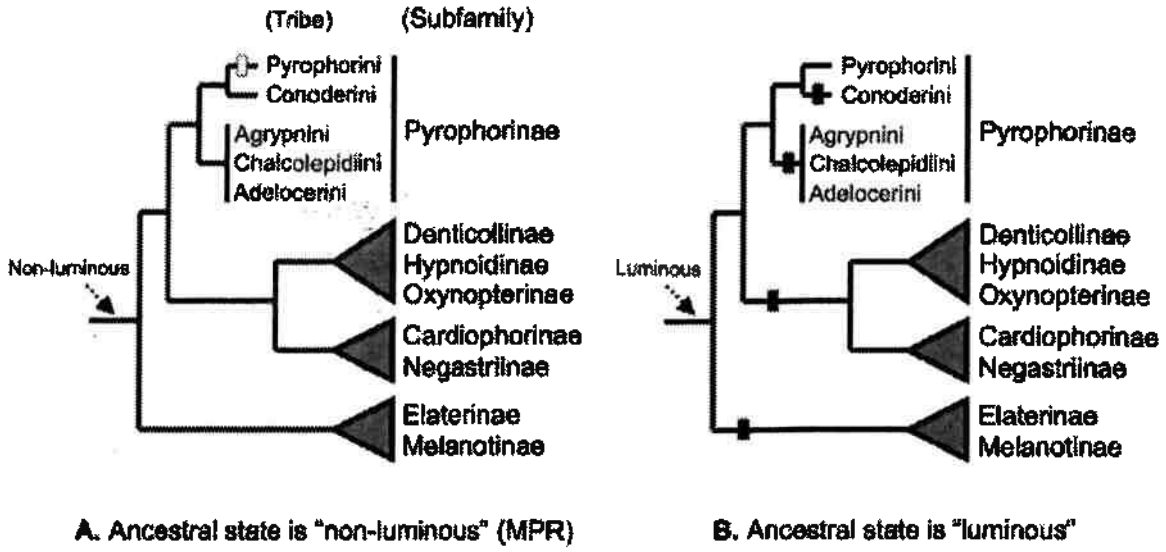


Figure 2. Character mapping of bioluminescence states on Elateridae phylogeny. The most parsimonious reconstruction (MPR) places the ancestral state as "non-luminous" (**A**). Luminosity as an ancestral state (**B**) requires at least 4 steps of loss of luminescence. Open rectangle indicates a gain of luminescence. Closed rectangles indicate a loss of luminescence.

of a fairly close affinity of Cantharoidea to Elateroidea, but nothing suggests any particular link of the former superfamily to Pyrophorini, so that it is unlikely that the luminosity of *Pyrophorus* and of various Cantharoidea derives from a common ancestor."

5. Origin of beetle luciferase

5.1. Genes similar to beetle luciferase

The origin of luciferases has not been clearly identified in any organisms, as most of the luciferases lack homology to any known proteins. It is remarkable that beetle luciferases share significant homology with adenylate-forming enzymes found in bacteria, fungi, vertebrates, and plants [26,27,40], but neither to luciferases of other organisms nor to any known proteins in insects. Thus, in order to elucidate the origin of beetle luciferase, we have focused on adenylate-forming enzymes in insects and their biochemical properties.

5.2. Firefly luciferase as a fatty acyl-CoA synthetase

Recently, we found that firefly luciferases, from *P. pyralis* and *Luciola cruciata*, exhibit the catalytic activity of fatty acyl-CoA synthetase in the presence of ATP, Mg^{2+}, and CoA (Eqs. 3 and 4) [41].

Fatty acid + ATP ⇆ Fatty acyl-AMP + PPi Eq. 3

Fatty acyl-AMP + CoA → Fatty acyl-CoA + AMP Eq. 4

Both luciferases were found to recognize a broad spectrum of saturated and unsaturated fatty acids, C8 – C20, and the most suitable substrate was lauric acid (C12:0) [42] (Fig. 3). The substrates for plant 4-coumarate:CoA ligase, 4-coumaric acid, caffeic acid, and ferulic acid, were not catalyzed by firefly luciferases. The results suggest that firefly luciferase is a bifunctional enzyme, catalyzing not only the luminescence but also fatty acyl-CoA synthesis.

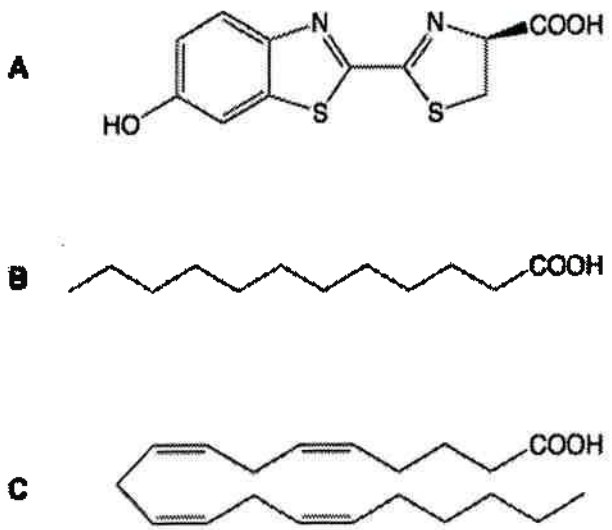


Figure 3. Are these structures similar? **A.** beetle luciferin. **B.** lauric acid. **C.** arachidonic acid. **B** and **C** are recognized as good substrates by firefly luciferases and their homologues in insects. (After Day *et al.* [10]).

5.3. CG6178 in *Drosophila melanogaster*

The most similar gene to beetle luciferase in the *D. melanogaster* genome database is CG6178 on chromosome 3R. The amino acid sequence identity to *P. pyralis* luciferase is 41% with a few gaps, which is higher than that between beetle luciferase and plant 4-coumarate: CoA ligase (34%, [26]) (Fig. 4). Notably, CG6178 contains a type 1 peroxisomal targeting signal sequence (PTS1, [43]) at the C-terminus, as do beetle luciferases [42]. We isolated the cDNA and characterized the recombinant protein. The results demonstrated that CG6178 is a fatty acyl-CoA synthetase but not luciferase [44], showing a broad spectrum of substrate specificity (various fatty acid of C8 – C20), and the most suitable substrate is lauric acid [42], as is the case with firefly luciferases (Fig. 3).

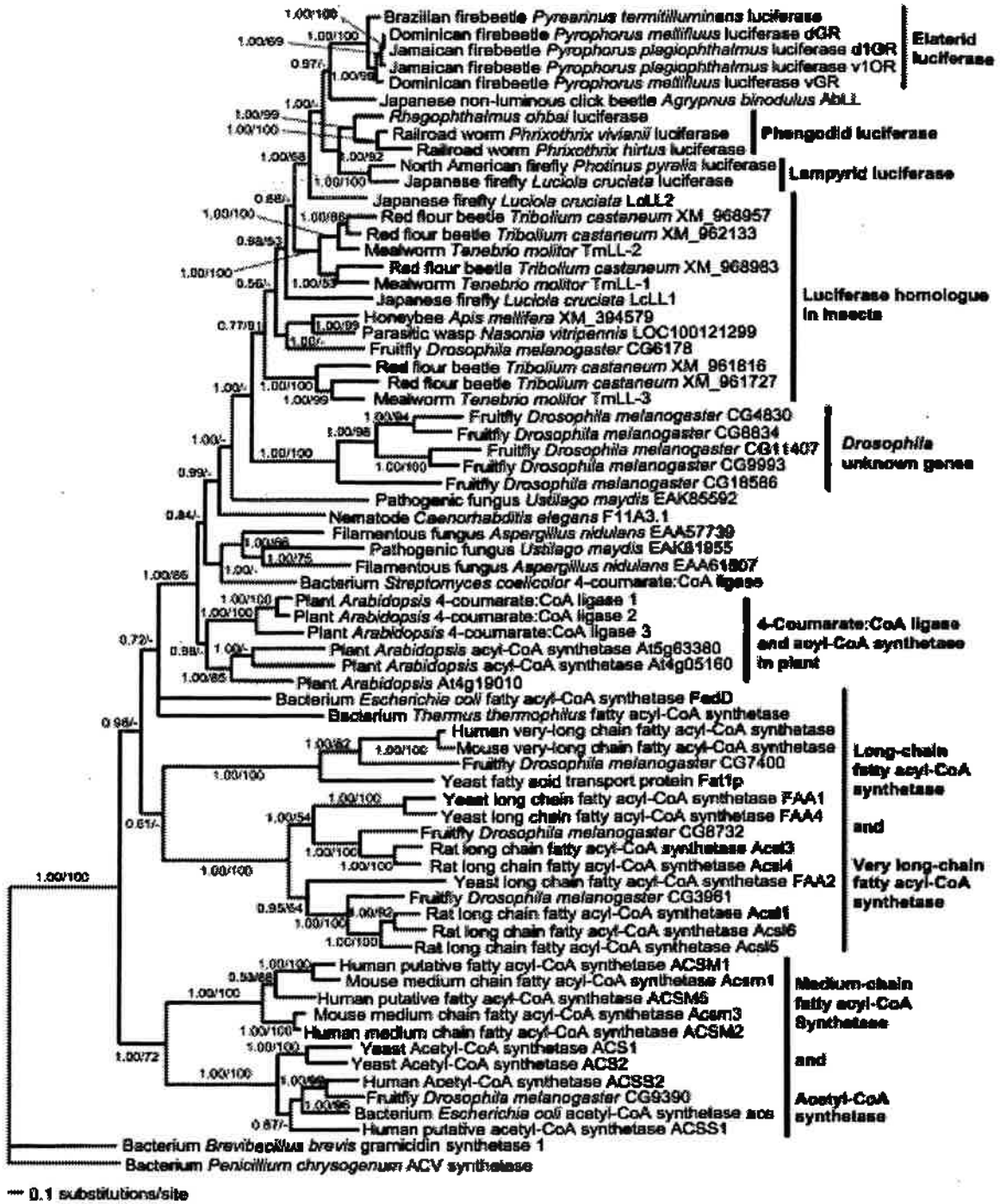


Figure 4. Bayesian phylogenetic tree of adenylate-forming enzymes (amino acids). Numbers on the node indicate Bayesian posterior probabilities/MP bootstrap values.

We constructed a chimeric protein that bears the N-terminal domain of *P. pyralis* luciferase and the C-terminal domain of CG6178. The recombinant chimera exhibited significant luminescence activity, approximately 4% of that of *P. pyralis* luciferase, with beetle luciferin [45]. This suggests that beetle luciferase and *Drosophila* CG6178 are similar in terms of protein structure.

Considering these findings, we previously proposed that beetle luciferase arose from a peroxisomal fatty acyl-CoA synthetase [46]. Recently, Schneider *et al.* [47] demonstrated that peroxisomal acyl-CoA synthetases of jasmonic acid precursors (At4g05160 and At5g63380, see Fig. 4) have activity with various fatty acids, and thus they are likely to have evolved from fatty acyl-CoA synthetase. Taken together with our findings of the properties of firefly luciferases, Schneider *et al.* [47] suggested high evolutionary plasticity of adenylate-forming enzymes with respect to their substrate utilization spectra (Fig. 5).

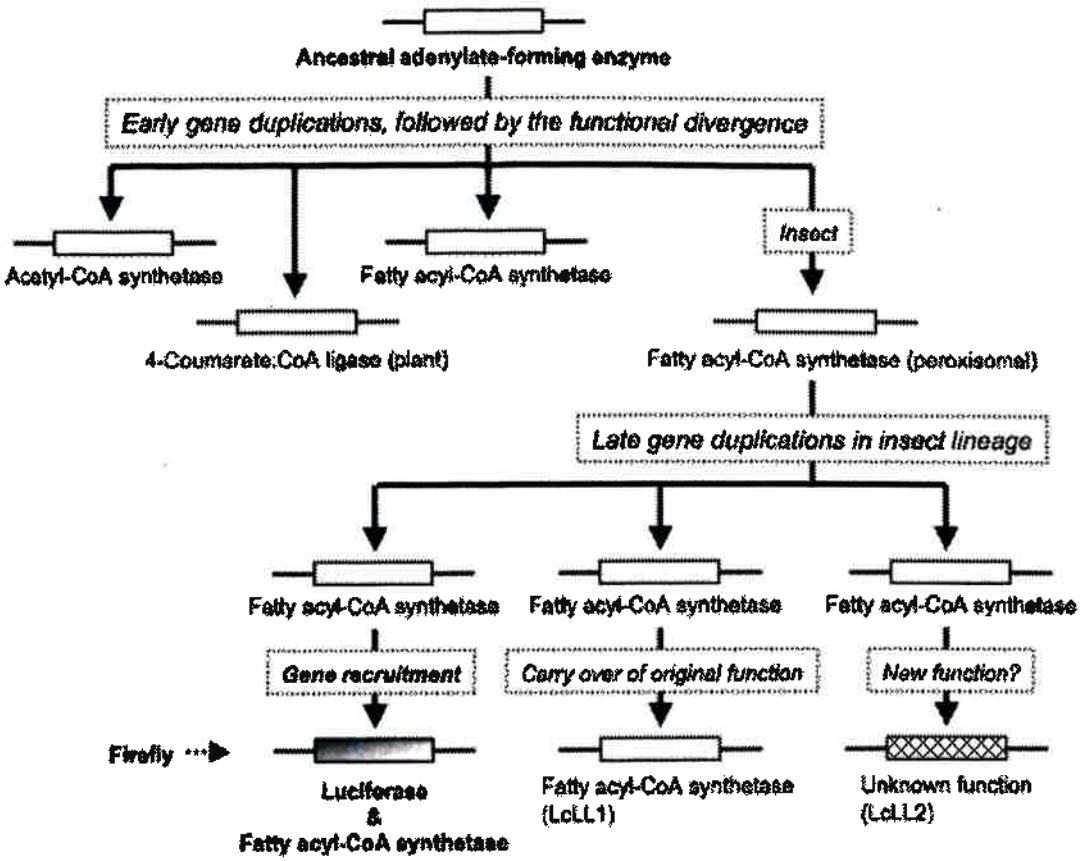


Figure 5. Schematic representation of evolutionary process of firefly luciferase.

5.4. Homologous genes of beetle luciferase in *Tenebrio molitor*

The mealworm beetle, *T. molitor* (Tenebrionidae, Coleoptera), is an outgroup taxon of all luminous beetles [48]. We isolated three homologous genes of beetle luciferase from *T. molitor* larvae and designated them as follows: *TmLL-1*, *TmLL-2*, and *TmLL-3* [49] (Fig. 4). TmLL-1 and TmLL-2, which are more closely related to beetle luciferases than is TmLL-3, possess a PTS1 signal at the C-terminus. Recombinant proteins of TmLL-1, TmLL-2, and TmLL-3 showed acyl-CoA synthetic activity with various fatty acids, but

not luciferase activity with beetle luciferin. These results suggest that non-luminous beetles utilize luciferase homologues for fatty acyl-CoA synthesis, a first step in fatty acid metabolism, in peroxisome, thus supporting the hypothesis that beetle luciferase arose from a peroxisomal fatty acyl-CoA synthetase (Fig. 5).

According to Viviani and Bechara [50], a mixture of a crude extract of larval *T. molitor* and beetle luciferin was found to emit ultra-weak luminescence, and the molecular size of the active component exceeded 100 kDa. Probably, the active protein is not related to beetle luciferase homologues, because beetle luciferase and their homologues, including TmLL-1 to -3, are approximately 60 kDa.

5.5. Paralogous genes of beetle luciferase in *L. cruciata*

Two paralogous genes of firefly luciferase, *LcLL1* and *LcLL2*, were identified in the Japanese firefly, *L. cruciata* [46]. The respective amino acid identities of LcLL1 and LcLL2 with beetle luciferase are approximately 40% and 45% (Fig. 4). Both proteins possess a PTS1 signal at the C-terminus. RT-PCR analysis showed that the transcript of *LcLL1* is abundant in the larva, but very low in the adult, and *LcLL2* was expressed in both the larva and adult. The recombinant protein of LcLL1 possessed fatty acyl-CoA synthetic activity, but not luminescence activity. The most suitable substrate was α-linolenic acid (C18:3) (Fig. 3). On the other hand, neither fatty acyl-CoA synthetic activity nor luminescence activity was detected in recombinant LcLL2. This result suggests that beetle luciferase evolved through gene duplications and subsequent divergence (Fig. 5). More recently, Day *et al.* [51] isolated a partial fragment of the paralogue of LcLL2 from the lampyrids, *Luciola italica* and *P. pyralis*, predicting that the genes of this clade underwent loss of bioluminescence activity.

5.6. Orthologous gene of beetle luciferase in a non-luminous elaterid

The non-luminous species, *Agrypnus binodulus,* is closely related to firebeetles belonging to the same subfamily (Pyrophorinae), but to a different tribe (Agrypnini) [39] (Fig. 2). A homologous gene of beetle luciferase was isolated from adult *A. binodulus* and its gene product, AbLL, was characterized [52]. The amino acid sequence identity between AbLL and elaterid luciferase is approximately 55%, which is higher than that between the luciferases of lampyrids and elaterids (Fig. 4). Recombinant AbLL has fatty acyl-CoA synthetase activity, but not luciferase activity. The substrate specificity of AbLL as a fatty acyl-CoA synthetase is selective for lauric acid

and α-linolenic acid (Fig. 3). This implies that elaterid luciferase evolved from a fatty acyl-CoA synthetase in the lineage of Elateridae.

6. Systematic distribution of beetle luciferin

The biosynthetic pathway of luciferin is an important issue that needs to be resolved in order to understand a complete picture of the evolution of bioluminescence [53], but the pathway remains obscure in the case of beetle luciferin [10,54]. Another remaining question regarding beetle luciferin biosynthesis concerns, when and where the pathway was acquired during the evolution of luminous beetles. Recently, we analyzed the luciferin contents in luminous beetles and non-luminous relatives by two different methods, i.e., by luciferin-luciferase reaction and HPLC using fluorescence detection [55]. The results showed that lampyrids and a luminous elaterid possess significant amounts of luciferin (2 pmol – 250 nmol/ insect). On the other hand, in non-luminous cantharoids (cantharids and lycids) and non-luminous elaterids (including the species of Pyrophorinae), no luciferin was detected. These data demonstrate that luminous beetles do possess luciferin, but that non-luminous relatives lack it. Our molecular phylogenetic analysis suggested that the ancestral state of the Elateridae was non-luminous [39] (Fig. 2). Therefore, it is expected that lampyrids and luminous elaterids independently developed the biosynthetic abilities of luciferin (Fig. 1). Alternatively, it may be assumed that the larvae of firebeetles, which are predacious [56], obtain luciferin by the ingestion of other luminous beetles such as Lampyridae and Phengodidae. However, this hypothesis will be rejected, because *Pyrophorus* specimens reared from egg to adult by feeding only mealworms were entirely luminous (Tama Zoological Gardens, Tokyo; personal communication).

7. Conclusion

Recent molecular phylogenetic data have suggested that the bioluminescence in click beetles has independently evolved in the lineage of Elateridae, despite a common mechanism of bioluminescence shared with Lampyridae and Phengodidae (Figs. 1 and 2). On the other hand, further studies will still be necessary to elucidate whether the bioluminescence exhibited in Lampyridae and Phengodidae has indeed a common origin or not (Fig. 1).

Gene duplication and gene recruitment (or gene sharing, in which a single protein shares both original and derived functions simultaneously) are major factors for enzyme diversification [57,58]. Our results, which revealed that *T. molitor* and *L. cruciata* possess at least 3 luciferase homologues, indicate that several gene duplications have occurred during the evolution of beetle

luciferase in the lineage of Coleoptera (Fig. 5). The functional duality of firefly luciferase, i.e., luminescence and fatty acyl-CoA synthesis, suggests that gene recruitment was involved in the evolution of firefly luciferase (Fig. 5).

François Jacob [59] proposed the idea that evolution is tinkering (or "bricolage"), not engineering. In this chapter, we suggested that beetle luciferases evolved from an adenylate-forming enzyme, most likely a peroxisomal fatty acyl-CoA synthetase (Fig. 5). This conclusion is consistent with the following: (i) the photocyte granule in the firefly lantern appears to be a peroxisome [60]; (ii) luciferase is localized in the photocyte granule [61]; and (iii) the peroxisome is the principal organelle in which fatty acids are degraded [62]. Accordingly, evolution of beetle luciferase does not rule out Jacob's "bricolage" concept, in which novel genes or proteins are readily made from pre-existing components.

Dubuisson *et al.* [63] characterized the antioxidant properties of beetle luciferin, and suggested that a primary function of beetle luciferin was to serve as an antioxidant. If the luminosity of firefly and firebeetles was inherited from a common ancestor, it appears, according to the "oxygen detoxification hypothesis," more plausible that extant species of non-luminous elaterids and cantharoids also possesses luciferin, as non-luminous beetles are generally diurnal and are thus exposed to relatively more reactive oxygen species under sunlight. However, our analysis demonstrated the presence of beetle luciferin in species of lampyrids and luminous elaterids, but not in their non-luminous relatives such as cantharids, lycids, and non-luminous elaterids. Thus, the "oxygen detoxification hypothesis" is unlikely to account for the origin of beetle luciferin, as Day *et al.* [10] have already noted, and luciferin biosynthesis was instead probably acquired independently in at least two different lineages, i.e., cantharoids and luminous elaterids (Fig. 1).

Taken together, the results that have accumulated to date suggest that cantharoids and a group of elaterids (firebeetles in Pyrophorini) independently developed identical bioluminescence systems (Fig. 1), which utilize the same luciferin compound and the luciferase sharing a common ancestry (Fig. 4); this is thus an example of "parallelism" or "parallel evolution" [64]. If this parallelism hypothesis is correct, our next questions then revolves around why fatty acyl-CoA synthetase was independently selected as a luciferase ancestor, and how different lineages could have acquired the same luciferin biosynthetic abilities.

References

1. Candèze, E. 1877, Les Aventures d'un Grillon (The adventure of Grillo), Magasin d'Éducation et de Récréation, Hetzel, Pari.
2. Crowson, R.A. 1972, Rev. Univ. Madrid, 21, 35.

3. Harvey, E.N. 1956, Q. Rev. Biol., 31, 270.
4. McElroy, W.D., and Seliger, H.H. 1962, Horizons in Biochemistry, Academic Press, New York.
5. Hastings, J.W. 1983, J. Mol. Evol., 19, 309.
6. Rees, J-F., de Wergifosse, B., Noiset, O., Dubuisson, M., Janssens, B., and Thompson, E.M. 1998, J. Exp. Biol., 201, 1211.
7. Lloyd, J.E. 1978, Bioluminescence in Action, Academic Press, New York.
8. Wood, K.V. 1995, Photochem. Photobiol., 62, 662.
9. Viviani, V.R. 2002, Cell. Mol. Life Sci., 59, 1833.
10. Day, J.C., Tisi, L.C., and Bailey, M.J. 2004, Luminescence, 19, 8.
11. Lawrence, J.F. 1982, Synopsis and Classification on Living Organisms, McGraw-Hill, New York.
12. Branham, M.A., and Wenzel, J.W. 2003, Cladistics, 19, 1.
13. Bertkau, P. 1891, Deutsche Ent. Zeitschr., 1, 37.
14. Burakowski, B. 1988, Bull. Entomol. Pologne, 58, 571.
15. Stibick, J.N.L. 1979, Pacific Insects, 20, 145.
16. Costa, C. 1984, Revta bras. Ent., 28, 397.
17. Lawrence, J.F., and Newton Jr., A.F. 1995, Biology, Phylogeny, and Classification of Coleoptera, Mus. Inst. Zool. PAN, Warszawa.
18. Bocakova, M., Bocak, L., Hunt, T., Teräväinen, M., and Vogler, A.P. 2007, Cladistics, 23, 477.
19. Costa, C., Vanin, S.A., and Neto, P.C. 1986, Revta bras. Ent., 30, 101.
20. Grimaldi, D., and Engel, M.S. 2005, Evolution of the Insects, Cambridge University Press, Cambridge.
21. Shimomura, O. 2006, Bioluminescence, World Scientific Publishing, Singapore.
22. Rhodes, W.C., and McElroy, W.D. 1958, J. Biol. Chem., 233, 1528.
23. Ribeiro, C., and Esteves da Silva, J.C.G. 2008, Photochem. Photobiol. Sci., 7, 1085.
24. McElroy, W.D., DeLuca, M., and Travis, J. 1967, Science, 157, 150.
25. de Wet, J.R., Wood, K.V., DeLuca, M., Helinski, D.R., and Subramani, S. 1987, Mol. Cell. Biol., 7, 725.
26. Schröder, J. 1989, Nuc. Acids Res., 17, 460.
27. Suzuki, H., Kawarabayasi, Y., Kondo, J., Abe, T., Nishikawa, K., Kimura, S., Hashimoto, T., and Yamamoto, T. 1990, J. Biol. Chem., 265. 8681.
28. Branham, M.A., and Wenzel, J.W. 2001, Florida Entomol., 84, 565.
29. Suzuki, H. 1997, Tokyo Metro. Univ. Bull. Nat. Hist., 3, 1.
30. Li, X., Yang, S., Xie, M., and Liang, X. 2006, Prog. Nat. Sci., 16, 817.
31. Stanger-Hall, K.F., Lloyd, J.E., and Hillis, D.M. 2007, Mol. Phylogenet. Evol., 45, 33.
32. Sagegami-Oba, R., Takahashi, N., and Oba, Y. 2007, Gene, 400, 104.
33. Beutel, R.G. 1995, J. Zoo. Syst. Evol. Res., 33, 145.
34. Pototskaja, V.A. 1983, Rev. Entomol. URSS, 62, 549.
35. Arnoldi, F.G.C., Ogoh, K., Ohmiya, Y., and Viviani, V.R. 2007, Gene, 405, 1.
36. Kobayashi, Y., Suzuki, H., and Ohba, N. 2002, J. Morphol., 253, 1.
37. Hyslop, J.A. 1917, Ann. Entomol. Soc. Am., 10, 241.

38. Ôhira, H. 1962, Morphological and taxonomic study on the larvae of Elateridae in Japan (Coleoptera), Aichi Gakugei Univ, Okazaki.
39. Sagegami-Oba, R., Oba, Y., and Ôhira, H. 2007, Mol. Phylogenet. Evol., 42, 410.
40. Toh, H. 1991, Protein Seq. Data Anal., 4, 111.
41. Oba, Y., Ojika, M., and Inouye, S. 2003, FEBS Lett., 540, 251.
42. Oba, Y., Sato, M., Ojika, M., and Inouye, S. 2005, Biosci. Biotechnol. Biochem., 69, 819.
43. Gould, S.J., Keller, G-A., Hosken, N., Wilkinson, J., and Subramani, S. 1989, J. Cell. Biol., 108, 1657.
44. Oba, Y., Ojika, M., and Inouye, S. 2004, Gene, 329, 137.
45. Oba, Y., Tanaka, K., and Inouye, S. 2006, Biosci. Biotechnol. Biochem., 70, 2739.
46. Oba, Y., Sato, M., Ohta, Y., and Inouye, S. 2006, Gene, 368, 53.
47. Schneider, K., Kienow, L., Schmelzer, E., Colby, T., Bartsch, M., Miersch, O., Wasternack, C., Kombrink, E., and Stuible, H-P. 2005, J. Biol. Chem., 280, 13962.
48. Hunt, T., Bergsten, J., Levkanicova, Z., Papadopoulou, A., St. John, O., Wild, R., Hammond, P.M., Ahrens, D., Balke, M., Caterino, M.S., Gómez-Zurita, J., Ribera, I., Barraclough, T.G., Bocakova, M., Bocak, L., and Vogler, A.P. 2007, Science, 318, 1913.
49. Oba, Y., Sato, M., and Inouye, S. 2006, Insect Mol. Biol., 15, 293.
50. Viviani, V.R., and Bechara, E.J.H. 1996, Photochem. Photobiol., 63, 713.
51. Day, J.C., Goodall, T.I., and Bailey, M.J. 2009, Mol. Phylogenet. Evol., 50, 93.
52. Oba, Y., Iida, K., Ojika, M., and Inouye, S. 2008, Gene, 407, 169.
53. Oba, Y., Kato, S., Ojika, M., and Inouye, S. 2002, Tetrahedron Lett., 43, 2389.
54. Niwa, K., Nakamura, M., and Ohmiya, Y. 2006, FEBS Lett., 580, 5283.
55. Oba, Y., Shintani, T., Nakamura, T., Ojika, M., and Inouye, S. 2008, Biosci. Biotechnol. Biochem., 72, 1384.
56. Costa, C. 1975, Arq. Zool. S. Paulo, 26, 49.
57. Ohno, S. 1970, Evolution by Gene Duplication, Springer-Verlag, New York.
58. Todd, A.E., Orengo, C.A., and Thornton, J.M. 1999, Curr. Opin. Chem. Biol., 3, 548.
59. Jacob, F. 1977, Science, 196, 1161.
60. Hanna, C.H., Hopkins, T.A., and Buck, J. 1976, J. Ultrastruct. Res., 52, 150.
61. Hopkins, T.A., and Hanna, C.H. 1972, The Physiologist, 15, 171.
62. Lodish, H.F., Matsudaira, P., Darnell, J.E., Baltimore, D., and Berk, A. 1995, Molecular Cell Biology, 3 rd Ed., W.H. Freeman Company, New York.
63. Dubuisson, M., Marchand, C., and Rees, J-F. 2004, Luminescence, 19, 339.
64. Gould, S.J. 2002, The Structure of Evolutionary Theory, Belknap Press, Cambridge.

Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 291-303
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

16 Rhythmic regulation of bioluminescence in glow-worms, *Arachnocampa*

David J. Merritt[1] and Arthur K. Clarke[2]
[1]School of Biological Sciences, The University of Queensland, Brisbane Qld, 4072, Australia; [2]School of Zoology, University of Tasmania Hobart, Tas 7001, Australia

Abstract

The glow-worms of Australia and New Zealand are the carnivorous, larval stages of a fly that uses bioluminescence to attract prey to its sticky webs. Here we briefly consider the sensory stimuli that can affect the light output of glow-worms, namely vibration, sound and light. We then consider the circadian regulation of bioluminescence in the genus. It has recently been shown that light output of epigean (surface-dwelling) glow-worms comes under true circadian control. Additional experiments are described

Correspondence/Reprint request: Dr. David J. Merritt, School of Biological Sciences, The University of Queensland, Brisbane, Qld, 4072, Australia. E-mail: d.merritt@uq.edu.au

in which the response of glow-worms to artificial shortening and lengthening of the dark period is assessed. Last, we present preliminary observations that the bioluminescence of cave-dwelling glow-worms is rhythmic and discuss the possible entraining agents.

Introduction

The glow-worms of Australia and New Zealand are the larval stages of a fly, genus *Arachnocampa*, Family Keroplatidae. Eight species are found in Australia and one in New Zealand (see chapter by C. Baker, this book). All produce light from a posteriorly-located light organ composed of the terminal cells of the malpighian tubules, closely apposed to a tracheal reflector [1,2]. The function of the bioluminescence is to attract prey [3]. This function is unusual in the insect world. A larval click-beetle found in termite mounds in Brazil bioluminesces for several hours through the night to attract prey [4] and some fireflies emulate the flashes of other species to attract them as prey items [5]. Most commonly, insect bioluminescence is used to find mates or has an aposematic function [6,7]. Unique to glow-worms, light output is bright and persistent throughout the night and has been reported to be continuous in caves [8,9]. Bioluminescence in glow-worms is produced by a reaction involving the protein luciferin, the enzyme luciferase and ATP in the presence of O_2 [10]. Given the ATP requirement and the many mitochondria in the light-producing cells [2], it is expected that bioluminescence would impose an appreciable energetic cost. The only study of the energetics of insect bioluminescence has shown that flashing in fireflies elevates the resting metabolic rate by one-third: a relatively minor increase when compared with flight and walking [11]. However glow-worms produce light continuously through the night rather than in spaced flashes, and in caves they are reported to glow continuously, so the daily energy expenditure could be significant. It is likely that the ability to down-regulate light output would be an energy-saving mechanism, for example, at times of nutritional stress or during daylight hours.

Regulation of the level of bioluminescence appears to be under neural control. Exposure to anaesthetics such as ether and CO_2 causes an acute release of light, even if larvae are not glowing at the time of exposure [12], suggesting that the default state is for the light organ to be active, and light output is inhibited by detection of daylight and severe disturbances. Ligation of larvae behind the head or removal of the head causes larvae to glow dimly for a long period, suggesting the inhibitory signal comes from the brain (8). The innervation of the light organ and presence of specific neurotransmitters have not been investigated beyond an electron-microscopy study that revealed that the bioluminescent cells are innervated by nerve terminals

containing both neurotransmitter and neurosecretory vesicles [2]. It has been suggested that modulation of oxygen supply to the light-organ is the means by which bioluminescence intensity is regulated [8,13]. The firefly flash appears to be activated firstly through octopaminergic innervation of the light organ that then activates nitric oxide and gates O_2 through an unknown pathway [14]. A role for these neurotransmitters in glow-worm bioluminescence has not been specifically investigated.

A number of sensory inputs have been shown to result in the up- or down-regulation of light output. Vibration induced by tapping their chamber caused larvae to rapidly increase their light output, followed by a return to undisturbed levels over about 10 min [12]. Controlled exposure to vibration of different frequencies and high-intensity sounds showed that *A. flava* are far more responsive to vibrations than to sounds, increasing their light output to a peak within 20-30 seconds and moving within their snare when vibration is detected (Popple and Merritt, in preparation). The fact that vibrational disturbances cause increased output when larvae are already glowing, suggests that energy or metabolites are held in reserve during the normal glowing cycle.

In New Zealand, tourism takes place in several caves that feature large glow-worm populations. Tours range from walk-through tours, to boat trips, to adventure-style blackwater rafting in which clients float through glow-worm chambers on inflated inner tubes [15]. Tour operators have noticed that sudden, sharp sounds can cause apparent increases in glow-worm light intensity and this has been confirmed photographically (Merritt, unpublished data).

The function of the light increase remains obscure: perhaps vibration causes resting insects, potential prey items, to take flight. A simultaneous increase in glow-worms' light output might increase the chances a prey item, disturbed by the same stimulus, will be attracted and caught. Other possibilities are that it is an aposematic response [6] or that it is simply a startle reaction. It is possible that the glow-worm detects vibrations through chordotonal organs, commonly found in the bodies of larval Diptera [16] and observed in the terminal segments of glow-worm larvae [8,17].

Exposure to light causes actively bioluminescing glow-worms to douse their light within a few minutes [8] and recover within 30 to 60 minutes]. Two-thirds of the individuals in a colony of *A. luminosa* larva extinguished their lights after exposure to 800 lux white light for 5 minutes [19]. The threshold ambient light intensity that causes dousing has not been carefully defined, however Richards [9] cited 0.5 foot candles (c. 5 lux) as the maximum light level that will not cause glow-worms to douse while Stringer [20] estimated the threshold at 1 to 0.1 foot candles based on observations of light

intensity at onset and termination of glowing at dawn and dusk in a forest setting. Light output is maintained under full moonlight [9,19].

Meyer-Rochow and Waldvogel [19] carried out preliminary observations of the dousing response by shining filtered light of various intensities and colours on glow-worms, showing that they perceive the light and respond by switch-off for an hour, and appear to be more sensitive to yellow, blue and green than to red. They showed morphological differences within the ommatidia of light- vs dark-adapted eyes, in accord with the light response seen in other insects. Electrophysiological investigations showed the adult eye is sensitive to UV and has two sensitivity peaks, one in the green and another in the blue-green spectrum, matching the spectrum of the adult and larval bioluminescence pattern.

Rhythmic behaviour of glow-worms

The foregoing results show that glow-worms have an ability to regulate the intensity of their light output in response to sensory input. In addition, the fact that rainforest glow-worms turn on every evening after dusk and switch off at dawn raised the prospect that the phenomenon could come under circadian regulation, just as the time of flashing in fireflies is regulated [21]. If that turned out to be the case, then it would be of interest to see whether cave glow-worms are also rhythmic. The ability to down-regulate bioluminescence in response to light and up-regulate in response to dark was taken as an indication that bioluminescence does not come under the control of a biological clock, but this was never tested [8]. Similarly, Stringer [20] mentioned that *A. luminosa* does not appear to show such a rhythm. The first signs of a circadian rhythmicity came from a study showing that *A. flava* placed in a dark incubator maintained rhythmicity for 7-8 days and subsequently began to glow more or less continuously [18]. Video-recording of *A. luminosa* in caves has revealed that they do not glow continuously, with indications of diurnal rhythmicity (Broadley and Stringer, this volume,22].

Merritt and Aotani [23] tested for a true circadian rhythmicity under tightly-controlled conditions by taking glow-worms (*A. flava*) from their typical rainforest setting, placing them in constant darkness (DD) and temperature, and recording the light output of individuals at 10-minute intervals using digital image capture over a period of several weeks. Individual glow-worms initially maintain on-off cycles for several days, however they free-run with a period of greater than 24 h. Each individual has a characteristic free-running period that remains relatively constant over several weeks. Progressively, each glow-worm extends the proportion of time spent glowing per subjective day, eventually resulting in them bioluminescing for most of the time, but the intensity fluctuates rhythmically, following the

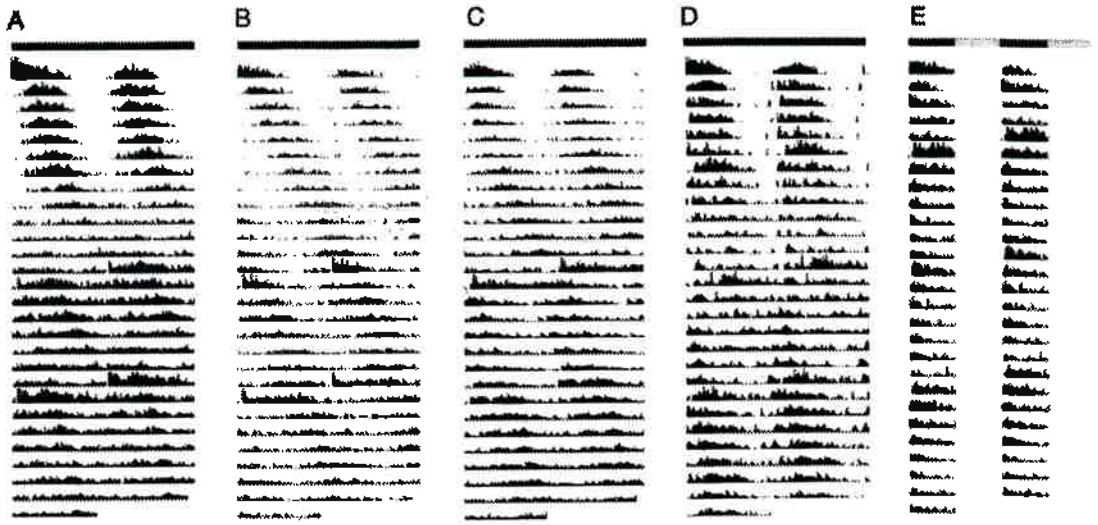


Figure 1. Circadian double-plots of the bioluminescence output of *Arachnocampa flava* larvae in the laboratory. **A-D**. Four individuals free-running under constant darkness for 28 days. **E.** A single individual exposed to 12:12 LD cycles.

free-running period. In Figure 1 the light output of 4 individuals is plotted over 28 days in constant darkness. The circadian double-plots show day 1 and 2 on the first line, day 2 and 3 on the second line, etc. This plot method graphically shows the departure of the free-running period from 24 h. A comparison of 4 individuals in DD (Fig. 1 A-D), with an individual that remains exposed to a 12:12 light:dark cycle (Fig. 1E), shows that the peaks align vertically when the rhythm is being re-entrained daily by exposure to light, but in the absence of re-entrainment the peaks drift to the right (a free-running period of greater than 24 h).

Light masks and entrains the rhythm of bioluminescence

The next question was what is the main entraining agent, also known as the zeitgeber? An entraining agent is usually predictable, regular and frequent: It resets the rhythm, usually daily. Frequent re-entrainment is why the innate period of an organism's internal clock is not rigidly required to be 24 h: an approximation is sufficient because external phenomena such as dawn and dusk periodically reset the rhythm each day. As expected, the rhythm of rainforest glow-worms is re-entrained by light. A cohort of rainforest glow-worms was exposed to constant darkness, allowing them to free-run for several days, and then exposed to artificial day:night cycles that were either synchronised with the original external period or maximally out of phase with it. In both treatments, the glow-worms immediately re-entrained to

the new light regime, and kept the new cycle upon return to constant darkness, indicating that light exposure resets the rhythm.

Light very commonly acts as an entraining agent and a masking agent in nocturnal animals [24]. As detailed above, light inhibits glow-worms' bioluminescence, and at the same time it resets the internal clock: light is a masking agent as well as a zeitgeber, i.e. it suppresses any sign of a bioluminescence rhythm because it causes the animal to switch off its light. Some masking agents don't necessarily affect the underlying rhythms, while others both mask and entrain: light has this latter function in glow-worms.

A notable change that occurs over time when rainforest glow-worms are placed in constant darkness is that the glow-worms gradually extend their glowing period so that they are glowing continuously. The absence of light-based masking has removed the switch-off period seen in a natural light regime and reveals the underlying bioluminescence rhythm, which appears to be approximately sinusoidal and is maintained for many weeks in constant darkness.

The response to exposure to skeleton photoperiods (1 h light pulses spaced 12 h apart) also showed the combined masking and entraining effects of light. If light were to act solely as a masking agent then a switch-off in response to the light pulses would be expected with resumption of preceding levels of light output between pulses. In fact the glow-worms interpreted the pulses as either dawn or dusk, depending on which was anticipated. They reduced their light output in the period of darkness that their clock determined as day and increased their light output in the period of darkness they subjectively determined as night.

One further attribute noted when glow-worms were placed in constant darkness is that, over time, the maximum daily intensity decreased and the time spent glowing increased. This is most likely due to energy conservation: the maximum light output decreases as the proportion of time spent glowing per day increases. The resulting curve of light output shows progressive damping. The combined effect of damping and the progressive desynchronisation of individuals under DD is that the plot of the combined light output becomes flat over time (Fig. 2). Incidentally, the peaks occurring at days 7 and 14 of Figure 2 are a response to the weekly feeding regime in which chilled *Drosophila* adults were flicked into the snare of each larva. The prolonged increase in light output is seen consistently. It is likely due to the stimulatory effect of food in the webs, the vibration induced by the prey (see above) and perhaps elevated bioluminescence while actually feeding.

The effect of changing the proportion of the 24 h day that glow-worms were exposed to darkness was determined, using the same methods as previously [23]. The duration of the dark period was (a) increased symmetrically, making artificial

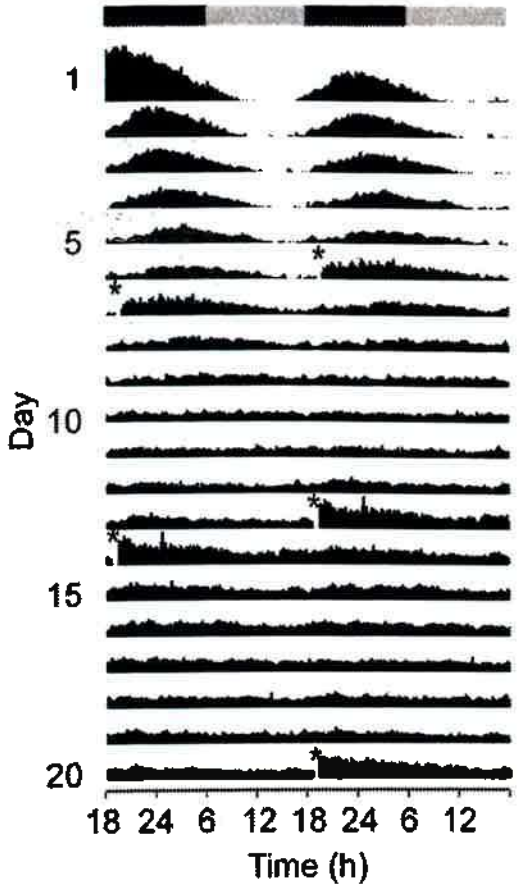


Figure 2. Circadian double-plot of the bioluminescence output of 15 *Arachnocampa flava* larvae under constant darkness for 20 days. Asterisks represent the time of feeding (see text). Data replotted from Merritt and Aotani [23].

dusk occur earlier each night and dawn later each morning, and (b) decreased symmetrically (Fig. 3A, B). Increasing the duration of the dark period caused the glow-worms to reach a lower maximum intensity each night and slowed the rate at which that intensity was reached (Fig. 3A). The latency between the switch-off of light and the beginning of glowing became longer as the length of the dark period increased (Fig. 3A). The reduced maximum light intensity in response to longer dark periods is similar to the response to the first 8 days constant darkness (Fig. 2). When larvae are exposed to progressively shorter nights, the maximum intensity of glowing does not change markedly compared to LD 12:12. It appears that the larvae do not show a rebound response to the delayed onset of darkness. The results of the treatments presented here and by Merritt and Aotani [23] are consistent with a circadian process of sensitization that increases during daylight when the bioluminescence is masked and decreases during active light output. Sensitization as an explanation for the onset of activity in nocturnal insects has been explored by Nielsen [25]. At the latitude the experimental animals

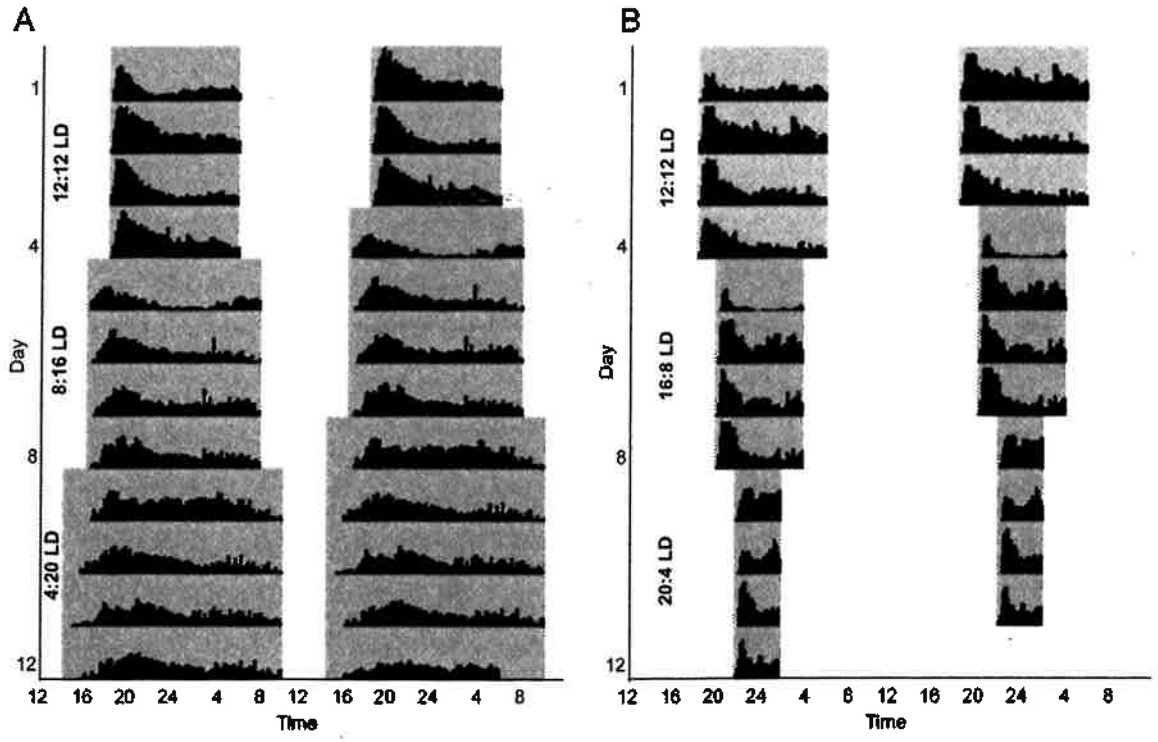


Figure 3. Circadian double-plots of the bioluminescence output of *Arachnocampa flava* larvae in the laboratory under altered LD conditions. **A.** The light output of ten individuals under extended darkness conditions. The hours of darkness were symmetrically increased to LD 8:16 on day 5 and to LD 4:20 on day 9. **B.** The light output of ten individuals under extended light conditions. The hours of darkness were symmetrically decreased to LD 16:8 on day 5 and to LD 20:4 on day 9.

were collected (-28° 12'), the extremes of the annual light:dark cycle are LD 14:10 in summer and LD 10:14 in winter, hence, with the exception of LD 12:12, the exposure conditions are well beyond the natural exposure regime.

Alternative entraining agents

In anticipation of investigating the rhythmicity of glow-worms in caves, alternatives to light as entraining agents were tested [23]. In general, temperature cycles are the most common entraining agent after light, and *A. flava* kept in constant darkness and exposed to temperature cycles were able to entrain to a 3°C temperature cycle. Exposure of to a square-wave daily thermal range of 3°C caused phase-locking of light output to the cryophase, the peak luminescence occurring around midnight. Exposure to triangular waves caused phase-locking to the middle of the falling phase, also corresponding to approximately midnight in a natural hypogean thermal cycle. These experiments showed that the phase of thermally-induced luminescence rhythms matches the phase of light-induced rhythms, in accord

with the thermophase/photophase linkage characteristic of nocturnal insects (21,26,25). Alternative entraining agents were tested, including feeding at the same time every day for several weeks, and daily disturbance for several weeks. Neither of these regular stimuli re-entrained the bioluminescence rhythm.

Potential bioluminescence rhythms in caves

In 1984, Erik Tetens Nielsen wrote; "*With very few exceptions, all free-living animals are exposed every single diel to the change from light to darkness; a change not only of enormous amplitude but also, and perhaps still more important, of inexorable regularity.*" [25].

Animals that themselves produce light would be expected to be tightly locked into this diel rhythmicity as their signals are effective only at night. However glow-worms provide us with an unusual case because members of several species can be found in caves where they are never exposed to light, as well as outside the cave where they encounter regular light cycles. These species of glow-worms have populations that accord with Nielsen's generalisation, i.e. they are exposed to a daily light cycle, as well as populations that comprise the few exceptions—they live in caves where they are never exposed to daylight (although they are exposed to each others' light). The question arises as to how cave populations respond to the lack of entrainment from external light. From the foregoing laboratory analyses of *A. flava's* rhythmicity, three possibilities arise: one is that glow-worms in caves would be arrhythmic because they have never been exposed to photic entrainment cues. Another is that the glow-worms in caves would be rhythmic but individuals in a colony would not be synchronised because they have different free-running periods, akin to placing rainforest glow-worms into artificial constant darkness (Fig. 2). A third is that they would be rhythmic, entrained to an as yet unknown stimulus.

Cave populations of *A. luminosa* in New Zealand give the impression that they glow continuously [8,9] however the only quantitative observations are two studies of *A. luminosa* in New Zealand, one by Ohba and Meyer-Rochow [22] and the other by Stringer and Broadley in this publication. Ohba and Meyer-Rochow [22] video-recorded cave populations during a two-hour block in the afternoon and another at night, the results indicating that the duration of prolonged glowing bouts is longer during the day and reductions in intensity or interruptions in light output were more likely to occur during the night, however phase and synchronisation of individuals was not investigated. Stringer and Broadley recorded the proportion of time 4 cave individuals spent glowing per hour over 24 h, finding evidence of a peak during the external day. Preliminary data based on time-lapse photography of

the light output of a colony of *A. tasmaniensis* larvae in a Tasmanian cave show that they maintain synchronised rhythmicity over several days (Merritt and Clarke, unpublished). A digital camera with external 12 volt power supply and a time-lapse controller was used to photograph a population of glow-worms in Mystery Creek Cave. The results showed that the number glowing and the total intensity of their light output varied through the day with peak of light output at about 13:00 h and a trough after midnight. The occurrence of the peak phase in the early afternoon was an unexpected finding, given the lack of light as an entraining agent and the very small temperature fluctuations experienced at the recording site (over 4 days, temperature varied between 5.4 and 6.9°C, with a mean daily range of 0.6 ± 0.2°C (± S.D.)).

At the time of writing, we don't know whether the phenomenon persists through the seasons, and indeed whether it is found in all colonies of all *Arachnocampa* species in all caves. It is possible that the phase of the bioluminescence rhythm varies throughout the year, just as it is possible that the synchronised rhythm was a temporary phenomenon, or is restricted to one particular cave. Currently, plans are underway to obtain a set of seasonal data to assess rhythmicity of several species across a wide geographic spread of caves. Bearing in mind these caveats about the universality of the phenomenon, we can discuss the functional significance of rhythmic bioluminescence in caves and explore the possible zeitgebers.

Rhythms in cave animals

Cave organisms are frequently seen as test cases for the universality of rhythmic phenomena [27]. One argument is that the invariability of the cave environment obviates the need for rhythmicity and that all cave-adapted animals are likely to be arrhythmic, having lost any rhythmicity present in ancestral cave colonisers. Another argument is that these ancestors had a long history of rhythmic control of internal physiological processes that has become such an integral part of their genetic and physiological makeup that the rhythmic phenomena are retained as internal coordinators, perhaps dissociated from recognisable behaviours such as locomotion, or— if linked—said behaviour would free-run due to the absence of entraining cues. The evidence from cave-dwelling animals is piecemeal and does not give firm, consistent answers [27]. Troglophilic arthropods characteristically show an ability to entrain to LD cycles in the laboratory [28], however, rarely has the phase of locomotory rhythms been established within a cave. We could find only one case where cave-restricted troglophiles or troglobites have been shown to possess synchronised, entrained circadian rhythms without leaving the dark zone. Small, guano-associated flies in a Jamaican cave show peak

flight activity during the external photophase [29]. The phase and synchronisation were attributed to physical disturbance due to the flight activity of bats during the external scotophase. In another case, the locomotory rhythms of troglobitic cave millipedes were monitored *in situ*. Approximately half of a sample showed circadian rhythmicity, but notably, the individuals were free-running [30].

We consider that the most likely functional explanation for the presence of a rhythm in *A. tasmaniensis* is that the light output peaks at the time of maximum prey availability, and that the cyclical presence of prey is the most likely zeitgeber. A number of studies have shown that glow-worms in caves primarily feed upon adult flies, especially chironomids and Ephemeroptera, that have been washed as larvae into caves from the external environment [9,31]. Diptera and Ephemeroptera very commonly show circadian rhythms of emergence [32] and require only brief exposure to light early in development to entrain the emergence rhythm [33], consequently a circadian rhythm of prey availability within the cave could entrain the rhythms of the glow-worms. On the other hand, prey-related stimuli would be intermittent: *A. luminosa* in rainforest are reported to catch prey items every 2.9 - 5.0 days and in caves every 19.2 - 36.5 days [3]. The sporadic nature would presumably reduce their effectiveness as zeitgebers. An experiment testing the phase-resetting ability of 14 days of daily feeding on *A. flava* [23] in constant darkness showed that the phase was not obviously altered, suggesting that the timing of prey availability is not a strong entraining agent, but it must be borne in mind that the specimens were not reared in caves—they were reared under natural light:dark cycles—and the rearing conditions could influence the sensitivity to alternative zeitgebers. Another possibility is that synchronisation is achieved through a response to light emitted by others—opposite to the masking effect of solar light. Perhaps a combination of periodicity in prey availability, increased light output in response to prey detection, and a sensitive, positive response to light emitted by adjacent glow-worms could eventually synchronise the population. It has been shown that even weak linkage between inherently oscillating phenomena will eventually result in synchronisation [34].

In summary, epigean glow-worms show true circadian regulation of their bioluminescence. While circadian control in cave glow-worms has not been formally demonstrated, it is considered likely. More observations are required to answer the questions posed here about the universality of rhythmic bioluminescence in cave glow-worms. Tests for correlation with the time of flight of prey items are needed. The possibility that light output in cave glow-worms is synchronised, rhythmic and out of phase with epigean glow-worms indicates that these fascinating animals are very well adapted to quite

different habitats. The duality of their preferred habitat and the phase of their light rhythm in each habitat makes them a potentially useful subject for examination of environmentally-based phase-switching of circadian rhythms.

Acknowledgements

We thank Peter Chandler at Spellbound Tours for his demonstration of glow-worm response to sound, and Cathie Plowman for her help in cave-recording in Tasmania. We thank the students in Dave Merritt's lab who have researched glow-worm bioluminescence, including Claire Baker, Julie-Anne Popple, Evan Davies and especially Sakiko Aotani for her work on rhythmicity.

References

1. Wheeler, W.M., and Williams, F.X. 1915, Psyche, 22, 36-43.
2. Green, L. 1979, Tissue Cell, 11, 457-465.
3. Broadley, R.A., and Stringer, I.A.N. 2001, Invert. Biol., 120, 170-177.
4. Viviani, V.R., Silva, A.C.R., Perez, G.L.O., Santelli, R.V., Bechara, E.J.H. and Reinach, F.C. 1999, Photochem. Photobiol., 70, 254-260.
5. Lloyd, J.E. 1966, Univ. Mich. Mus. Zool. Misc. Publ.,130, 1-96.
6. De Cock, R. and Matthysen, E. 1999, Evol. Ecol., 13, 619-639.
7. Buck, J. and Case, J. 2002, J. Insect Behav., 15, 51-68.
8. Gatenby, J.B. 1959, Trans. Roy. Soc. N.Z., 87, 291-314.
9. Richards, A.M. 1960, Trans. Roy. Soc. N.Z., 88, 559-574
10. Viviani, V.R., Hastings, J.W. and Wilson, T. 2002, Photochem. Photobiol., 75, 22-27.
11. Woods, W.A., Hendrickson, H., Mason, J. and Lewis, S.M. 2007, Am. Nat., 170, 702-708.
12. Lee, J. 1976, Photochem. Photobiol., 24, 279-285.
13. Meyer-Rochow, V.B. 2007, Luminescence, 22, 251-265.
14. Ghiradella, H. and Schmidt, J.T. 2004, Integr. Comp. Biol., 44, 203-212.
15. Pavlovich, K., 2003, Tourism Manage., 24, 203-216.
16. Wong, D.C. and Merritt, D.J. 2002, Int. J. Dev. Biol., 46, 475-481.
17. Ganguly, G. 1960, J. R. Micr. Soc., 79, 137-154.
18. Baker, C.H. 2002, A biological basis for management of glow-worm populations of ecotourism significance, Wildlife tourism research report (Cooperative Research Centre for Sustainable Tourism), No. 21.
19. Meyer-Rochow, V.B., and Waldvogel, H. 1979, J. Insect Physiol., 25, 601-613.
20. Stringer, I.A.N. 1967, Tane, 13, 107-117.
21. Dreisig, H. 1976, Physiol. Ent., 1, 123-129.
22. Ohba, N., and Meyer-Rochow, V.B. 2005, Luminescent behaviour in the New Zealand Glowworm, *Arachnocampa luminosa* (Insecta; Diptera; Mycetophilidae). In Bioluminescence & Chemiluminescence, A. Tsuji, M. Matsumoto, M. Maedo, L.J. Kricka, and P.E. Stanley, (Eds.), World Scientific Publishing Co. Pte. Ltd, Singapore, 23-26.

23. Merritt, D.J., and Aotani, S. 2008, J. Biol. Rhythms, 23, 319-329.
24. Mrosovsky, N. 1999, Chronobiol. Int., 16, 415-429.
25. Nielsen, E.T. 1984, Behaviour, 89, 147-173.
26. Dreisig, H. 1978, Behav. Ecol. Sociobiol., 3, 1-18.
27. Sharma, V.K. 2003, Chronobiol. Int., 20, 901-919.
28. Lamprecht, G. and Weber, F. 1992, Spontaneous locomotion behaviour in cavernicolous animals: The regression of the endogenous circadian system. In The Natural History of Biospeleology, A.I. Camacho, (Ed.), Monografias del Museo Nacional de Ciencias Naturales, Madrid, 225-262.
29. Stringer, I.A.N. and Meyer-Rochow, V.B. 1997, Invert. Biol. 116, 348-354.
30. Koilraj, A.J., Sharma, V.K., Marimuthu, G. and Chandrashekaran, M.K. 2000, Chronobiol. Int. 17, 757-765.
31. Pugsley, C.W. 1984, J. Roy. Soc. N.Z., 14, 387-407.
32. Pinder, A. M., Trayler, K.M., Mercer, J.W., Arena, J. and Davis, J.A. 1993, Aust. J. Entomol., 32, 129-135.
33. Beck, S.D. 1980, Insect Photoperiodism, Academic Press, New York.
34. Strogatz, S. 2003, Sync: The Emerging Science of Spontaneous Order, Hyperion, New York.

Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 305-324
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

17

Australian glow-worms (Diptera: Keroplatidae: *Arachnocampa*): An overview of their distribution, taxonomy and phylogenetic relationships

Claire Baker
Research work was conducted at: The School of Integrative Biology
The University of Queensland, Mansfield Place, Brisbane QLD 4072, Australia

Abstract

The genus Arachnocampa is an unusual and economically important insect taxon. They are unusual in that the larvae use bioluminescence to attract their prey, thereby creating attractive displays. This in turn is utilised by tour operators in Australia and

Correspondence/Reprint request: Dr. Claire Baker, Research work was conducted at: The School of Integrative Biology, The University of Queensland, Mansfield Place, Brisbane QLD 4072, Australia
E-mail: bugsyclaire@yahoo.com.au

New Zealand, who have made a viable and unique industry from this natural asset. Despite these attributes, limited studies have focussed on Australian Arachnocampa *and many species have important conservation issues surrounding their long-term sustainability. This chapter identifies the distribution of Australian glow-worms and discusses their phylogenetic relationships, as they are currently understood, with references to sustainable management of each species.*

Taxonomic history of *Arachnocampa*

Arachnocampa (Edwards) currently comprises nine described species, eight of which are endemic to Australia [1,2] (Figure 1). Five new species have recently been described following detailed analysis of mitochondrial DNA, mating trials, morphological identification and descriptions [2-4]. The ninth species, *A. luminosa*, is endemic to New Zealand [5].

Arachnocampa taxonomy has been continually reviewed since the first specimen was described in the literature. In 1886, Meyrick gave mention of a luminous insect from New Zealand that he considered a coleopterous larva possibly from the Staphylinidae [6]. In a footnote the journal editors requested further verification of this identification and a follow-up article identified the New Zealand larvae as dipteran Mycetophilidae based on the appearance of the larval snare and the larva's ability to travel along the snare and retreat into crevices [7]. This was reinforced the following year with a classification based on the larval head and mouth structures [8]. Skuse described the New Zealand species as *Bolitophila luminosa* [5]. The name persisted until Edwards included the species in his new Mycetophilidae genus, *Arachnocampa*, based on the morphology and characteristics of the larvae [9].

The first description of an Australian glow-worm was that of a specimen taken from the Ida Bay Caves, Tasmania [10]. The description of the Tasmanian endemic species, *A. tasmaniensis*, was based on the adult form, with a brief mention of larval biology. Confusion was created when several authors reverted to *Bolitophila* [11-15]. However, Harrison [1, 16] reaffirmed the name as *A. luminosa* from the Mycetophilidae and placed it in subfamily Ceroplatinae (= Keroplatinae), based primarily on larval morphological characteristics. Harrison identified and described other documented Australian *Arachnocampa* colonies as two new species [1]. The two species, *A. flava* from southeast Queensland, and *A. richardsae*, from New South Wales, were placed in a new subgenus, *Campara*, based on wing venation [1]. Matile further reviewed the taxonomy of the Mycetophiloidea and *Arachnocampa* and a number of other bioluminescent genera were placed in the family Keroplatidae [17].

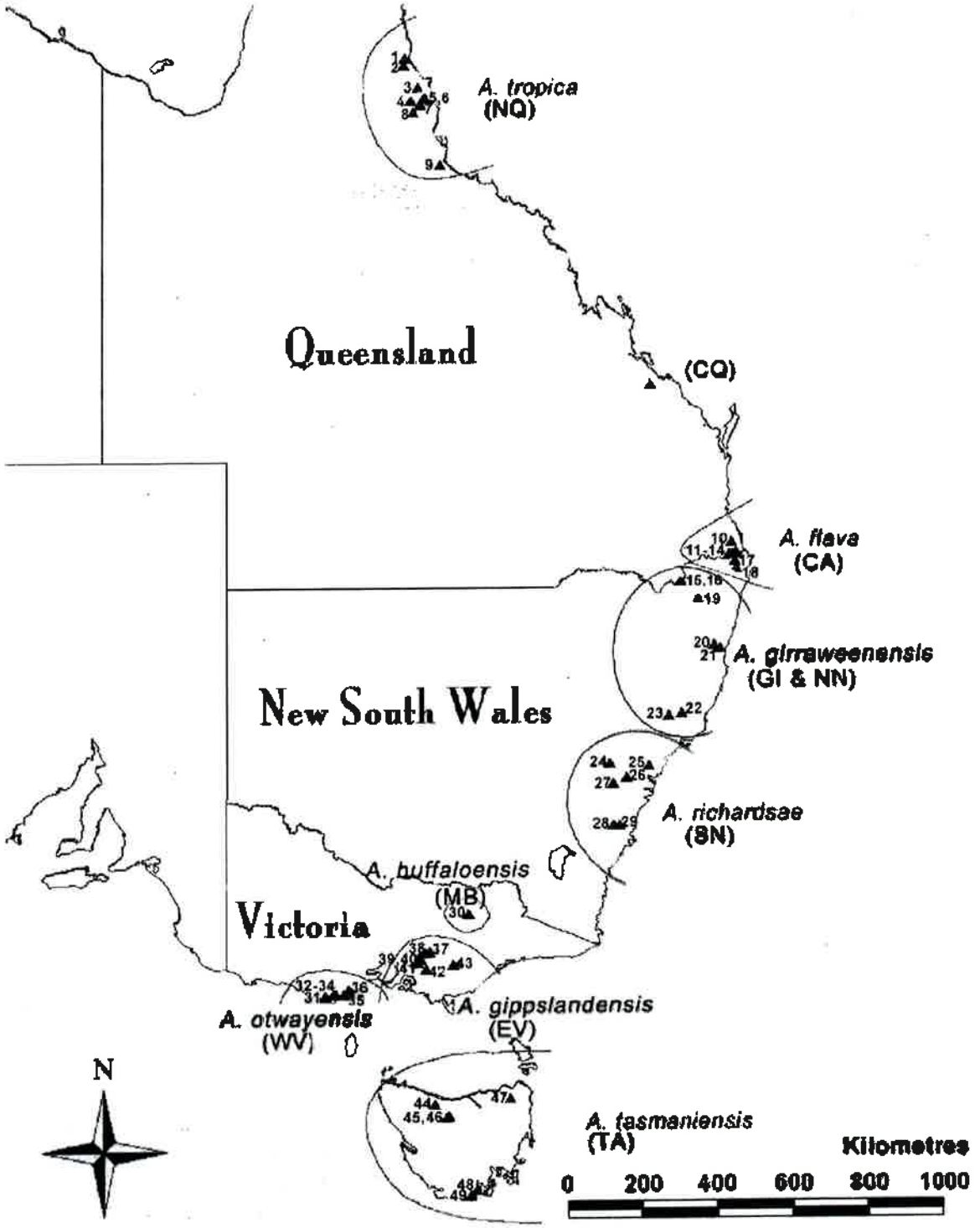


Figure 1. Map of the Australian distribution of *Arachnocampa* glow-worms (East coast of Australia). The known limits for species distributions are indicated by lines. Letters in parenthesis indicate the regional code for each species. CQ is a rainforest site within Kroombit National Park and this population is tentatively included in CA species group. Each species is limited to wet caves or rainforest habitat within these regions. The small triangles indicate specific colony locations (numbers refer to Table 1).

Table 1. Locality names, codes and habitat types for numbers on Figure 1. These sites were visited and *Arachnocampa* were sampled during a taxonomic study from 2000-2004 [3]. The codes refer to the phylogeny presented in Figure 4.

Site name	#	Code	Habitat	Site name	#	Code	Habitat
Mossman Gorge	1	NQ1	Rainforest	Waterfall Springs Conservation park	25	SN2	Rainforest
Mt Lewis Road	2	NQ2	Rainforest	Upper Kurrajong	26	SN	Rainforest
Lamb Range National Park	3	NQ3	Rainforest	Grand Canyon walk, Blue Mtns NP	27	SN4	Rainforest
Dinner Falls, Mt Hypipamee National Park	4	NQ4	Rainforest	Bundanoon	28	SN	Rainforest
Bartle Frere cave, Wooroonooran National Park	5	NQ5	Cave	"The Grotto" Fitzroy Falls National Park	29	SN3	Rainforest
Bartle Frere stream	6	NQ6	Rainforest	Underground River Cave, Mt Buffalo National Park	30	MB1	Cave
Mungalli falls	7	NQ7	Rainforest	Melba Gully State Park	31	WV1	Rainforest
Charmilllan walking trail, Tully Gorge State Park	8	NQ8	Rainforest	Beauchamp Falls, Otway National Park	32	WV	Rainforest
Birthday Creek Falls, Paluma National Park	9	NQ9	Rainforest	Hopetoun Falls, Otway National Park	33	WV	Rainforest
Kroombit Tops National Park	#	CQ	Rainforest	Beauty Spot Reserve	34	WV	Rainforest
Tamborine Mountain	10	CA	Rainforest	Grey River Picnic area, Angahook-Lorne SP	35	WV2	Rainforest
Natural Bridge	11	CA1	Rainforest	She-oak Picnic area, Angahook-Lorne State Park	36	WV	Rainforest
Springbrook Plateau	12	CA	Rainforest	Upper Yarra Valley mine tunnel	37	EV1	Mine shaft
Springbrook National Park	13	CA	Rainforest	O'Shannassy Weir	38	EV2	Manmade weir tunnel
Lamington National Park	14	CA	Rainforest	Britannia Creek Cave, State Forest	39	EV3	Cave
South Bald Rock, Girraween National Park	15	GI1	Cave	Shining star gold mine, Warburton	40	EV4	Mine shaft
Ramsey Creek Cave, Girraween National Park	16	GI2	Cave	Shiprock Falls, Kilnkurth State Forest	41	EV5	Cave
Korrumbyn Creek Picnic area, Mt Warning National Park	17	CA2	Rainforest	Labertouche Cave	42	EV6	Cave

Table 1. Continued

Protestors Falls, Nightcap National Park	18	CA3	Rainforest	Walhalla Mine tunnel	43	EV7	Mine shaft
Washpool walk, Washpool National Park	19	NN1	Rainforest	Gunns Plains Caves	44	TA	Cave
Cleavers Bridge, New England National Park	20	NN2	Rainforest	Marakoopa Cave	45	TA1	Cave
Crystal Shower Falls, Dorrigo National Park	21	NN3	Rainforest	Sassafras Cave	46	TA2	Cave
Gloucester River Camping area, Barrington Tops National Park	22	NN	Rainforest	Derby Mine tunnel	47	TA3	Mine shaft
Gloucester Cave	23	NN	Cave	Bates Creek, Dover,	48	TA4	Rainforest
Newnes Railway tunnel	24	SN1	Railway tunnel	Entrance (Mystery Creek) Cave, Ida Bay	49	TA5	Cave

The five new Australian species are: *A. tropica* from tropical north Queensland; *A. girraweenensis* from two isolated caves on the QLD/NSW border; *A. buffaloensis* from a sub-alpine cave in Victoria; *A. gippslandensis* from the Gippsland region of south eastern Victoria; *A. otwayensis* from the small fragmented patches of rainforest on Victoria's south western edge [4]. *A. otwayensis* had previously been tentatively identified as *A. richardsae* [18]. One new subgenus, *Lucifera*, was described due to differences in adult wing venation, campaniform sensilla placement and specific differences in pupal suspension. *A. tasmaniensis* and *A. buffaloensis* belong to the newly erected subgenus [4]. The Keroplatidae now comprises more than 800 species within 80 genera worldwide [19].

Habitat requirements of *Arachnocampa*

Arachnocampa glow-worms are endemic to Australasia where they are physically limited to moist caves and rainforest habitat in Australia and New Zealand [1,2,9,17]. The susceptibility of *Arachnocampa* to desiccation restricts them exclusively to areas of high humidity or direct moisture contact within their habitat (>94%) [20]. Colonies of *Arachnocampa* larvae have been reported in a diversity of humid micro-habitats, including stream banks, waterfall edges, mines, limestone and granite boulder caves, overhangs, mossy outcrops, railway tunnels and sea caves [1,4,6,10,18,20-27]. Within their microhabitat, larvae typically inhabit small crevices in rock or earth into which they can retreat during dry periods or when disturbed [7,20, 22,23, 28,29]. Overhangs are necessary for the larval snares to be hung as the

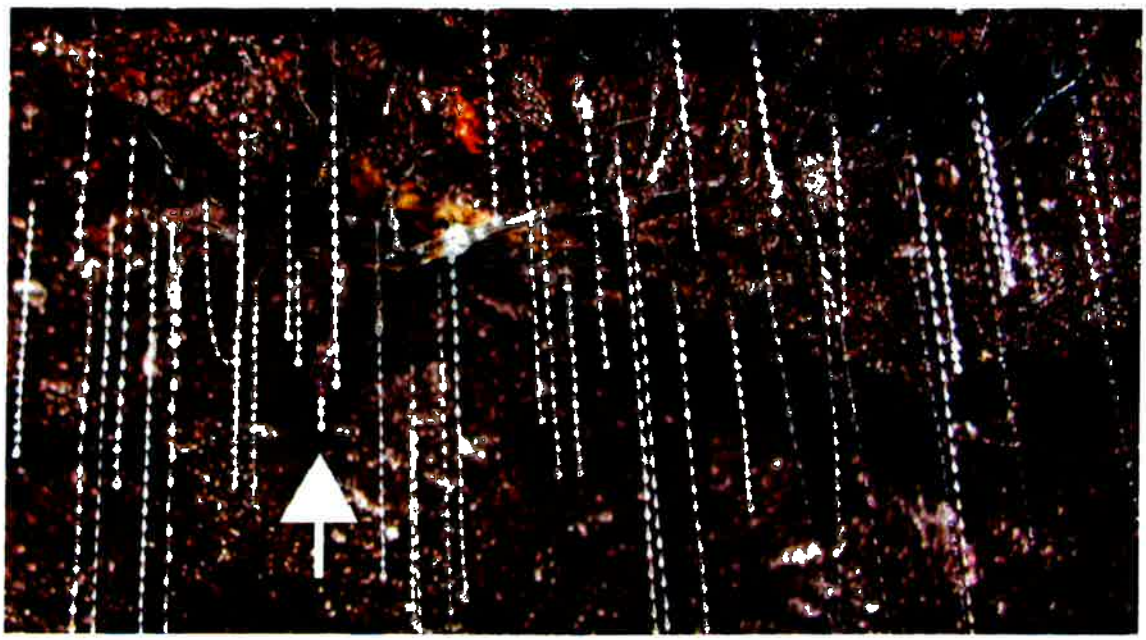


Figure 2. *Arachnocampa* larval snare. Each glow-worm constructs a web of silken threads and sticky mucous droplets to capture insects drawn to the bioluminescence. The glow-worm pictured above is in the midst of making a new fishing line for the snare (arrow).

Arachnocampa glow-worm larvae hang vertical 'fishing lines' attached to the substrate or from within the mucous tube on which they spend much of their time (Figure 2). It has been demonstrated that if habitat conditions (i.e. micro-climate, including crevices) are suitable for larval survival, *A. luminosa* and *A. flava* can remain in a dormant state and persist during periods of low food abundance [30-32]. Restricted to this specific, often fragmented and isolated habitat, over time *Arachnocampa* populations have become entirely allopatric, being physically unable to disperse across the large geographic barriers separating suitable habitat.

Arachnocampa dispersal

Adult *Arachnocampa*, particularly the emergent, gravid females, are poor fliers [20,33] (Figure 3). Laboratory observations of the flying ability of gravid adult female *Arachnocampa* suggest they have a short flight range due to their relatively high egg load [20,33]. By contrast, *Arachnocampa* males have greater flight range dispersal abilities, presumably due to the lack of egg production. Observations in the field and laboratory studies indicate females mate with awaiting males upon emergence from the pupal case and generally oviposit in the immediate vicinity (Richards 1960, pers. obs). As adult females have a shortened life stage (2 days) in comparison to males (4 – 6 days),

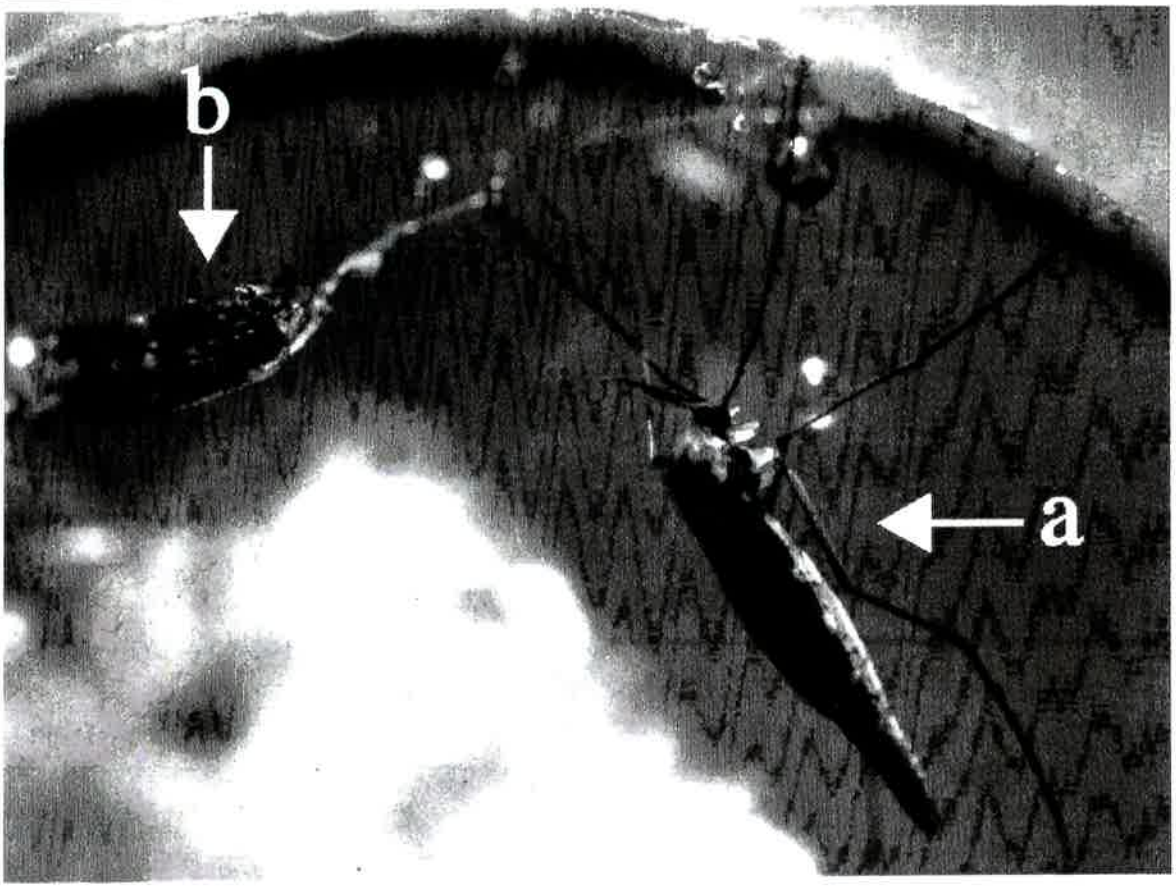


Figure 3. A gravid adult female *A. flava* (a) after emerging from her pupal case (b). The females usually mate with an awaiting male or remain near their pupal case until a male arrives. It is thought that pheromones play a major role in mate attraction in *Arachnocampa*. Photo courtesy of Anthony O'Toole, University of Queensland.

this strategy is thought to improve reproductive output in a species where females have restricted dispersal abilities and adult life spans are relatively short [33].

As the dispersal strategies of *Arachnocampa* are not yet well understood, physiological and morphological similarities between adult *Arachnocampa* and species of Culicidae suggest that extrapolations from mosquitoes may prove informative. An Australian-based study on the medically significant species, *Aedes notoscriptus* (Skuse) (Diptera: Culicidae), showed a flight range of between 105 and 238 metres per day [34], while *Aedes aegypti* (Linnaeus) has been shown to fly between 100 and 500 metres per day [35,36]. As with *Arachnocampa*, flight range in the Culicidae is thought to be largely dependant on habitat suitability and availability of harbourage sites [34]. Another factor affecting the dispersal of adult *Arachnocampa* is their inability to feed [16,20] and therefore their reliance on nutrient stores derived from the larval stage. Flying would greatly decrease these stored energy

resources and in this respect, it is possible that females have made a trade-off for egg production through decreased flight abilities.

Arachnocampa larvae have been shown to travel short distances in the wild, recolonising habitat denuded of larvae [37]. The potential for larval dispersal along watercourses associated with their habitats has yet to be determined. Cultured larvae have survived immersion in water for several days in the laboratory [3]. It is possible that larval dispersal along waterways, especially during flooding events, could be an important dispersal stage for this genus.

Australian *Arachnocampa* distribution and phylogeography

A recent morphological, taxonomic, behavioural and genetic study largely increased the known geographic range of *Arachnocampa* [2-4] (Figure 3). Preliminary dating places species divergences within the subgenus Campara between 3.17-6.21 mya [2]. These dates coincide with cooling and aridification during the Pleistocene/Pliocene, resulting in massive contractions of rainforest to coastal and montane regions of the eastern coastline of Australia. Movement between these species groups is improbable today due to the distance between the sites, unsuitable intervening habitat and the poor flying ability of adults. Larval dispersal, if occurring, would rely on suitability of the habitat for colonisation further downstream and the ability of the larvae to survive immersion for these periods.

Arachnocampa tropica – Far North Queensland, wet tropics region (NQ)

The wet tropics region of North Queensland is 7910 km^2, the largest continuous region of rainforest within Australia [38]. Classified as a tropical, seasonal, wet rainforest this region is World Heritage-listed and has extraordinary biodiversity significance, while also being recognised as crucial habitat for many rare, threatened and endemic flora and fauna [39,40]. The push for recognition of the conservation importance of this area has been aided by recent studies focused on speciation events in species reliant on this habitat for survival [e.g. frogs: 41-43; snails 44; birds: 45,46; mammals: 47; and other vertebrates: 48]. Evidence of severe rainforest contractions in the wet tropics during glacial periods in the Pleistocene is indicated from pollen cores [49-51] and fossil charcoal [52]. Investigations of low-vagility invertebrates have shown patterns of refugia and speciation [53,54]. Evidence of past glaciation periods, current rainforest distribution data and recent studies indicate effective dispersal barriers in the north Queensland Wet Tropics, including a faunal barrier over the relatively low-lying, dryer Black

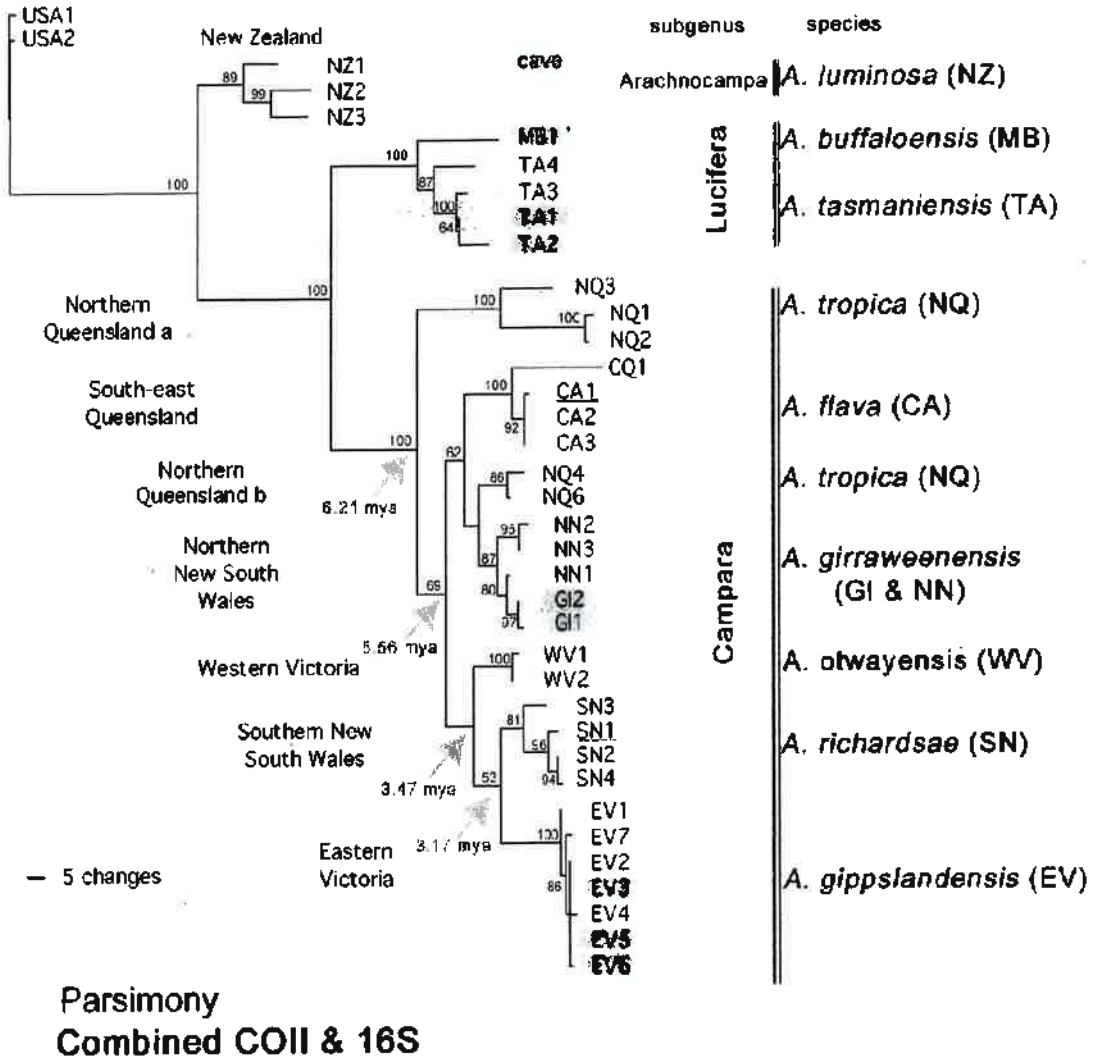


Figure 4. Phylogeny of Australian *Arachnocampa* using combined analysis of partial 16S and COII mitochondrial DNA modified from [2]. Codes for each locality are listed in Table 1.

Mountain corridor (BMC) [42,43,55] (Figure 1). Another barrier is evident for species groupings within vertebrate and invertebrates found on the Atherton tablelands [e.g. 56].

North Queensland *Arachnocampa* colonies currently represent one new species, *A. tropica*, based on their extreme geographic isolation from other Australian *Arachnocampa* populations, morphological examination and molecular studies of sections of mitochondrial DNA, 16S and COII [2-4]. It is hypothesised from the initial study that another species or subspecies separation may be necessary after further population level genetic studies [2] (Figure 4). Genetically there is strong evidence that either *Arachnocampa* made more than one movement into the tropics or barriers made it impossible

for gene flow between populations, eventually leading to allopatric speciation [2,3] (Figure 4). *A. tropica* are widespread in the wet tropical rainforest regions of north Queensland, however, the density and overall colony sizes within each documented NQ population is low in relation to other rainforest sites throughout Australia [3]. The low numbers of this species may be directly due to more sporadic rainfall patterns in north Queensland (ie most of the rainfall occurs between November and March) or a species of wasp, recently discovered parasitising *A. tropica* [3]. The wasp is yet to be formally described but has been tentatively identified as a new species of *Megastylus* from the Ichneumonidae (Chris Burwell pers. comm.). The gravid adult female wasp lays an egg on or into the larval stage of the glow-worm. Wasp development occurs within the body of the *Arachnocampa* host before the wasp finally encases the glow-worm larva in a silken cocoon within which the wasp pupates [3]. As yet, this wasp is endemic to far north Queensland. The movement of this wasp, via movement of infected *Arachnocampa*, to southern populations would be catastrophic to the glow-worm tourism in Australia.

A. flava – South east Queensland/northern New South Wales (CA)

Further south, the rainforests of southeast Queensland are classified as warm, sub-tropical, seasonal, moist rainforests [39]. The core region of rainforest here covers the Border Ranges, extending both north along the Main Range, and east along the McPherson Range [57]. One outlying population, located in central Queensland in Kroombit Tops National Park (CQ), forms a clade with *A. flava*, indicating a close genetic relationship (Figure 4). This population may prove to be a new species through further fine-scale genetic analysis and morphological identification. No adult specimens have yet been procured for morphological descriptions.

Arachnocampa colonies in southeast Queensland and northern New South Wales are located in rainforest hinterland areas and caves. Colonies are widespread through Springbrook National Park and Lamington National Park, but are only found in pockets of rainforest directly associated with water and sheltered from direct sunlight and drying conditions. The area has volcanic origins, situated on the northern side of the once active Mt Warning crater. Averaging 900 m elevation, Springbrook National Park receives an annual average of 3000 mm rainfall [58]. These rainforest habitats formed over remnant soil and rock from the Mt Warning volcano that erupted ~22 million years ago [59]. Mt Warning harbours rainforest *Arachnocampa* in sheltered areas around the mountain's base and lower slopes surrounding Korrumbyn Creek. Historically, the region between Tweed Heads and the

Richmond River, once known as the "Big Scrub" or "Red Scrub", covered >75,000 ha until logging reduced the area to 300 ha, 0.4% of its original size by 1900 [59]. This has drastically reduced the available habitat for many rainforest restricted flora and fauna including *Arachnocampa* and may have once provided an important habitat corridor for dispersal between colonies.

Tourism has also increased dramatically over the past 14 years to Natural Bridge in Lamington National Park, where glow-worms occur in high densities [3]. Natural Bridge is now subject to nightly ranger visits to assess permits (320 allocated permits for tour operators each night) and monitor visitor behaviour. A fenced boardwalk limits the accessible area for tourists and protects the glow-worms. A luminous sign at the entrance to the large overhang indicates impacts humans have on glow-worms in an effort to increase visitor awareness and decrease vandalism at the site. As our own human population expands, more controls will undoubtedly need to be put in place at other heavily visited sites to ensure the long-term sustainability of these fragile insects.

A. girraweenensis – Isolated caves and tentatively also in rainforest in northern New South Wales (GI)

The sister species *A. flava* and *A. girraweenensis* are located in close proximity to each other geographically, yet are morphologically and genetically distinct species [2,4]. Mating trials between *A. girraweenensis* and *A. flava* indicated no mate recognition between the species [3]. The two documented *Arachnocampa* populations in Girraween National Park are entirely restricted to the cave environment, as this is the only perpetually moist habitat found in this region. The annual rainfall for this area is 850 mm, falling predominantly between November and March, leaving much of the year dry [60]. This region is much dryer than the rainforest habitats in which other southeast Queensland populations are found. The park is predominantly made up of open eucalypt forests (dry sclerophyll) that are able to withstand the drier months. It is likely that the two cave *Arachnocampa* populations are relicts of historical rainforest retractions during the Pleistocene. Many rainforest-associated plants (e.g. Mutton wood, sweet pittosporum and possum wood) are found within sheltered and moist gullies in the same eroded granite formations that give rise to granite boulder infill caves [60]. The survival of these plants in these areas indicates the moist caves and gullies have provided important refugia through rainforest retractions [60].

The cave populations at Girraween National Park are geographically closest to, and genetically affiliated with rainforest populations documented in Washpool National Park, northern New South Wales [2]. More fine scale genetic sampling is needed to resolve this further. Fragmented rainforest and

mixed vegetation patches exist between the Mt Warning caldera populations and Washpool National Park. However, these are yet to be surveyed. It is doubtful that *Arachnocampa* adults or larvae are able to utilise these fragmented patches for dispersal between these two regions due to the large uninhabitable distances between them [3].

A. richardsae – New South Wales (SN)

Within New South Wales, each *Arachnocampa* colony is located in a pocket of fragmented rainforest along the eastern coastline. These rainforests are defined as cool sub-tropical, submontane cloudy and moist. Most rainforest pockets in New South Wales are situated in moist, fire-protected gullies at elevation above 650 m along the Great Dividing Range [57,59]. Many of these gullies have become fragmented over time and now represent important isolated habitat for rainforest flora and fauna including *A. richardsae*.

Other northern New South Wales populations are found in the World Heritage-listed New England National Park, Dorrigo National Park and Barrington Tops National Park. Together these areas encompass 156, 962 ha of pristine rainforest but are not linked by continuous rainforest corridors. *A. richardsae* larvae found in various locations in these rainforest areas were always associated with water and areas of high humidity. Barrington Tops National Park is located at the initial southern limit of the World Heritage listed rainforests of Eastern Australia [61], which was further extended in 2000 to include areas surrounding Sydney (the Greater Blue Mountains area) [62]. Further south, a population at the Waterfall Springs Conservation Park is located deep within farming land on one of the last remaining stands of rainforest in this area. The colony here relies on its water source being unpolluted from nearby intensive farming. The Newnes railway tunnel has very high numbers of *A. richardsae*. This man-made structure was built between April 1906 and November 1907 to facilitate the movement of shale oil [63]. The operation ceased in 1912 and the tunnel has since been colonised by *A. richardsae* from surrounding rainforest gullies. A small stream now flows through the tunnel, providing humid conditions and is thought to have increased prey availability for the colony. The colony has previously been targeted as a source for collection of large numbers of specimens for scientific research purposes and more recently for a display in a Japanese zoo [64-66]. It is likely that *A. richardsae* are widespread in the moist sandstone canyons of Wollemi National Park, based on information received from bush-walkers and climbers.

Bundanoon and Fitzroy Falls are the most southern colonies so far identified in New South Wales. A boardwalk has been constructed at the

Bundanoon colony (Glow-worm Glen) to keep the nightly flow of tourists on a designated track away from the delicate creek banks where the *A. richardsae* colony resides. "The Grotto" overhang at Fitzroy Falls harbours a large number of *A. richardsae* surrounding the waterfall. Other colonies are likely in rainforest within and surrounding the Australian Capital Territory and rainforest areas in southern New South Wales including Deua, and Budderoo National Park, although no documentation exists for glow-worm records in these areas and the sites were not surveyed in the most recent taxonomic project [3]. Genetic analysis has thus far provided evidence of a close affinity with the two Victorian species, *A. otwayensis* and *A. gippslandensis* [2]. Many of the New South Wales colonies have no rainforest corridors linking them to other colonies, and without available harbourage sites, movement between colonies is unlikely.

Laboratory mating trials between *A. richardsae* and *A. otwayensis*, *A. gippslandensis* and *A. girraweenensis* all indicated no mate recognition between these geographically isolated species [3]. Three attempts were made to cross *A. richardsae* with *A. flava*, however on two occasions there was no mate recognition, and the third cross resulted in a mating with non-viable eggs [3].

A. gippslandensis – Eastern Victoria (EV)

The Central Highlands region, north east of Melbourne, contains an isolated group of *A. gippslandensis* colonies. Found in a mixture of cave, mineshaft and rainforest habitats, the sites are all fringed with rainforest. Mineshafts were relatively recently built by humans and *A. gippslandensis* inhabitancy of these sites shows the species' ability to move short distances into new habitats from the surrounding rainforest. The small patches of cool temperate and montane wet rainforest found in Victoria are recognised as remnants of the Gondwana break up, that had not been destroyed by European settlement, logging or recent fires [67]. The area surrounding the Strzelecki Ranges in Victoria was substantially cleared during the 1900's and rainforest is now restricted to small pockets in Tarra-Bulga National Park [67]. No glow-worms were found in Tarra-Bulga National Park [3].

Further east of Melbourne, the small town of Walhalla hosts high numbers of *A. gippslandensis* in abandoned mine shafts. The town is surrounded by dense rainforests, however, the man-made tunnels have created ideal habitat for *A. gippslandensis* and may denote modern refugial niches in times of dry weather or may reflect the preferences of *Arachnocampa* for living in darkened environments.

Arachnocampa otwayensis West Victoria (WV)

As part of the southern clade, *A. otwayensis* is found in the rainforests of the Otway Ranges and Angahook Lorne State Forest where they receive a relatively evenly distributed annual rainfall of more than 1500 mm [68].

The European history of clearing and burning native vegetation in Australia may have impacted on current distributions of *A. otwayensis* as these are insects reliant on moisture for survival. Warm temperate forests are typically found in fire-protected moist gullies, however, exceptionally dry weather has led to some pockets being infiltrated by fire and consequently destroyed. This is particularly evident in the rainforest regions of the Otway Ranges and Central Highlands affected by the severe fires during Ash Wednesday, 1983. However, there is the possibility that areas have been recolonised by *A. otwayensis* larvae or adults after fire or that they survived in small, moist refugia. Mating trials between populations within this region indicate a need for further differentiation as no mate recognition occurred between geographically isolated populations in the Otway National Park and Angahook Lorne State Forest [3].

Arachnocampa tasmaniensis - Tasmania (TA)

Logging and burning of Tasmania's cool temperate rainforest has been extensive since European settlement [39,69]. The largest remaining area of rainforest in Tasmania is in the northwest where it is heavily interspersed with mixed forest [69]. Further south, mature rainforest is more fragmented, separated by scrub and sedgeland-heaths [69]. Rainforests in Tasmania often fringe the multitude of cave systems found throughout the state. These systems may represent important refugial habitats for *A. tasmaniensis* during changing climatic conditions.

The Tasmanian species, *A. tasmaniensis* is one of two species of Australian *Arachnocampa* protected under the National Parks and Wildlife Act 1970 (Statutory Rule No. 88 of 1976) and cannot be collected without permits from either private or conservation land areas [70] (All species are protected within Australian National Parks).

A. tasmaniensis occur in mineshafts, rainforest and karst regions. However, the common limestone cave habitats contain the densest and most abundant colonies. Currently all Tasmanian *Arachnocampa* are listed as the same species [4,10,25]. Geographically, these populations may represent separate species or sub-species as the rainforest found in northeast Tasmania surrounding Derby is completely isolated from the larger rainforest stands along the western side of the state [3,25]. In Australia, colony sizes and densities of *Arachnocampa* are greatest in Tasmanian populations, rivalling New Zealand *A. luminosa* colonies for their bioluminescent displays. Stream-

fed limestone caves have been shown to provide highly suitable habitat for larvae due to the high levels of seepage within these systems and the large numbers of prey found associated with the streams [30,71]. A thorough review and investigation of Tasmanian cave systems, cave fauna, and relevant management issues is provided by Eberhard [72,73].

Sister species, *A. tasmaniensis* and *A. buffaloensis* are the only two species in the subgenus *Lucifera*. Morphological and genetic data support this designation [2].

A. buffaloensis - Mt Buffalo, Victoria (BUFF)

Arachnocampa buffaloensis is a clear geographically, morphologically and genetically distinct population [2-4]. The population is listed as a threatened species due to its isolation to one sub-alpine cave at 1300 m altitude, with no present rainforest contact zone. This colony is entirely restricted to the cave in winter months, but *A. buffaloensis* have been sighted living deep in the canyons surrounding the cave during warmer summer nights [74]. Cave access in late winter/early spring is impossible due to high water levels from melting snow. *A. buffaloensis* metabolism and thus their development is likely slowed in the cold winter months, enabling them to survive the presumably lower prey abundance. The numbers of *A. buffaloensis* are said to increase dramatically during spring after water levels within the cave subside [75]. The inclusion of *A. tasmaniensis* and *A. buffaloensis* in one subgenus, *Lucifera*, raises some interesting evolutionary questions. The geographic separation of these two species could have occurred as cool-adapted ancestors retracted to refugial caves during arid conditions or by using land bridges may have provided dispersal corridors during the Pleistocene [4].

Caves as refugia for *Arachnocampa*

Many of the *Arachnocampa* species used for the genetic analysis were sampled directly from caves [2,3]. Caves are well documented in their suitability as *Arachnocampa* habitat [1,10,20,24,25,64,76] with *Arachnocampa* displaying significant adaptations for cave life. The evolution of *Arachnocampa* to a purely carnivorous diet from fungivorous ancestors raises many interesting evolutionary questions and is succinctly reviewed by Meyer-Rochow [77] Their use of bioluminescence for prey attraction is well adapted for a continuously dark cave environment [2]. *A. flava* individuals from rainforest colonies were shown to adapt to total darkness over an eight day period, through increased bioluminescence displays and finally by exhibiting continuous bioluminescence if fed every few days [3]. The larval

requirement of exceptionally high humidity [20,76] restricts them to limestone and granite boulder caves with perennial stream flow or seepage through the rock ceiling. The availability of suitable habitat for larval snare building (i.e. where snares can hang down without obstruction) is significantly increased in a cave environment, where undulating rock walls and the rock ceiling immediately increase the viability of a larger colony. Despite these characteristics, *Arachnocampa* are not classified as troglobites as they are not entirely restricted to the cave environment. Rather, classified as troglophiles, *Arachnocampa* are able to live and reproduce outside the cave environment if conditions are suitable for survival.

Karst regions in Australia vary greatly in size and location [78]. Many caves are located within or on the fringes of rainforest pockets but can represent a habitat with lower day-to-day humidity and temperature variation than epigean habitat due to thermal inertia [79]. Although caves show lower variable daily changes than surrounding epigean environments, seasonal fluctuations have been shown to have considerable influence on cave thermodynamics [80]. However this seasonal variation is expected to have less impact on *Arachnocampa* colonies than daily variation in epigean environments. Therefore, caves may harbour large colonies of *Arachnocampa* due to the availability of suitable habitat and food resources and may have represented important refugial habitats during past glaciation cycles. Management of cave populations requires detailed plans specific to each site. Severe weather events such as major flooding can significantly impact on cave colonies by flushing them out of the cave environment. If conditions are suitable for survival outside the cave system, then larvae and adults may be able to recolonise a cave system from outside or from small numbers that survived the flooding within the cave system. Human visitation to cave systems needs to be carefully managed, given the delicate nature of the cave environment and the organisms that rely on them for survival.

Conclusions

The primary driving force for speciation events within *Arachnocampa* is indicated as geographic isolation over time [3]. Many *Arachnocampa* sites have large geographical distances between their closest colonies. These allopatric populations appear to be completely isolated due to the adult's poor flight ability. Important conservation issues arising from geographic isolation between populations, indicates the need for more detailed analysis of individual regional groups of *Arachnocampa* species. Limited mobility and geographic separation are major factors that may limit gene flow between populations, and result in potential speciation events (i.e. within specific regional groups). There is potential for future work using bio-climatic

modelling [44]. These systems would, however, still be limited in that although they could pinpoint areas to assess as potential habitats, it would take further extensive fieldwork to determine if *Arachnocampa* larvae currently inhabit these areas. Many suitable *Arachnocampa* habitats were identified only to find through fieldwork that they contained no *Arachnocampa* colonies [3]. This is most likely due to past environmental and climatic changes eliminating colonies and their inability to recolonise over distances.

Many caves inhabited by *Arachnocampa* were found within or near rainforest pockets. However, three caves are identified as isolated refugia where rainforest contractions are proposed to have left relict colonies. A major threat to *Arachnocampa* in Australia is habitat loss. *Arachnocampa* in both cave and rainforest habitat are extremely specialised in their habitat requirements, making them highly susceptible to future extinction. Site densities and overall colony sizes are subject to considerable seasonal variation [37]. Therefore, to obtain informative monitoring data, counts of colonies need to be recorded consistently and correlated with climate data for each site.

It is hoped that taxonomic and distribution studies continue to expand our understanding of *Arachnocampa* speciation in Australia. As prime rainforest habitat continues to be degraded or destroyed, it is imperative to document new species and push for increased protection of Australia's natural ecosystems.

References

1. Harrison, R.A. 1966,Pacific Ins., 8, 877-883.
2. Baker, C.H., Graham, G. C., Scott, K. D., Cameron, S. L., Yeates, D. K., and Merritt, D. J. 2008, Molec. Phylogen. Evol., 48, 506-514.
3. Baker, C.H. 2004, PhD Thesis, University of Queensland, Brisbane.
4. Baker, C.H. 2009, Mem. Qld. Mus. (in press)
5. Skuse, F.A.A. 1890, Proc. Linn. Soc. N. S.W., 2nd ser., 5, 677-679.
6. Meyrick, E. 1886, Entomol. Mthly. Mag., 22, 266-267.
7. Osten-sacken, C.R. 1886, Entomol. Mthly. Mag., 22, 133.
8. Osten-sacken, C.R. 1887, Entomol. Mthly. Mag., 23, 230-231.
9. Edwards, F.W. 1924, Ann. Mag. Nat. Hist., ser. 9, 14, 175-179.
10. Ferguson, E. W. 1925, Proc.Linn. Soc. N.S.W., 50, 487-488.
11. Gatenby, J.B., and Cotton, S. 1950, Trans. Roy. Soc. N. Zld., 88, 149-156.
12. Gatenby, J.B. 1959, Trans. Roy. Soc. N. Zld., 87, 291-314.
13. Gatenby, J.B. 1960a, Trans. Roy. Soc. N. Zld., 88, 577-593.
14. Gatenby, J.B. 1960b, Tuatara, 8, 86-92.
15. Gatenby, J.B., and Ganguly, G. 1958, J. Roy. Microsc. Soc., 76, 146-148.
16. Harrison, R.A. 1961, Trans. Roy. Soc. N. Zld., 1, 197-201.
17. Matile, L. 1981, Annals Soc. Entomol. France, n. ser. 17, 99-123.

18. Crosby, D.F. 1978, Victor. Entomol., 8(6), 56-59.
19. Matile, L. 1960, Reserches sur la systematic et l'evolution des Keroplatidae (Diptera, Mycetophiloidea). Mem. Mus. Nat. D'Hist. Natur., Paris.
20. Richards, A.M. 1960, Trans. Roy. Soc. N. Zld., 88, 559-574.
21. McKeown, K.C. 1935, Insect Wonders of Australia. Angus and Robertson, Sydney.
22. Perkins, F.A. 1935, The Qld. Naturalist, 9, 84- 85.
23. Hudson, G.V. 1950, The natural history of the New Zealand glowworm. In: Fragments of New Zealand Entomology, 15-37, Ferguson and Osborne, Wellington.
24. Renwick, K 1954, N. Zld. Speleol. Bull., 1, 17.
25. Geode, J.B. 1967, Helictite, 71 - 86.
26. May, B.M. 1971, N. Zld. Speleol. Bull., 3, 169-172.
27. Anonymous 1994, Department of Conservation, Melba Gully State Park draft management plan, National Parks Service - South West Area, Melbourne, 21.
28. Hudson, G.V. 1886, Entomol. Mthly. Mag., 23, 99-100.
29. Stringer, I.A.N. 1967, Tane, 13, 107-117.
30. Broadley, R.A. 1998, MSc Thesis, Massey University, Palmerston-North.
31. Baker, C.H. 1999, BSc Hons. Thesis, Dept. Zool. and Entomol., University of Queensland, Brisbane.
32. Broadley, R.A., and Stringer, I.A.N. 2001, Invert. Biol., 120 170-177.
33. Baker, C. H., and Merritt, D.J. 2003, Austral. Entomol., 30, 45-55.
34. Watson, T.M., Saul, A., and Kay, B.H. 2000, J. Med. Entomol., 37, 380-384.
35. Trpis, M., and Hausermann, W. 1986, Am. J. of Trop. Med. Hyg., 35, 1263-1279.
36. Muir, L.E., and Kay, B.H. 1998, Am. J. Trop. Med. Hyg., 58, 277-282.
37. Baker, C.H. 2002, Wildlife Tourism Report Series 21, Sustainable Tourism CRC, Gold Coast, 76.
38. Bell, F.C., Winter, J.W., and. Pahl, L.I 1987, Proc. Roy. Soc. Qld., 98, 27-39.
39. Web L.J., and Tracey, J.G. 1981 Australian Rainforests: Pattern and Change. In Ecological Biolgeography of Australia, vol. 2, A. Keast (Ed.), Junk, The Hague.
40. Webb, L.J. 1987, Conservation Status of the rainforest of north Queensland. In *The rainforest legacy. Australian national rainforests study Vol. 1*, G. L. Werren and A. P. Kershaw (Eds.), Australian Government Publishing Service, Canberra.
41. Dennis, A., and Trenerry, M. 1987, The Rainforest Legacy: Australian National Rainforests Study. Canberra: Australian Government Publication Service, Canberra.
42. Schneider, C.J., Cunningham, M., and Moritz, C. 1998, Mol. Ecol., 7, 487-498.
43. Schneider, C., and Moritz, C. 1999, Proc. Roy. Soc. Lond. B, 266, 191-196.
44. Hugall, A., Moritz, C., Moussalli, A., and Stanisic, J. 2002, Proc. Natl. Acad. Sci. U.S.A., 99, 6112-6117.
45. Kikkawa, K. 1968, J. Animal Ecol., 37, 143-165.
46. Kikkawa, J., Monteith, G.B., and Ingram, G. 1981, Cape York Peninsula: Major region of formal interchange. In *Ecological biogeography of Australia*, J. Keast (Ed.), Junk, The Hague ,1697
47. Winter, J.W., Bell, F.C., Pahl, L.I. and Atherton, R.G. 1987, Distributions of selected north-eastern Australian rainforest mammals. In The Rainforest Legacy.

Australian National Rainforests Study Vol. 2, G. L. Werren and A. P. Kershaw (Eds.), Australian Government Publishing Service, Canberra.
48. Williams, S.E., and Pearson, R.G. 1997, Proc. Roy. Soc. Lond. B, 264, 709-716.
49. Kershaw, A.P. 1983, New Phytologist, 94, 669-682.
50. Kershaw, A.P. 1993, A quaternary vegetation history of northeastern Queensland from pollen analysis of ODP site 820. In *Proceedings of the Ocean Drilling Program, Scientific Results*, J.A. McKenzie, P.I. Davies, and A. Palmer-Julson (Eds.) 107-114.
51. Kershaw, A.P. 1994, Palaeogeography Palaeoclimatology Palaeoecology, 109, 399-412.
52. Hopkins, M.S., Ash, J., Graham, A.W., Head, J. and Hewett, R.K. 1993, J.Biogeography, 20, 357-372.
53. Yeates, D.K., Bouchard, P., and Monteith, G.B. 2002, Invert. Systematics, 16, 605-619.
54. Yeates, D.K. and Monteith, G.B. 2008, The invertebrate fauna of the Wet Tropics: diversity, endemism and relationships. In: Living in a Dynamic Tropical Forest Landscape, N. Stork, and S. Turton (Eds). Blackwell Publishing, Oxford 178.
55. Moritz, C., Patton, J.L., Schneider, C.J., and Smith, T.B. 2000, Ann. Rev. Ecol. Systematics, 31, 533-563
56. Bell, K.L., Yeates, D.K., Moritz, C., and Monteith, G.B. 2003 Mol. Phylogenetics Evol., 41, 741-753.
57. Adam, P. 1992, Australian Rainforests. Oxford Science Publications, Clarendon Press, Oxford.
58. Anonymous, 1998, Queensland National Parks and Wildlife Service, Visitor Information Springbrook National Park: Department of Environment and Heritage, Brisbane, 2.
59. Floyd, A.G. 1990, Australian Rainforests in New South Wales. Chipping Norton: Surrey Beatty and Sons Pty Ltd. in association with National Parks and Wildlife Service of New South Wales, Sydney.
60. Anonymous, 2000,Queensland National Parks and Wildlife Service, Park Guide: Girraween National Park, Toowoomba: Queensland Government, Queensland Parks and Wildlife Service, 8.
61. Anonymous 1998, New South Wales National Parks and Wildlife Service, Hurstville: New South Wales Government National Parks and Wildlife Service, 2.
62. Anonymous 2003, Department of Environment and Conservation, World Heritage-listed areas, Sydney.
63. Anonymous 1976, New South Wales National Parks and Wildlife Service. NSW National Parks and Wildlife service, Blackheath, 1-19.
64. Lee, J. 1976, Photochemistry and Photobiology, 24, 279-285.
65. Richards, A.M. 1977, The glowworm tunnel. In The Wolgan Valley: A study of land-use and conflicts with proposals for future managment, R. Breckwoldt (Ed.), The National Trust of Australia, Sydney, 39-43.
66. Takaie, H. 1997, Insectarium, 34, 336-342.
67. Busby, J. R., and Brown, M. J. 1994, Southern Rainforests. In Australian Vegetation second edition, R. H. Groves (Ed.), Cambridge University Press, Cambridge, 131-155.

68. Anonymous 1996, Parks Victoria, Mt Buffalo resource information, Parks Victoria, Melbourne, 4.
69. Jarman, S.J., Brown, M.J., and Kantvilas, G. 1984, Rainforest in Tasmania. Tasmania: National Parks and Wildlife Service, Hobart.
70. Skinner, A., 1977, Helictite, 15, 38.
71. Pugsley, C.W. 1980, PhD Thesis, Auckland University, Auckland, 107.
72. Eberhard, S.1999, Cave fauna management and monitoring at Ida Bay, Tasmania. Nature Conservation Report 99/1, Tasmania Parks and Wildlife Service, Hobart,Tasmania, 37.
73. Eberhard, S. 2000, Reconnaissance survey of cave fauna management issues in the Mole Creek Karst National Park, Tasmania. Nature Conservation Report 2000/1: Department of Primary Industry, Water and Environment, Hobart, 38.
74. Elery Hamilton-Smith, cave rescue team, personal communication.
75. Dave Chitty, Adventure Caving operator, personal communication.
76. Pugsley, C.W. 1984, J. Roy. Soc. N. Zld., 14, 387-407.
77. Meyer-Rochow, V.B. 2007, Luminescence, 22, 251-265.
78. Gillieson, D., and Spate, A. 1998, Karst and caves in Australia and New Guinea. In *Global Karst Correlation*, Y. Daoxian(Ed.), VSP Press, Utrecht, 299-254.
79. De Freitas, C.R., Littlejohn, R.N., Clarkson, T.S., and Kristament, I.S. 2, J. Climatol., 2, 383-397.
80. De Freitas, C.D., and Littlejohn, R.N. 1987, J. Climatol., 7, 553-569.

Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 325-355
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

18

Larval behaviour of the New Zealand glowworm, *Arachnocampa luminosa* (Diptera: Keroplatidae), in bush and caves

Adam Broadley[1] and Ian A.N. Stringer[2]
[1]Australian Quarantine & Inspection Service, P.O. Box 1006, Tullamarine Victoria 3043, Australia; [2]Department of Conservation, P.O. Box 10420 Wellington, New Zealand

Abstract

Larval behaviour of the New Zealand glowworm Arachnocampa luminosa was investigated by videotaping with infrared light, five larvae in bush (total 424 h), five in the Demonstration Chamber of Glowworm Cave (410 h), four in Reserve Cave (369 h) and two in Waitomo Waterfall Cave (48 h). Glowworms in bush usually began making and repairing snares

Correspondence/Reprint request: Adam Broadley, Operational Science Program, Australian Quarantine & Inspection Service, P.O. Box 1006, Tullamarine, Victoria 3043, Australia. E-mail: adam.broadley@aqis.gov.au

in late afternoon and started glowing up to 1.5 h later. When commencing, bioluminescence took <15 s to ca. 1 min to become fully bright and turning off involved a gradual fading over several minutes. In bush, larvae did not glow or glowed intermittently during cold nights (~ < 6° C) and on average, glowed 82.3% of the time at night and 0.13% during the daytime. During the night they spent 16.6% of the time making fishing lines, 0.79% capturing and feeding on prey, 0.18% fighting and 0.08% defaecating. During the day they spent 1.7% of the time making fishing lines and 0.05% defaecating. No feeding or fighting was observed. In Glowworm Cave and Reserve Cave they spent, respectively, 68.7% and 55.5% of the time glowing, 10.1% and 6.3% making fishing lines, 0.17% and 0.19% capturing prey and feeding, 0.04% and 0.01% defaecating, and 0.67% and 0.14% fighting. In Glowworm Cave, which is visited by tourists, they glowed brightly between 18:00 and 09:00 but often did not glow when artificial lighting was used. In Reserve Cave, which is rarely visited, they usually glowed between 8:00 and 12:00 and glowed less often when it was night-time outside the cave. Overall, 12 small winged insects were observed being caught and in bush at night spiders, harvestmen, mites, millipedes, slugs and other small unidentified invertebrates moved through glowworm snares but were not caught. Fighting larvae glowed brilliantly and tried to bite and dislodge each other. Cannibalism was not observed. Defaecating was observed 17 times. The larvae either voided defaecatory droplets from the snare or hung them on fishing lines and either left them hanging, or lengthened the fishing lines until the droplets contacted the substrate, or dropped the fishing lines. Faecal droplets contained a variety of insect sensillae, spines and cuticle and sometimes entire insects or larger parts. Small gastropod shells or parts of millipedes were occasionally present.

1. Introduction

Information on the behaviour of larvae of the glowworm *Arachnocampa luminosa* (Skuse) (Diptera: Keroplatidae), was previously obtained by observing them with visible light [1-5]. Both larvae and adults are sensitive to visible light [6, 7] and anecdotal reports suggest that visible light affects their behaviour [1-5, 8]. Here we describe the behaviour of undisturbed glowworm larvae recorded using time-lapse video and infrared light in both bush and cave habitats at Waitomo, New Zealand. We also examined if glowworms were affected by disturbance caused by repeated use of lights during the daytime in a tourist cave, Glowworm Cave. This was done by comparing the behaviour of these glowworms with those in nearby Reserve Cave where they were undisturbed.

Larvae of *A. luminosa* are widespread throughout much of New Zealand where they occur in caves or under banks alongside streams or roads wherever the humidity is high and they are sheltered from wind. Each larva lives separately under a horizontal gallery of silk and mucus attached by a silken web beneath overhanging substrate. The larvae are also carnivorous and catch prey using numerous vertical 'fishing lines' hung from the web. Evenly spaced sticky droplets on the fishing lines snare the prey [see reviews: 9-11]. Blue-green bioluminescence produced from a light organ formed from the distal ends of four Malpighian tubules at the posterior end of the body [12] attracts prey in the dark. Most prey are dipterans but other flying insects are also caught [13] together with crawling invertebrates that presumably fall from above. The prey is caught by the droplets on fishing lines and are hauled up and eaten. *Arachnocampa luminosa* larvae possess chewing mouthparts with which they break off pieces of prey and ingest them [9]. When food is scarce, a larva will consume its prey entirely, but if well fed it eats the body contents and discards the uneaten cuticle [1-3, 5, 8, 14, 15]. Some authors have also reported that the larvae are cannibalistic and that they will feed on other larvae, pupae, and adults that become caught in fishing lines [1, 3, 16, 17].

2. Methods

2.1. Infrared video-recording

Recordings were made between 21 February 1995 and 26 June 1998 with a time-lapse video cassette recorder (Panasonic AG6040E, 48 h onto each 3 h tape), an infra-red sensitive video camera (Panasonic WV-BP504E) and two custom-made LED light sources (~ 900 nm). All components were powered by batteries. All locations were in the Waitomo Caves area (38°16'S, 175°05'E) (Figure 1). Two sites in bush at Ruakuri Caves and Bush Scenic Reserve were used together with the ceiling of the Demonstration Chamber, Glowworm Cave (Figure 2), one site within Reserve Cave (Figure 3); and one in Waitomo Waterfall Cave (Figure 4). Each recording was usually made of an area encompassing two or three large larvae with the camera lens positioned close without touching any fishing lines. Behaviour was timed to the nearest second. The equipment was visited once a day to exchange batteries and replace video cassettes.

Temperature data recorded both at the jetty in Glowworm Cave and in bush at the cave entrance was obtained from the National Institute of Water and Atmospheric Research. Spot temperatures were also recorded at the Reserve Cave sites in 1995 and 1998 using a Sundo 5300 dial thermometer. Sunrise and sunset times were obtained from the Carter Observatory, Wellington.

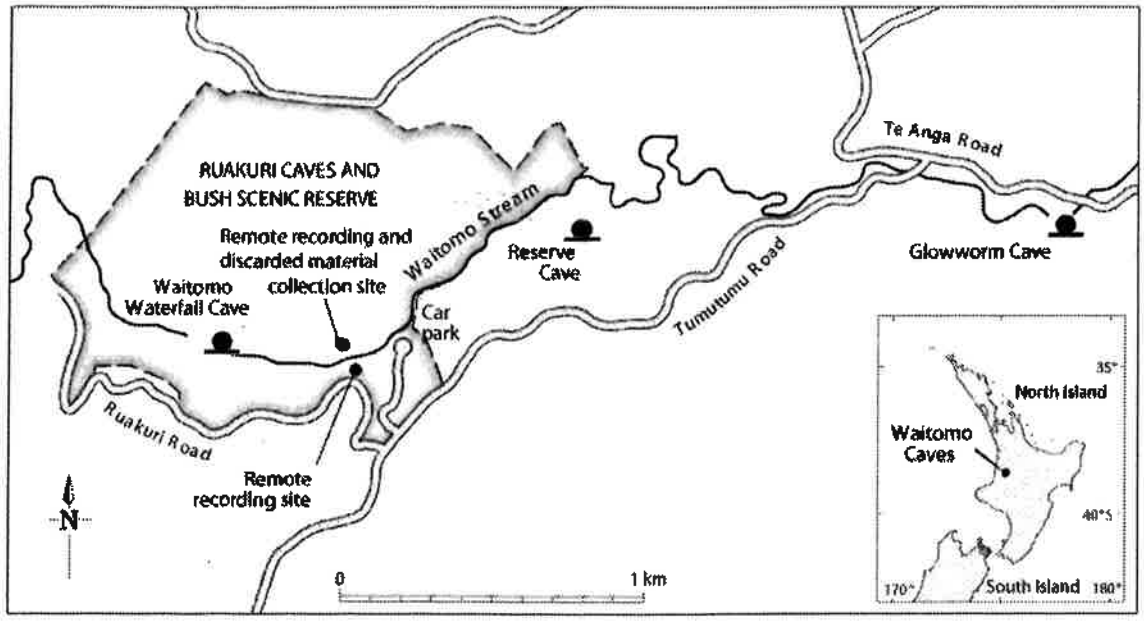


Figure 1. Map of the Waitomo area, showing locations of bush and cave sites where remote recordings were made of *Arachnocampa luminosa* larvae. The site in bush where material discarded by glowworms was collected is also shown.

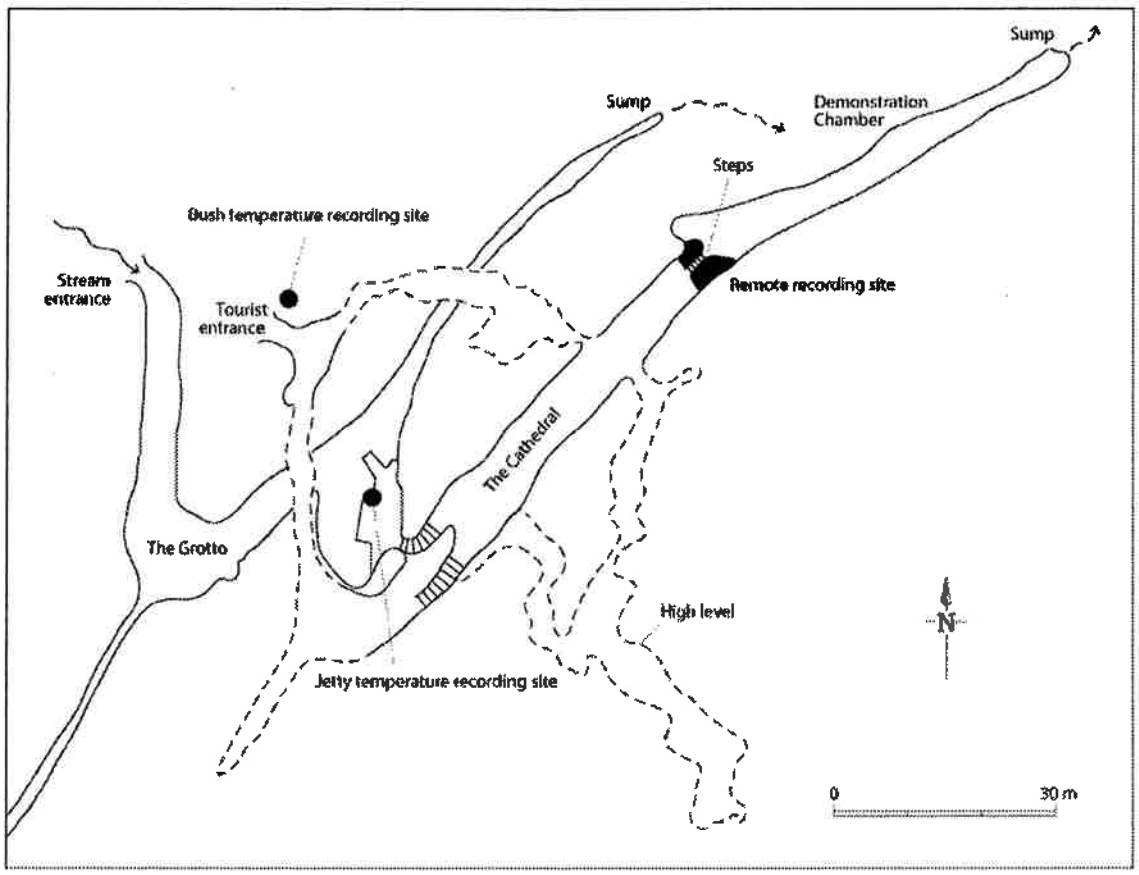


Figure 2. Map of Glowworm Cave showing the location of recording sites.

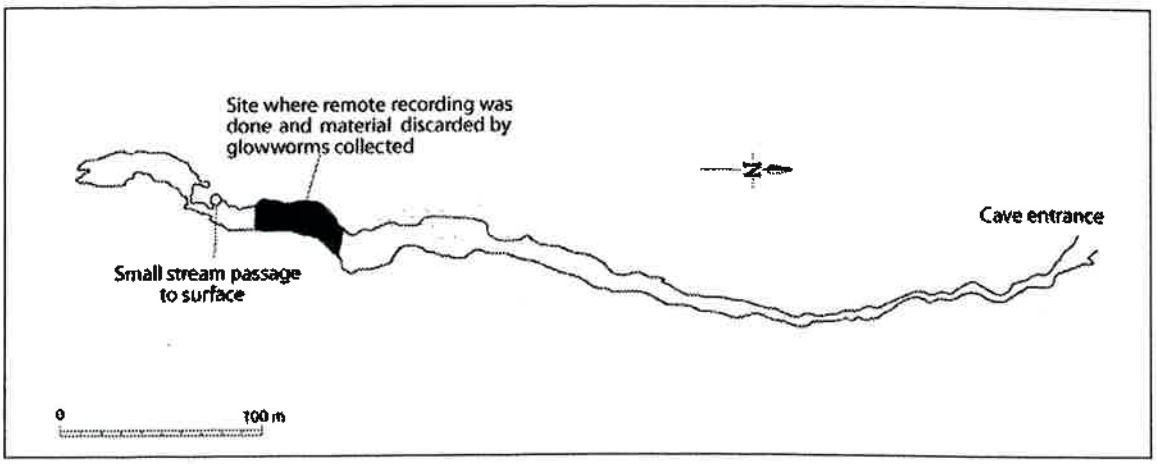


Figure 3. Map of Reserve Cave, Waitomo.

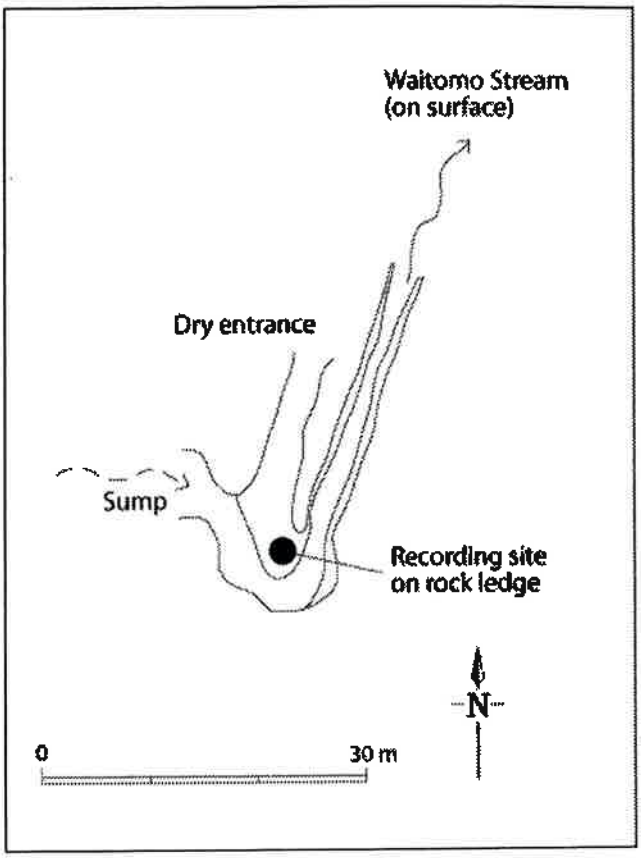


Figure 4. Map of Waitomo Waterfall Cave.

2.2. Collection of material discarded by glowworms

Faeces and other material discarded by glowworms were collected on sheets of blotting paper (570 mm long x 220 mm wide) supported by aluminium foil beneath the glowworm webs. This was done under one group of three glowworms and one of 10 glowworms in Reserve Cave and under

two groups of six glowworms in bush. All were within 20 m of the glowworms that were video-recorded. The blotting paper was left in place for 62 days from 9 September 1995 ("spring" collection), and for 78 days from 10 November 1995 ("summer" collection). The faecal droplets were counted under a dissecting microscope and, where possible, the contents of the droplets and any discarded material were identified.

2.3. Statistical reporting

Data are presented as minutes or seconds per day or hour, and unless stated otherwise all results are given as means ± standard errors (SE).

3. Results

3.1. Infrared video-recording

A total of 424 h of recordings were taken from five glowworms in bush; 410 h from five glowworms in Glowworm Cave, and 369 h from four glowworms in Reserve Cave. Two glowworms in Waitomo Waterfall Cave were recorded over a 48 h period.

Overall, glowworms spent a large proportion of the time glowing and much less time making fishing lines, whereas defaecating, fighting and feeding were relatively rarely observed. The time spent in these behaviours varied depending on where the glowworms were located, although the greatest differences depended on the amount of light the glowworms were subjected to. Thus bush glowworms glowed on average for 82.3% of the night but only glowed 0.13% of the day, whilst those in Glowworm Cave and Reserve Cave spent, respectively, 68.7% and 55.5% of the entire time glowing. In bush, making fishing lines occupied 16.6% of the night and 1.7% of the day (10.6% overall), whereas it occupied 10.1% of the time of glowworms in Glowworm Cave and 6.3% of those in Reserve Cave. Defaecatory droplets were produced during 0.08% of the night and 0.05% of the day by glowworms in bush, 0.04% of the entire time by glowworms in Glowworm Cave and 0.01% of the time by those in Reserve Cave. Fighting occurred during 0.18% of the night and was not observed during the day in bush, 0.67% of the time in Glowworm Cave and 0.14% in Reserve Cave. Glowworms also spent little time capturing prey and feeding: in bush this was only observed at night for 0.79% of the time (0.47% overall), in Glowworm Cave for 0.17% of the time and in Reserve Cave for 0.19% of the time (Table 1).

3.2. Turning around in galleries

Glowworms turned around in their galleries by folding back upon themselves to face the opposite direction. Each turn took ca. 3 s to complete.

Bush glowworms turned around more frequently at night (mean: 16.52 ± 1.82 times) than they did during the day (6.93 ± 1.66 times). At night they turned around when invertebrates contacted the snare, or while mending the snare or defaecating (see *Prey capture in bush*, *Interactions between glowworms and crawling invertebrates,* and *Defaecation*). They also turned around for no obvious reason, particularly during the day in bush when the glowworms were mostly inactive. Compared with bush glowworms those in the caves turned around less frequently with those in Reserve Cave turning around least (Table 1).

3.3. Bioluminescence behaviour in bush

Glowworms in bush usually glowed only at night, up to 1.5 h after becoming active. Most commenced glowing at or after sunset (20 min ± 3 min; range 0–43 min after sunset) and stopped again before sunrise (6 h 13 min ± 2 h 4 min; range 4 min to 9 h 33 min before sunrise). On only two occasions did individual larvae glow just before sunset. The first glowed brightly for 9 min when a small insect flew into its snare (see *Prey capture in bush* below). It then stopped glowing and started again 5 min after sunset. The second glowed brightly for 3 min when a slug (Gastropoda) touched its snare 14 min before sunset and stopped glowing when the slug moved away. This glowworm next started glowing 43 min after sunset. On four nights, pairs of larvae started glowing 15–47 min apart and on four other nights all larvae began glowing within about one minute. Bright light was produced from a non-glowing state over a period of less than 15 s to about one minute. When ceasing to glow the light faded over several minutes. Periodically, the glowworms also appeared to glow less brightly for short periods when their light organs were partially obscured by their bodies or when they retreated along their galleries into crevices or holes in the substrate.

The three glowworms that were observed in February and November produced bioluminescence between 20:30 and 06:42 the next day (Figure 5). They appeared to glow continuously and at about the same brightness except for one that stopped glowing when a millipede moved through the fishing lines at 02:43 on 24 November. This larva resumed glowing brightly 22 min after the millipede had moved away.

Two glowworms video-taped in Autumn (9–15 May 1995), produced bioluminescence between 16:52 and 07:05 (Figure 6). They glowed constantly when the temperature was ~ 12–18° C at sunset and dropped to a minimum of ~ 6° C at sunrise whereas on the night of 10 May 1995 when the temperature was ~ 2–9° C, both larvae glowed intermittently before stopping altogether. The first glowed for 2 h 43 min, followed by two short periods of

Table 1. Means, Ranges and Standard Errors for behavioural observations made of *Arachnocampa luminosa* larvae in bush, Glowworm Cave and Reserve Cave, Waitomo. Numbers of faecal droplets disposed of on blotting paper sheets are also shown for comparison.

ACTIVITY	Mean	Range	SE
Time spent glowing (s per h);			
Bush	1707	0–3600	82
Glowworm Cave	2472	0–3600	167
Reserve Cave	1998	0–3600	384
Time spent making fishing lines (s per h);			
Bush	383	0–2832	28
Glowworm Cave	363	0–2897	109
Reserve Cave	227	0–2428	110
Numbers of fishing lines made per h;			
Bush	0.89	0–6	0.06
Glowworm Cave	0.60	0–5	0.04
Reserve Cave	0.24	0–2	0.02
Time taken to make each fishing line (s);			
Bush	344	36–1204	0.36
Glowworm Cave	577	21–1926	1.07
Reserve Cave	964	188–1968	1.73
Time larvae spent fighting (s per h);			
Bush	2	0–1837	2
Glowworm Cave	24	0–1403	24
Reserve Cave	5	0–1203	5
Lengths of time pairs of larvae were observed fighting (s);			
Bush	920	2–1837	918
Glowworm Cave	908	215–2298	201
Reserve Cave	366	2–1203	219
Time larvae spent defaecating (s per h);			
Bush	2.21	0–180	0.82
Glowworm Cave	1.56	0–120	0.54
Reserve Cave	0.32	0–120	0.32

Table 1. Continued

Lengths of time taken to produce faecal droplets (s);			
Bush	115	50–180	15
Glowworm Cave	75	60–120	8
Reserve Cave	120	-	-
Numbers of droplets collected per day from each glowworm in spring;			
Bush	0.175	0.03–0.40	0.036
Reserve Cave	0.134	0.05–0.21	0.014
Numbers of droplets collected per day from each glowworm in summer:			
Bush	0.150	0.09–0.26	0.017
Reserve Cave	0.289	0.22–0.41	0.022
Time larvae spent feeding on individual prey items (min);			
Bush	36	12–77	21
Glowworm Cave	12	7.5–15	1.5
Reserve Cave	22	13–31	9
Time larvae spent feeding (s per h)			
Bush	17	0–3600	10
Glowworm Cave	6	0–900	3
Reserve Cave	7	0–1860	5
Numbers of times per hour glowworms turned around in their galleries			
Bush	1.04	0–12	0.07
Glowworm Cave	0.71	0–4	0.04
Reserve Cave	0.46	0–5	0.03

9 min and 1 min within the next hour whereas the other larva stopped after 2 h and 23 min and only glowed again briefly for 10 min nearly an hour and a half later. Both larvae finally stopped glowing within 25 min of one another, at 21:34 and 21:59 respectively when the temperature dropped below ~ 6° C.

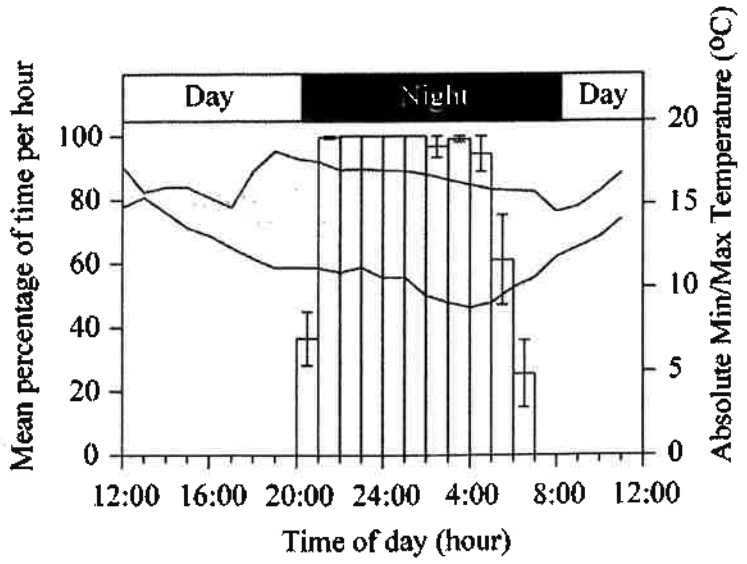


Figure 5. Bioluminescence production by three *Arachnocampa luminosa* larvae in bush between 23–25 February and 19–24 November 1995. (Data are mean hourly percentage of time spent glowing per hour ± SE).

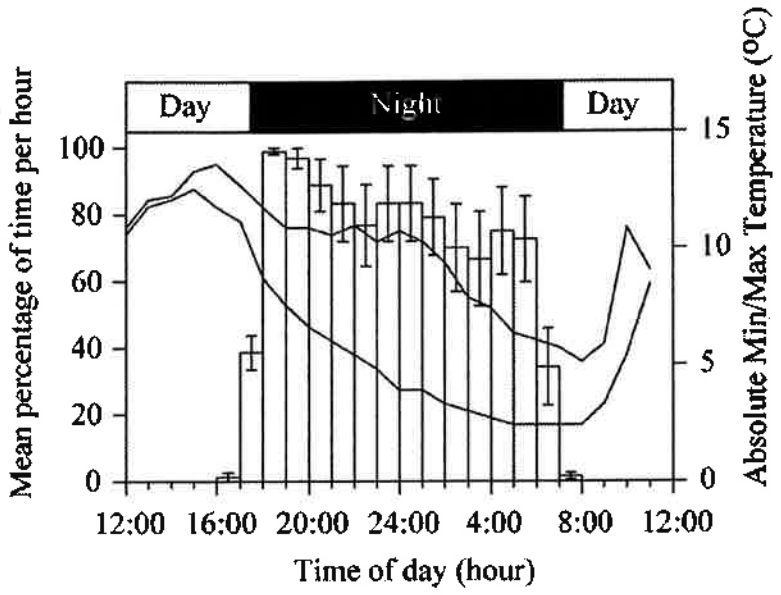


Figure 6. Bioluminescence production of two *Arachnocampa luminosa* larvae in bush between 9–15 May. (Data are mean percentage of time glowing per hour ± SE).

3.4. Bioluminescence behaviour in Glowworm Cave

Larvae in the Demonstration Chamber of Glowworm Cave glowed brightly most of the time between 18:00 and 09:00 (Figure 7) and once glowing, they continued for relatively long periods (mean 10 h 7 min ± 58 min; range 1 h 6 min to 15 h 57 min). These larvae glowed brightly or weakly

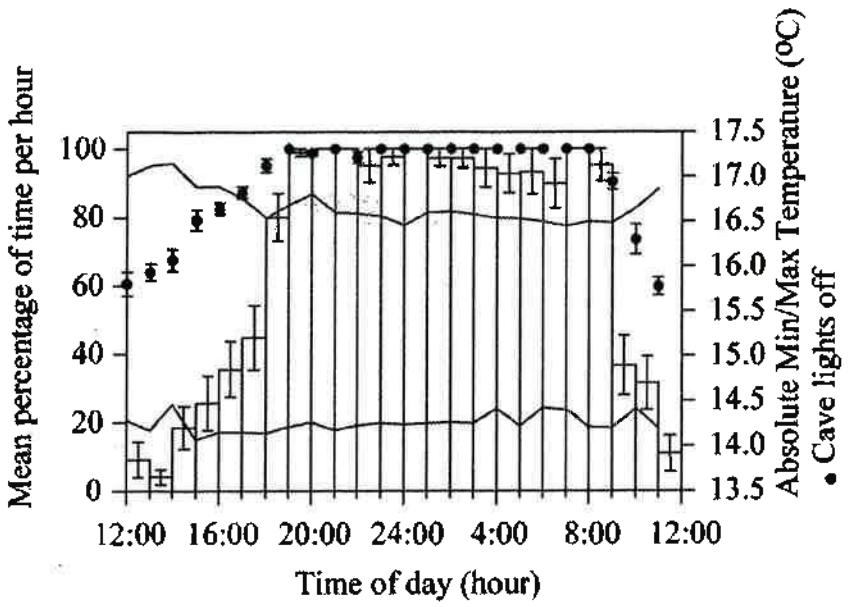


Figure 7. Relationship between bioluminescence production of five larvae in the Demonstration Chamber of Glowworm Cave, and the proportion of time the lighting was turned off. Observations were made between 2–5 April 1995; 18–23 May 1995; and 9–13 February 1996. (Data are mean percent time glowing per hour ± SE).

for shorter periods (34 min ± 5 min; range 1 min to 2 h 50 min), or ceased glowing altogether between 09:00 and 18:00 (Figure 7) when artificial lighting was switched on most frequently for tourists (Figure 7). The glowworms were generally inactive and seldom glowed during this period and the fishing lines, which were normally stationary, always began swaying and sometimes became tangled about one minute before the lights were switched on. The lights were automatically switched off 3–5 minutes after they were switched on but the fishing lines often continued swaying for some time afterwards. This was especially noticeable between 10:00 and 15:00 each day when they swayed almost constantly. Such movement probably resulted from the breathing of people leaning forwards to examine the insects and not by convection currents generated by the lights because swaying commenced before the lights were switched on.

3.5. Bioluminescence behaviour in Reserve Cave

The four larvae observed in Reserve Cave generally glowed weakly compared with larvae observed at other locations. The proportion of time spent glowing was at a minimum (14–33%) between 04:00 and 05:00 but increased to a maximum (94–100%) between 10:00 and 11:00 (Figure 8). Following this, the amount of time spent glowing gradually decreased to a minimum at 04:00 (Figure 8).

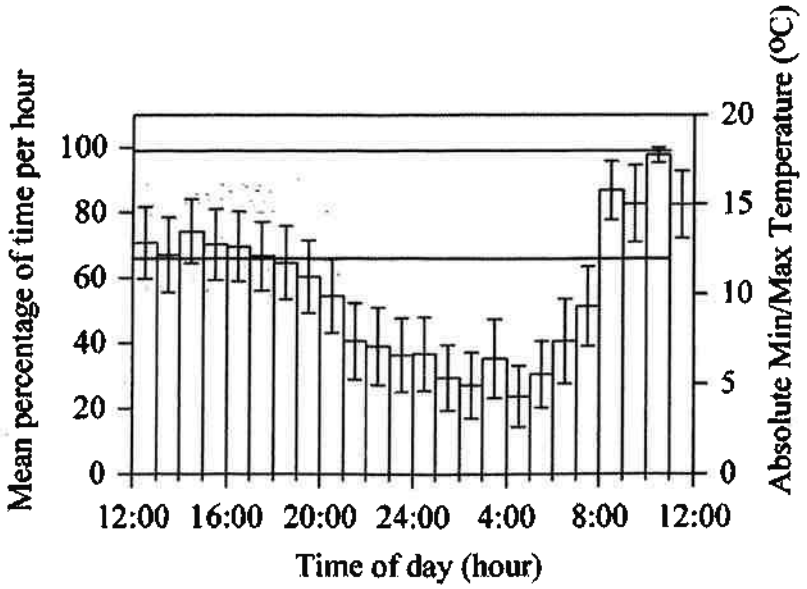


Figure 8. Bioluminescence production of four *Arachnocampa luminosa* larvae in Reserve Cave between 6–7 September, 7–19 November and 19–26 June 1998. (Data are mean percent time glowing per hour ± SE).

Most larvae observed in Reserve Cave glowed between 8:00 and 12:00 when it was daytime outside the cave and the least number glowed when it was dark outside the cave (Figure 8). Individual periods spent glowing varied from 8 min–31 h 38 min with an average of 4 h 50 min ± 55 min.

One initially dimly glowing larva on 24 June 1998 increased its brightness 45 s after a large dipteran flew through its fishing lines at 11:46 (see *Prey capture in Reserve Cave*). It continued to glow brightly until 16:00 when it was exposed to artificial light while the batteries were being exchanged and then it glowed dimly for 3 h 15 min before stopping. Another larva observed from 7–19 November 1995 only glowed dimly except on five occasions when it glowed brightly while fighting another larva.

3.6. Construction of fishing lines

Larvae were observed on 744 occasions making fishing lines (Table 1). Prior to making a fishing line a larva usually attached a suspensory thread between the substrate above or beside the snare and the gallery. The glowworm then extended out of its gallery on the new suspensory thread and hung the anterior half of its body vertically (Figure 9). A large globule of mucus then appeared between its mandibles while the head was rocked backwards and forwards about 20 times over a period of 70.4 s ± 4.6 s (range: 40–100 s). The globule was then lowered a short distance on a silk strand and subsequent droplets, about half the size of the initial droplet, were then produced

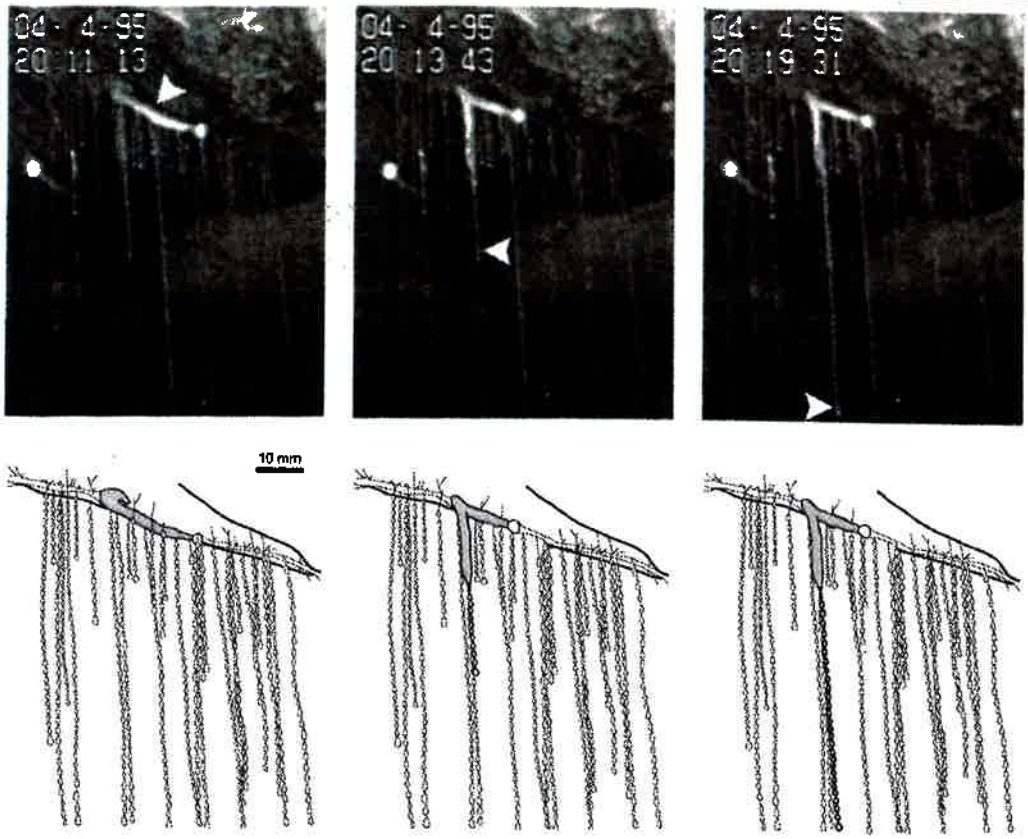


Figure 9. A sequence showing an *Arachnocampa luminosa* larva (~ 25 mm long) constructing a fishing line in the Demonstration Chamber, Glowworm Cave. The light organ is brightly glowing while the larva hangs from its gallery. From left to right: making the suspensory filament, stages during the construction of the fishing line showing the typical posture of larvae while engaged in this behaviour.

after adding short lengths of silk. Each droplet took 13.8 s ± 0.2 s (range: 12–16 s) to produce while the head was rocked back and forth two to four times. When a fishing line was nearing completion the glowworm slowly reversed backwards into its gallery whilst still adding droplets to the length of the fishing line. Finally, it attached the line to the suspensory thread and withdrew into its gallery.

In Glowworm Cave, larvae were observed to haul in tangled fishing lines before replacing them with new ones on 11 occasions and on eight occasions they moved fishing lines up to ~ 2 mm from their original attachment points to new positions on the web.

3.7. Constructing fishing lines in bush

Glowworms in bush made more fishing lines than larvae in either of the caves, although they spent less time making each one (Table 1). They began making fishing lines up to 1 h 18 min before sunset (mean 39 min ± 7 min (± SE),

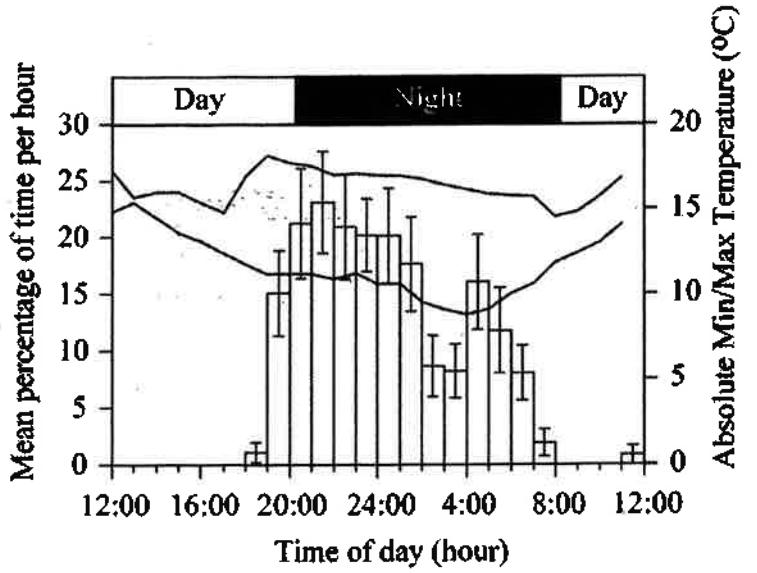


Figure 10. Percentage of time spent by three *Arachnocampa luminosa* larvae constructing fishing lines in bush between 23 and 25 February and 19 and 24 November 1995. Bars show standard errors.

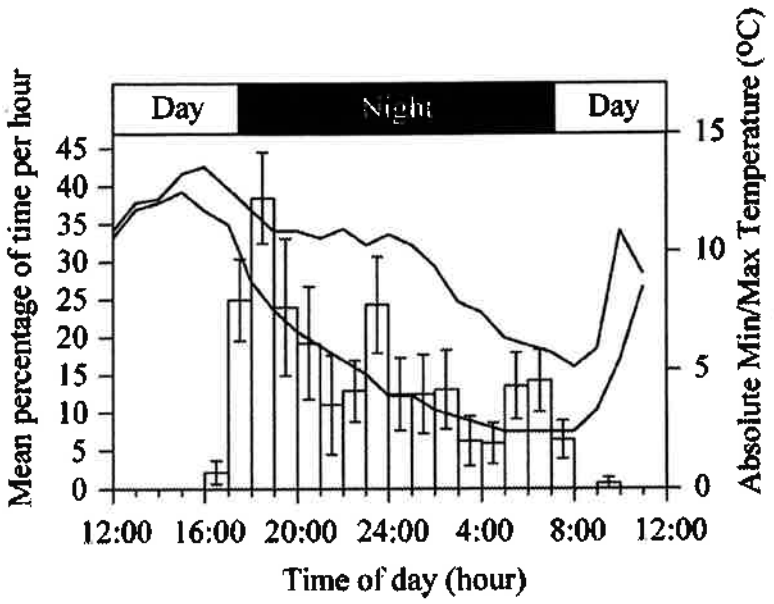


Figure 11. Percentage of time spent by two *Arachnocampa luminosa* larvae constructing fishing lines in bush between 9 and 15 May 1995. Bars show standard errors.

range: 11 min to 1 h 18 min) and stopped making them up to 5 h 18 min after sunrise (mean 1 h 15 min ± 36 min, range: 13 min to 5 h 18 min). In February and November, glowworms made fishing lines between about 18:00 and 08:00 and on one occasion in November a glowworm was observed making a

fishing line between 11:00 and 12:00 (Figure 10). The time spent making fishing lines increased to a maximum between 21:00 and 22:00 when 19–28% of the time was thus occupied. Thereafter the proportion of time spent diminished with a small increase in activity after 04:00 followed by a gradual decrease again until dawn (Figure 10).

In Autumn, individual larvae made fishing lines between about 16:00 and 10:00 (Figure 11). The time spent making fishing lines increased rapidly to a maximum between 18:00 and 19:00 when this occupied 32–44% of the time. Between 21:00 and dawn 3–18% of the time was occupied making fishing lines except for a small increase in this activity to 18–31% between 23:00 and midnight (Figure 11).

3.8. Constructing fishing lines in Glowworm Cave

Larvae in Glowworm Cave constructed fishing lines at almost any time, although they were most active doing this between 18:00 and 21:00 when it occupied 19–41% of their time (Figure 12). After 21:00, fishing line construction diminished to 0–15% per hour. Glowworms did not appear to change the amount of time they spent making fishing lines when the lights in the cave were switched on for less than about 30% of the time per hour but if

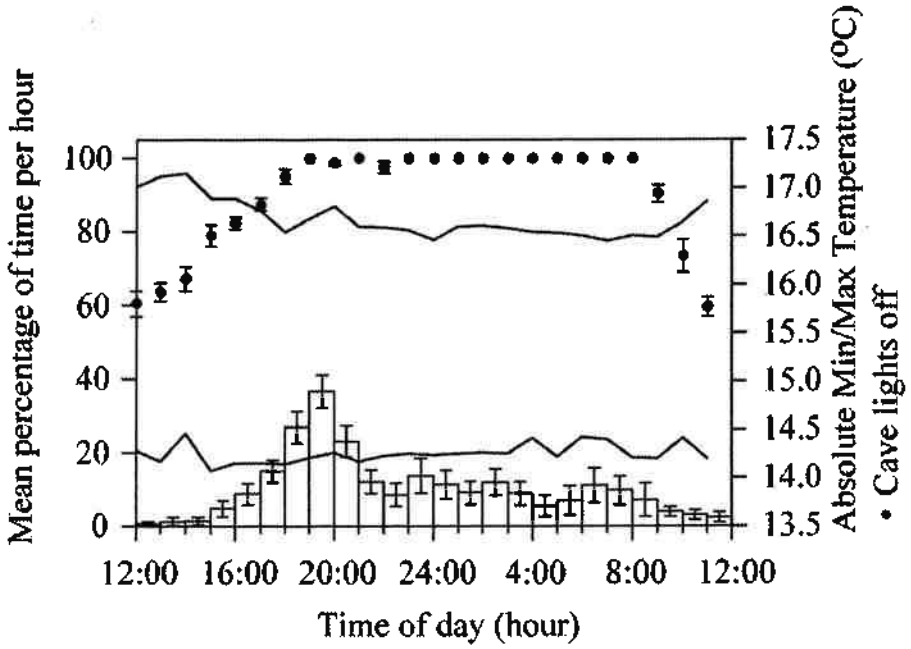


Figure 12. Relationship between the proportion of time spent by five larvae constructing fishing lines (histogram), the temperature and the proportion of time that lights were switched off in the Demonstration Chamber, Glowworm Cave. Observations were made between 2–5 April 1995; 18–23 May 1995; and 9–13 February 1996. Bars show standard errors.

they were switched on for more than this then fishing line construction diminished to 0–5% of the time (Figure 12).

3.9. Constructing fishing lines in Reserve Cave

Four glowworms observed in Reserve Cave constructed fishing lines throughout most of the day except between 23:00 and 24:00 and 08:00 and 09:00 when none were made (Figure 13). They spent the greatest proportion of time making fishing lines between 12:00 and 17:00 (8–24% per hour) except for a small decrease in activity between 15:00 and 16:00 (5–12% per hour) and they spent little or no time doing this (0–13% per hour) between 18:00 and 12:00. These glowworms made fewer fishing lines than larvae in bush or in Glowworm Cave, and took longer on average to make each fishing line (Table 1).

3.10. Prey capture in bush

Capture of prey in bush was observed only three times, which equates to one potential prey capture in bush every 6.1 days. The first (Figure 14) occurred after what appeared to be a ~ 2 mm long dipteran flew into several fishing lines about 20 mm below the glowing larva. The larva began turning around within its gallery 3 s later, extended its anterior end about half-way from its gallery until it was hanging vertically and had reached the struggling

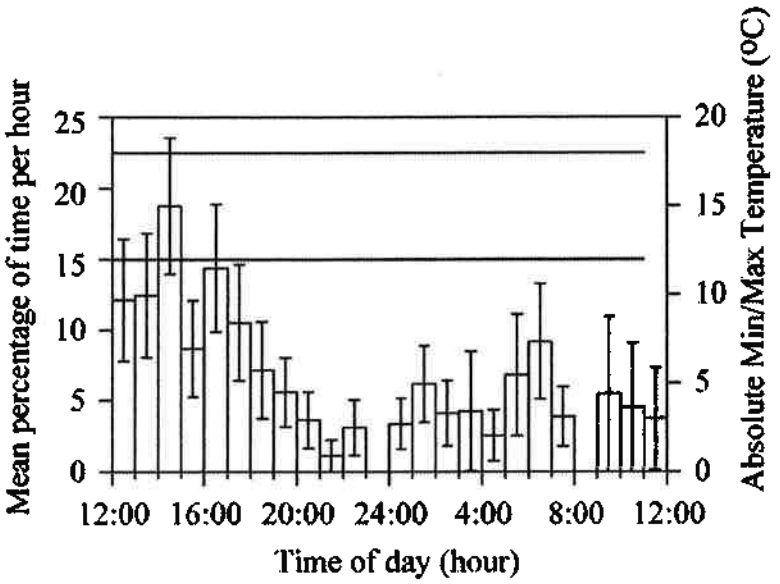


Figure 13. Mean hourly percentage of time (histogram) spent by four larvae constructing fishing lines in Reserve Cave in relation to temperature. Observations were made between 6–7 September, 7–19 November and 19–26 June 1998. Bars indicate standard errors.

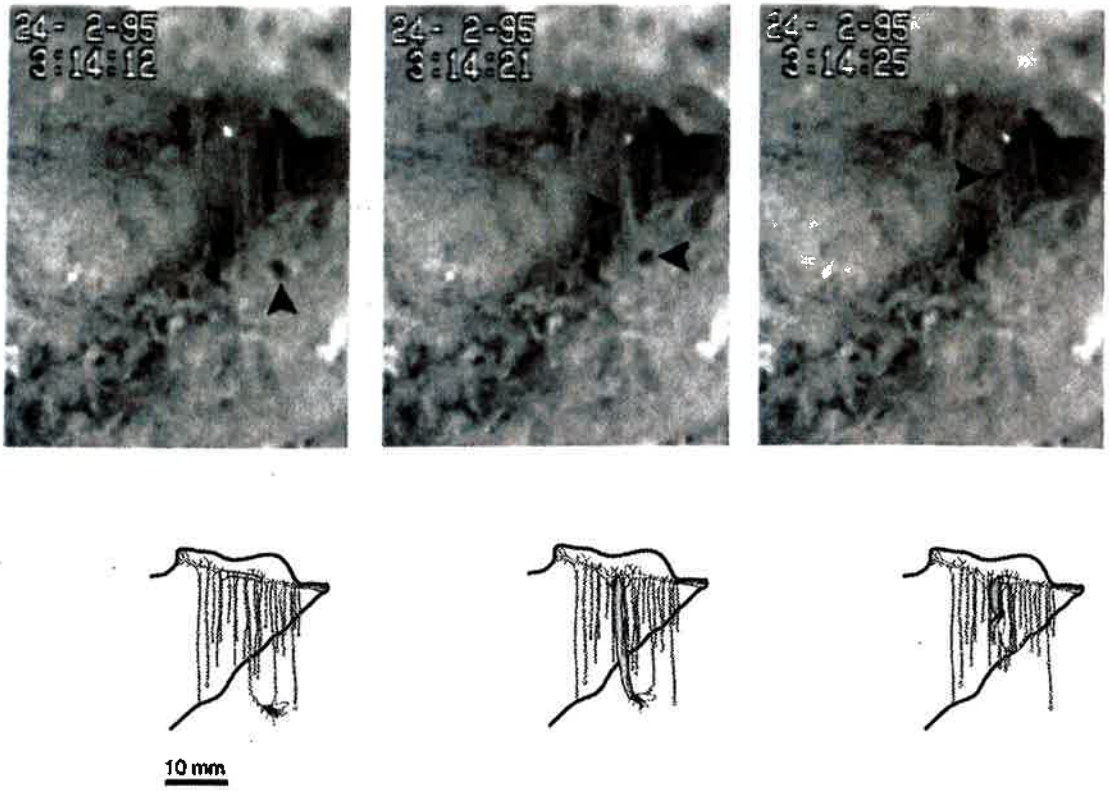


Figure 14. Capture of an insect by a glowworm in bush at night. Left to right: glowworm starting to reach down; biting the insect (9 s after it had flown into the fishing lines); eating the insect after it was pulled up nearer the gallery.

insect. The glowworm then appeared to bite the insect 9 s after it was snared and then pulled it about 15 mm up. Feeding occurred over the next 1.5 h while the glowworm was hanging part way out of its gallery. The larva glowed brightly while capturing the insect and glowed less brightly while feeding. It stopped feeding once for ~ 13 min, glowed brilliantly and turned to face a mite that presumably contacted some part of the web when it moved behind the fishing lines. Once the larva finished feeding it withdrew into its gallery and 1 min later, began repairing its snare and constructing new fishing lines.

Nearly two hours later (05:03:55) another insect flew into a fishing line approximately 10 mm below the same web while the glowworm was hanging head first out of its gallery making a fishing line. The larva started retreating backwards into its gallery within 2 s and then reached down to bite the struggling insect 20 s later. This glowworm then spent 17 min feeding but stopped when something touched part of the web just out of view. The larva glowed brightly and turned around in its gallery to face the disturbance but did nothing until 15 min later when it started making a fishing line. The prey appeared to have been entirely eaten.

The last complete prey capture in bush was observed (02:00, 20 November) when an insect less than 1 mm long flew into a fishing line about 10 mm below a brightly glowing larva. The glowworm moved backwards along its gallery within 2 s, reached down to the insect and appeared to bite it 32 s after it was caught. The glowworm then spent 12 min feeding before withdrawing into its gallery. It continued to glow brightly and started making another fishing line six minutes after ceasing to feed.

Three unsuccessful captures were observed in bush. The first occurred 29 min before sunset (16:52, 14 May) when a tiny winged insect flew towards a glowworm snare and apparently touched it because the glowworm glowed brightly for 9 min and turned around in its gallery several times. However, nothing further happened and the insect was too small to see clearly what happened to it. The second occurred at night in November when a small insect appeared to fly into a fishing line about 10 mm below a glowing larva. The glowworm reached down but the insect broke free and flew away.

The last unsuccessful capture occurred when a small (~ 1 mm) flying insect appeared to hover near a glowworm snare and either touched it or caused it to vibrate because the glowworm glowed brightly, turned around and moved towards the possible contact. It then reached out of its gallery and appeared to attempt to bite the insect. However, the insect continued to hover close by and eventually flew away.

3.11. Prey capture in Glowworm Cave

One complete and three partial prey captures were observed in Glowworm Cave. Just after midnight on 4 April a tiny insect flew into and became snared approximately 30 mm below a glowing glowworm. The captured insect did not appear to struggle but the glowworm immediately glowed more brightly and, 30 s later, turned around, moved to the fishing line with the prey and extended part way down the fishing line. It then spent 36 s hauling the line up before feeding for 7 min 30 s.

Three partial observations of prey capture by the same glowworm were recorded but the reaction times were not available because the insects were caught out of view. The first occurred on 20 May when the glowworm took 2 min to haul up a tiny winged insect (~ 2–3 mm long) and then spent 15 min eating it. The next day this glowworm hauled up another struggling winged insect and spent 13 min feeding on it. The third observation occurred on 22 May when the glowworm was observed to feed on a struggling insect for 11 min and then 7 min later begin to make a new fishing line. On all three occasions no parts of the insect were visible after the glowworm had finished feeding.

3.12. Prey capture in Reserve Cave

Two complete prey captures were recorded in Reserve Cave (one capture every 8.3 days). The first (04:45, 14 November 1995) occurred when a small insect flew into the snare but it was unclear if the insect became caught on a fishing line or on some other part of the web. This glowworm had not glowed since the previous afternoon but it immediately commenced glowing brightly, turned around to face the insect and fed for 13 min whilst continuing to glow brightly. It turned around in its gallery and stopped glowing when it finished feeding and did not start glowing again until five and a half hours later (10:27). The second capture occurred at 15:06 on 22 June 1998 when a small insect was caught approximately 15 mm below a dimly glowing larva. The glowworm turned around within 2 s, reached down the fishing line to bite the struggling insect 20 s after capture and then spent 31 min feeding before retreating back into its gallery. Nothing remained of its prey and the glowworm did not appear to glow any brighter during the capture or while feeding. The glowworm started making a new fishing line 11 min after finishing feeding.

One partial capture and two prey escapes were also recorded in Reserve Cave. The partial capture, at 16:10 on 17 November, took place at the edge of the camera frame and was only partially visible. The brightly glowing glowworm was observed to take 1 min 30 s to haul up ~ 15 cm of fishing line with a struggling insect on it. It was not clear how long the insect had been caught or how long the glowworm fed on its meal because both the glowworm and its prey were at the edge of the field of view. The first escape occurred at 16:05 on 23 June 1998 when a tiny insect escaped from a fishing line when a glowworm started hauling it up. The second escape occurred at 11:46 on 24 June after a large dipteran flew through the fishing lines and landed on the cave wall next to a dimly glowing larva. The larva glowed brightly 45 s later and moved towards the insect. Nine minutes later the glowworm appeared to touch the insect, which flew away, knocking several fishing lines as it went.

3.13. Prey capture in Waitomo Waterfall Cave

In Waitomo Waterfall Cave where a dense concentration of glowworms inhabited the ceiling, insects were commonly observed caught in their snares in summer. On 21 February 1995 the camera was directed to a large mayfly (Ephemeroptera) entangled in a fishing line about 15 cm below a larval gallery. The mayfly often moved its wings and legs. Two hours later the glowworm took 5 min to haul the mayfly up to the gallery but feeding was obscured by a projection from the cave roof. A day and a half later another

glowworm was observed taking 4 min to haul up a mayfly that did not struggle, but it was not known when the capture occurred because it was out of view. This glowworm stopped glowing shortly after commencing to feed and started glowing again 45 min after it had finished feeding. The uneaten remains of the mayfly were left hanging in the snare.

A glowing adult *A. luminosa* was observed to fly into and be caught by fishing lines in Waitomo Waterfall Cave. It flew upwards towards the cave ceiling, while still attached to the end of the fishing lines and on two occasions it managed to break free but got caught again before it disappeared from view.

3.14. Interactions between glowworms and crawling invertebrates

On one occasion (26 June 1995) in Waitomo Waterfall Cave a dead cave weta (Orthoptera: Rhaphidophoridae) was observed hanging by its hind legs from the remains of a larval snare with no glowworm. The next day the weta had disappeared from the snare and the larva was still missing.

On seven occasions in bush at night spiders (Araneae) were observed moving over or through glowworm snares. Millipedes (Diplopoda) and mites (Acarina) did likewise on three occasions; followed on two occasions each by harvestmen (Opiliones) and slugs (Gastropoda). All appeared to do this accidentally and were too large and powerful to become caught in the fishing lines. None of the arachnids were observed preying on glowworm larvae. On two occasions other small crawling invertebrates that were not identified were observed moving over the substrate near glowworm snares but were not captured.

Glowworms usually reacted to their snares being touched by crawling invertebrates at night by glowing brightly from a few seconds and up to 6 min (9 occasions), turning around or moving towards the contact (2 occasions) then either extending part-way out of the snare to either check fishing lines (2 occasions), start hauling in the one that was touched (1 occasion), or extending from the web and attacking the intruder (6 occasions). During one of these attacks a glowworm fastened onto the leg of a mite with its jaws for 68 s and became stretched to about 1.5 times its normal length before the mite escaped and the glowworm rebounded back into its snare (Figure 15). All of the other invertebrates that were attacked also escaped. Less frequently a glowworm apparently did nothing (1 occasion), moved only slightly (2 occasions), turned around in its gallery and faced away from the contact (1 occasion), or retracted back along its gallery (1 occasion). In 4 cases the glowworm commenced repairing its web or began making a new fishing line up to 30 min after the intruder had left.

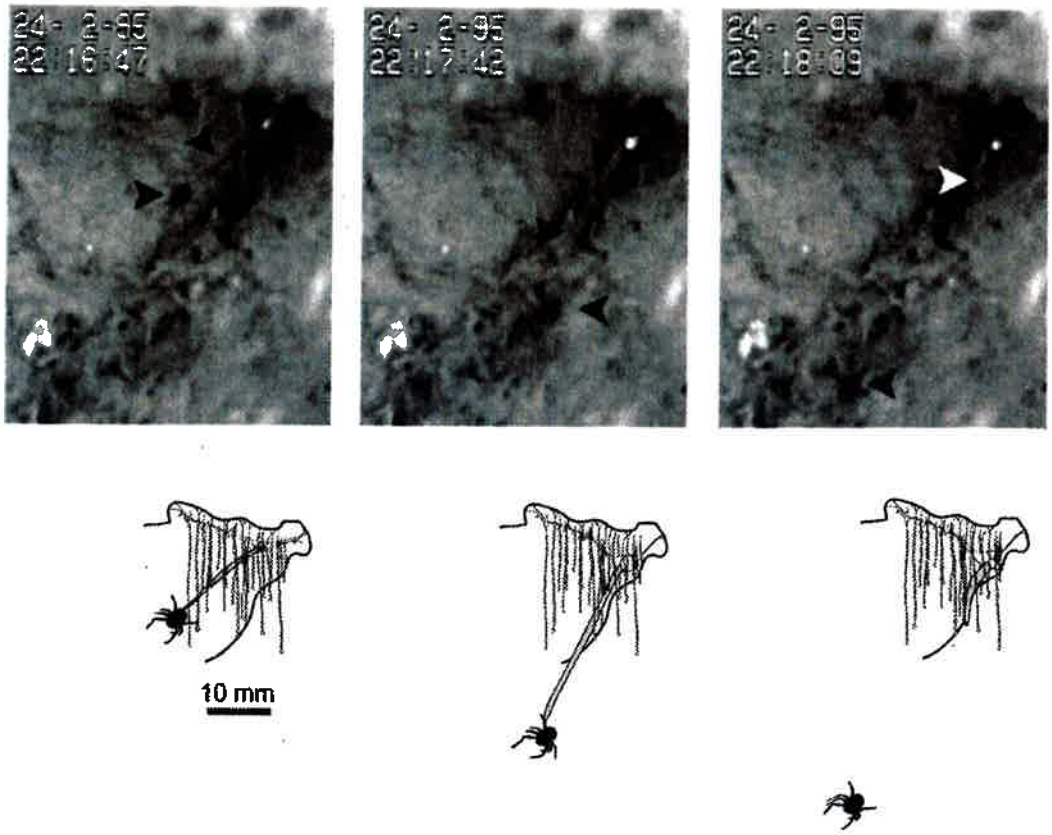


Figure 15. A mite in bush at night that had walked into a crevice behind a curtain of fishing lines and was attacked by the glowworm as it moved back out. This mite subsequently broke free, and moved quickly away.

During daytime, the reactions of glowworms appeared to be defensive and, with the exception of two contacts that occurred within 30 min of sunset, none glowed when their web was touched. On one occasion, just after sunrise a glowworm rapidly moved away along its gallery each time when a harvestman twice touched its snare. Another glowworm turned around and faced a small crawling arthropod (~ 1 mm) which then moved away. On the last occasion, a glowworm moved backwards in its gallery and reached towards a millipede that had contacted its web and appeared to bite it because the millipede recoiled and moved quickly away.

3.15. Larval fighting

Fighting between larvae was uncommon even though most glowworms observed were large and their webs were close to each other to allow simultaneous observation (Table 1). Most fighting commenced when a larva moved part-way out of its gallery to attach a new suspensory thread (see *Construction of fishing lines*), and touched the snare of a neighbouring glowworm. The neighbour glowed brilliantly and moved part-way out of its

gallery towards the intruder and made snapping movements with its jaws. Fighting then commenced, which generally comprised glowworms making biting movements at each others heads. On several occasions fighting pairs of glowworms were observed pulling a silk strand strung between their mandibles as in a tug-o-war. Each appeared to try to pull the other from its snare, at the same time biting at each other when they came within reach. Cannibalism was not observed, but on one occasion a larva was injured by its attacker. This occurred in Glowworm Cave after a larva left its snare and moved into another snare (Figure 16). The intruder bit the resident larva behind its head and the resident larva quickly recoiled, but appeared to survive the attack. The intruder eventually moved back in the direction it had come.

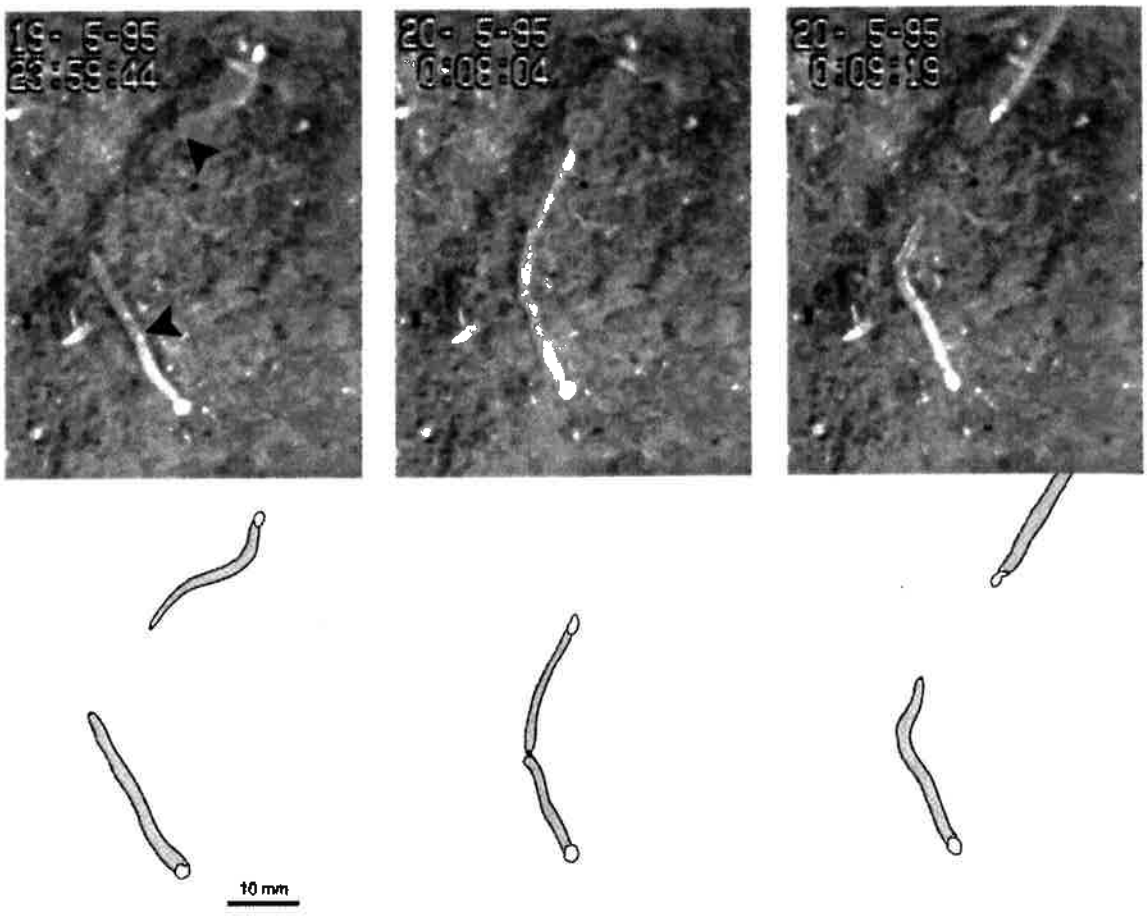


Figure 16. A pair of *Arachnocampa luminosa* larvae fighting in Demonstration Chamber of Glowworm Cave. Note the intensity of their lights. The larva at top had moved out of its own snare, and into its neighbour's. The two glowworms began to fight, but the glowworm at top bit the body of the other glowworm with its jaws. The lower larva immediately recoiled from this attack. The larva at top then moved back in the direction it had come from. The lower larva appeared to survive the attack, although it did not move while glowing only faintly for many hours afterwards.

3.16. Defaecation

Arachnocampa luminosa larvae were observed defaecating on eight occasions in bush, nine occasions in Glowworm Cave and once in Reserve Cave. Details of the behaviour varied but the entire process took up to three min (Table 1). Defaecation was first observed in bush (21:00 on 23 February 1995, 49 min after sunset). A dark mass of faecal material was clearly visible within the posterior quarter of this glowworm's body. The glowworm first moved half-way out of its gallery as if starting to make a fishing line, then it retracted until about a third of its body remained hanging and turned around, lowered the posterior half of its body out of its gallery and began making peristaltic muscular contractions along its body. Next, the larva moved back up into the gallery again, turned around, and moved head first down to the same point. The larva appeared to be checking the fishing lines hanging from this part of the snare. It then retracted into its gallery, moved further along its snare, and repeated the behaviour, except that on this occasion the faecal material was expelled as a large droplet over a period of ca. 50 s (Figure 17). This faecal droplet then fell onto the substrate below the nest, where at least four other droplets were also clearly visible (Figure 17). The larva then turned around and, hanging head first again, appeared to check that the droplet had not become tangled in the fishing lines. The same glowworm was observed defaecating again 24 h later. It carried out the same behaviour as before except that this time it expelled its droplet at the first attempt after a period of 1 min of muscular contractions, onto a fishing line ~ 25 mm below the gallery. The larva then turned around and lowered the fishing line until the droplet contacted the substrate.

Another larva was observed defaecating 24 min after sunrise (07:30, 10 May 1995) onto a fishing line at one end of its snare. This faecal droplet took 180 s of muscular contractions to produce, and this time the droplet went out of view when it was discarded. Other defaecations were observed at night. One was produced 20 mm below the gallery on a fishing line after 112 s of muscular contractions. This droplet was left hanging and 48 h later the same glowworm repeated this behaviour, onto another fishing line taking 140 s to produce the droplet. This faecal droplet fell when the fishing line broke. Another faecal droplet (14 May 1995) took 140 s of muscular contractions to produce and this time it ran part-way down a fishing line. The glowworm then lengthened the fishing line until the droplet contacted the substrate below. The last two observations of defaecations were made in November. The first occurred just before sunrise (05:12) with the droplet taking 115 s to produce, then the same glowworm made another two days later (05:18) onto a fishing line after 2 min of muscular contractions. This droplet ran down the fishing line until it went out of view.

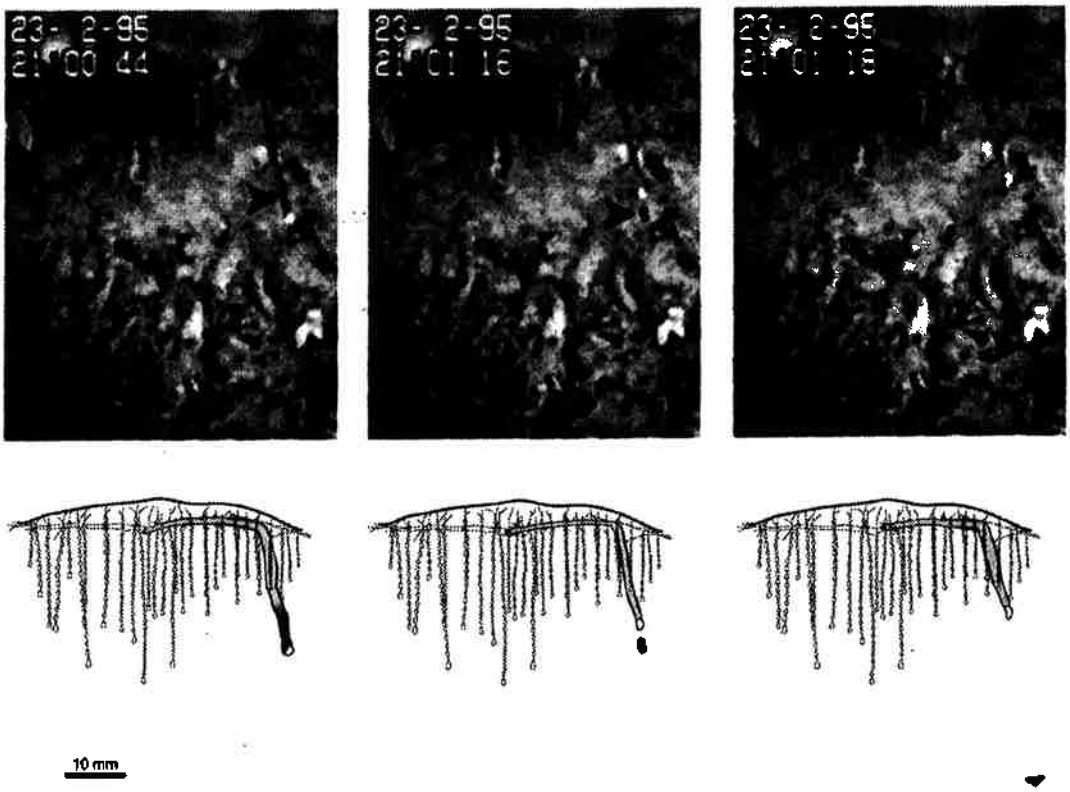


Figure 17. Defaecation. The sequence shows: left – a brightly glowing larva with the posterior part of its body hanging from its gallery at night in bush. Middle – releasing a faecal droplet. Right – the faecal droplet on the substrate below. Other faecal droplets from previous occasions also litter the substrate and are clearly visible. Note the spider (arrow) sitting motionless at left.

The first defaecation observed in Glowworm Cave (15:39, 2 April 1995) occurred after a brightly glowing larva reached part way out of its gallery to haul up a tangled fishing line, then reversed so it hung part way out of its gallery on a short fishing line. The faecal droplet took 75 s to produce, then the larva turned around, ceased glowing and appeared to briefly check the fishing line. However, it left the droplet hanging for nearly 1.5 h, before cutting it off and letting the droplet fall. Faecal droplets were produced by other glowworms during 1 min (4 occasions), 1 min 10 s, 1 min 36 s and 2 min of muscular contractions. All faecal droplets were deposited on fishing lines and on three occasions the faecal droplet ran down these fishing lines. On two occasions the larvae glowed brightly but either stopped glowing when it turned around to check the fishing line attached to the droplet or stopped glowing a few minutes later. In two cases the larvae turned around and bit off the fishing line attached to the droplet but on one occasion it was not possible to see whether the droplet was dropped or not. Once the droplet swung sideways when the fishing line was bitten off and became tangled with

another fishing line ca. 5 mm below the gallery where it was subsequently left alone. On one occasion the camera batteries were changed part way through defaecation and the entire process was not recorded. On this occasion (08:02, 22 May 1995) a larva was hauling up one of its fishing lines when it stopped and constructed a very short fishing line, turned around and began to defaecate when recording ceased. No droplet was visible when recording recommenced about 3 min later.

Defaecation was observed only once in Reserve Cave (21:25, 10 November 1995). The faecal droplet was produced onto a fishing line during ca. 2 min of muscular contractions and the glowworm then cut the fishing line with its mandibles so it dropped.

3.17. Material discarded from glowworm webs

Material found stuck together in clusters beneath glowworms was presumed to be from faecal droplets. Most of this material consisted of well-chewed parts of insects (Table 2). Small parts of insect sensillae and spines were always present and cuticle including that from compound eyes was found in most clusters. Legs, antennae and wings that appeared to have been discarded separately from the faecal droplets were also sometimes present, as were entire wings of mycetophilid, psychodid and sciarid flies under glowworms in bush and psychodid and sciarid wings under glowworms in Reserve Cave (Table 2). Occasionally, entire or fragmented millipedes, and insect head capsules, thoraxes and abdomens were also present. Several small empty snail shells (Gastropoda) were found under bush glowworms and three entire Diptera were found under cave glowworms in summer. All of the three latter insects (one psychodid, one empidid and one unidentified) lacked any body contents.

4. Discussion

We have confirmed that *Arachnocampa luminosa* larvae in bush glow mostly at night as was first reported by Hudson [18]. Indeed, most of the glowworms we observed in bush started glowing at or after sunset and stopped glowing before sunrise. The exceptions were two larvae that glowed briefly when disturbed up to half an hour before sunset but in both cases it would have been relatively dark in the bush when these larvae glowed. Both larvae started glowing again after sunset. We have also confirmed that glowworms were largely active at night in bush and that during the day they were generally inactive, except when they occasionally turned around in their galleries or occasionally made fishing lines. Stringer [2] also reported this behaviour during the day and noted that they moved only when alarmed.

Table 2. Material identified from blotting paper sheets placed under bush and cave glowworms. Numbers indicate the numbers of such fragments found.

Discarded material	Bush - spring	Bush - summer	Cave - spring	Cave - summer
Antennae	+		+	
Cuticle	+	+	+	+
Cuticle - eye	+	+	+	+
Sensillae + spines	+	+	+	+
Leg parts	+	+	+	+
Mandibles				+
Wing fragments			+	
Wing membrane			+	
Whole wings;				1
Mycetophilidae	1			
Psychodidae	2		1	
Sciaridae		2	1	
Arthropod abdomen	1			1
Arthropod head	1	3	2	1
Arthropod thorax	1	1		
Whole exuviae;			1	1
Empididae				1
Psychodidae				1
Coleoptera elytra	1			
Gastropoda shells	1	3		
Millipedes		1	+	3

+ indicates material that was present but not able to be counted.

In addition, we found that glowworms turn around in their galleries during the day for no apparent reason. Our results concur with the observations by Stringer [2] that in bush glowworms usually became active late in the afternoon, when they began making fishing lines and repairing snares. Our observations that glowworms began to glow up to an hour and a half after becoming active, that bioluminescence takes less than 15 s to about one

minute for a bright light to be visible, whereas at dawn, it took several minutes for the light to fade out, are also in accord with those of Gatenby [3].

In bush, temperature appears to influence whether the larvae glow or not. Hudson [18] first reported that they "cease to shine on very cold nights" and other authors observed that larvae in bush may stop glowing for some periods [2-4, 14]. Our results showed that in February and November, when it is warm, glowworms in bush generally glowed with the same approximate brightness all night whereas on three nights in May, when it was cold, they either glowed faintly or stopped glowing altogether. In addition, two larvae that began glowing after sunset ceased glowing after nearly four hours when the temperature dropped below about 6° C. Exact temperatures were not available because the readings were taken in bush outside the entrance to Glowworm Cave about 2.5 kilometres away but it is unlikely that the temperatures at the two sites differed much. Low temperatures also appear to similarly affect the glowworm *Arachnocampa flava* Harrison because Baker [19] reported that "the strongest single correlation is with minimum temperature: on cold nights fewer glow worms bioluminesce" at Natural Bridge in Springbrook National Park, Queensland.

One factor that appeared to affect glowworms in the Demonstration Chamber of Glowworm Cave was human activity because the larvae there only glowed for short periods or stopped glowing altogether and they also spent little time making fishing lines when the artificial lighting was switched on most frequently. Possibly the slight breezes created by breathing or the movements of people during this period may also have disturbed the larvae. The wind was sufficient to tangle the fishing lines and larvae were observed occasionally moving whole fishing lines short distances (~ 2 mm) around their snares, perhaps to prevent them tangling further. Such human disturbance does not appear to be detrimental to the overall glowworm population which appeared healthy despite about half a million visitors passing through the Demonstration Chamber each year. The lighting system was installed in 1992 (K. Banbury, personal communication) so there has been plenty of time for any adverse effect of this to have shown. The numbers of glowworms present do, however, fluctuate in response to flooding and food availability [20].

We have demonstrated that cave-dwelling glowworms do not always glow continuously as reported by Gatenby [3] even when undisturbed. Glowworms in both Glowworm Cave and Reserve Cave – a cave that is rarely visited by humans – ceased glowing for many hours. The latter also did not glow as brightly as glowworms at the other locations but, despite this, their bioluminescence certainly still attracts prey [13]. Glowworms in caves can also survive for long periods – at least 78 days – with little or no food

[13]. Our results indicate that glowworms in Reserve Cave generally glow more during the daytime than during the night time indicating that there may be a circadian component to this behaviour. A similar pattern was reported for glowworms living in a part of Glowworm Cave not lit by artificial lights [21]. In the case of Reserve Cave, the prey is mostly small dipterans [13] which are probably carried in as larvae by a small stream which enters the cave nearby through a sink hole.

Circadian activity rhythms have been reported for a number of cave dwelling animals, including the millipede *Glyphiulus cavernicolus* Sulu [22] and a cricket, *Strinatia brevipennis* Chopard [23]. These animals live in perpetual darkness in caves which are characterised by almost constant ambient temperatures and high relative humidities. Certainly in Reserve Cave it is completely dark and temperatures do not fluctuate much (< 1° C per day). So, in the absence of obvious zeitgebers such as day-night light patterns and daily temperature cycles, the question which we cannot answer is – how do cave-inhabiting glowworms maintain a circadian component to their behaviour?

Four out of five of the partial and complete prey captures and escapes by potential prey that we observed in Reserve Cave occurred during the day time, between 11:46 and 16:10. Perhaps prey capture is a cue for the larvae to glow; if their insect prey is more active inside the cave when it is daytime outside the cave, then this is the best time for them. In support of this, Biswas and Ramteke [24] suggested that "periodic restricted feeding [of the cave loach *Nemacheilus evezardi* (Day)] could act as a powerful zeitgeber of circadian rhythms in subterranean organisms." However, Merritt and Aotani [25] found during a recent study of the Australian glowworm *A. flava,* that larvae taken from bush and kept in constant darkness in the laboratory could not be entrained by a daily feeding regimen. However, they did not investigate this with cave-dwelling glowworms. It therefore remains to be determined if there is a relationship between food availability and glowworm bioluminescence behaviour in dark undisturbed caves.

Disturbance certainly appears to influence fishing line construction. Thus larvae in Reserve Cave made fewer fishing lines than those in Glowworm Cave or in bush, but they spent longer on average making each fishing line than glowworms at the other two locations. Larvae in Reserve Cave did not have to contend with frequent disturbances such as breezes tangling their fishing lines, or with spiders and other invertebrates moving through their snares, as happens in bush.

Prey capture appears to be a relatively rare event. This was observed only 12 times during the 1250 'larva-hours' of observation. All but two of the captures appeared to be small dipterans, and these were previously reported to be the predominant food in both caves and in the bush at this location [13].

However, other authors have reported that glowworms capture non-dipteran invertebrates – Coleoptera, Lepidoptera, Crustacea [15]; red ants, amphipods, millipedes, small land snails [2]; caddis flies, mayflies, stoneflies, immature isopods and a silphid beetle *Necrophilus prolongatus* Sharp [1]. During a previous investigation using adhesive trapping we estimated that glowworms catch one potential prey item every 2.9–5.0 days in the bush-clad entrance to Reserve Cave and every 19.2–36.5 days in the cave itself [13]. Our video recordings show one prey capture every 6.1 days in the bush, which is close to our previous bush estimate. However, in the cave we estimate that one prey capture was made every 8.3 days. A possible explanation for why we previously underestimated the prey capture rate of glowworms in the cave could be that the traps reduced the light intensity of glowworm bioluminescence by about 80% [13], and since glowworms in the cave do not appear to glow brightly compared to those outside the cave the reduction in light intensity may have had a greater effect on reducing their attractiveness and thus overall catch rates.

Spiders, harvestmen, mites, millipedes, slugs and other small unidentified crawling invertebrates were observed to blunder into glowworm snares at night in bush, but they were never observed to be captured. Most were apparently too large and powerful to become ensnared in the sticky fishing lines although a single large dead cave weta was found in an unoccupied glowworm web in Waterfall Cave. The remains of a millipede and four empty snail shells were found amongst discarded material under glowworms in bush, which suggests that they may occasionally be captured and eaten, as previously reported by Stringer [2]. On one occasion in bush at night a glowworm was observed attacking a mite that had wandered into its snare but the mite escaped.

Spiders have never been reported to either form part of the glowworms' diet or to prey upon *A. luminosa*. They do, however, often spin their webs in front of glowworm snares [personal observation; 2, 3, 18, 26] and this may indicate that spiders exploit the prey attracted to glowworm bioluminescence. A similar association was also reported by Baker [19] with the Australian glowworm, *A. flava*, while Heiling [27] and Adams [28] reported a similar use of artificial lights by at least two nocturnal orb weaver spiders. We suggest that such behaviour by spiders may explain why more spiders were caught on transparent adhesive traps over glowworms in bush (27) compared with empty traps (16) [13] when determining what prey is attracted by glowworms. Finally, spiders have been reported to live in close association with a mycetophilid larva (*Macrocera stigma* Curtis). These small predaceous non-bioluminescent mycetophilids live under logs and boulders in the south and south-east of England and the spiders move about within the snare without getting entangled [29].

Fighting between glowworm larvae was relatively uncommon despite the many hours of observation at each location. Fighting probably occurs even less often overall in a population of glowworms because we video-recorded small groups of glowworms that were close together to increase the data collection rate. Pugsley [20] suggested that glowworm "larvae maintain uniform spacing by aggressive territorial defence, extending occasionally to cannibalism." However, we never observed cannibalism, although other authors have reported that it does occur [1, 3, 16, 17]. Several authors also suggested that young larvae are more likely to cannibalise each other [3, 10] but we were unable to confirm this because we only observed large larvae.

Glowworms usually consume their entire prey as evidenced by their faecal material which consisted mainly of finely cut pieces of cuticle. This is in agreement with Wheeler and Williams [12] who reported that the larval gut contents were "loosely filled with small pieces of chitin, often covered with hairs. Some of the pieces belonged to small gnats allied to the Chironomidae. In one case a mandible of some small insect larva was found among the fragments." This suggests that glowworms can consume all edible parts of their prey, an advantage when there are often long periods between prey captures.

Overall, this investigation of undisturbed glowworm larvae using infra-red remote video-recordings has confirmed that most of the behaviour previously reported for these insects also occurs when the insects are disturbed by short periods of artificial illumination. Artificial light seemed to affect them most during defaecation [e.g. 2] so this had not been described in detail previously. Overall, our study has provided the first detailed activity time-budgets for this species and it has demonstrated that even in bush, where flying insect prey can be expected to be more readily available, glowworms feed relatively infrequently despite their bioluminescence.

Acknowledgements

Thanks to former manager Dave Williams, and staff of the Waitomo Caves Hotel; the late Peter Dimond (Director) and staff of the Waitomo Museum of Caves; Pete Chandler and John Ash of the Black Water Rafting Company; Bruce Rapley, Dexter Muir and Wyatt Page of the Department of Production Technology, Massey University; Allan Singleton of the National Institute of Water and Atmospheric Research Limited; the Carter Observatory, Wellington; Jonathan Ravens (New Zealand Speleological Society) for permission to use NZSS cave maps and Chris Edkins for preparing maps for publication. Financial support for this research was provided by the T.H.C. Waitomo Caves Hotel, Waitomo Caves Management Committee, Ruapuha Uekaha Hapu Trust, Ecology Group (Massey

University) and a graduate research award from the Massey University Graduate Research Fund.

References

1. Richards, A.M. 1960, Trans. Roy. Soc. N.Z., 88, 559-574.
2. Stringer, I.A.N. 1967, Tane, 13, 107-117.
3. Gatenby, J.B. 1959, Trans. Roy. Soc. N.Z., 87, 291-314.
4. Hudson, G.V. 1950, The natural history of the New Zealand glow-worm. In Fragments of New Zealand Entomology, Ferguson & Osborn, Wellington, 15-37.
5. Gatenby, J.B., and Cotton, S. 1960, Trans. Roy. Soc. N.Z., 88, 149-156.
6. Meyer-Rochow, V.B., and Waldvogel, H. 1979, J. Insect Physiol., 25, 601-613.
7. Meyer-Rochow, V.B., and Eguchi, E. 1984, N.Z. Entomol., 8, 111-119.
8. Gatenby, J.B. 1960, Tuatara, 8, 86-92.
9. Kermode, L. 1974, N.Z. Speleol. Bull., 5, 313-328.
10. Meyer-Rochow, V.B. 1990, The New Zealand Glowworm, Waitomo Caves Museum Society Inc, Waitomo.
11. Meyer-Rochow, V.B. 2007, Luminescence, 22, 251-265.
12. Wheeler, W.M., and Williams, F.X. 1915, Psyche, 22, 36-43.
13. Broadley, R.A., and Stringer, I.A.N. 2001, Invert. Biol., 120, 170-177.
14. Edwards, F.W. 1933, Proc. Linn. Soc. Lond., 146, 3-10.
15. Norris, A. 1894, Entomol. Mon. Mag., 30, 202-203.
16. Richards, A.M. 1964, Stud. Speleol., 1, 38-41.
17. Hudson, G.V. 1926, Ann. Mag. Nat. Hist., 9, 667-670.
18. Hudson, G.V. 1890, Trans. N.Z. Instit., 23, 43-49.
19. Baker, C.H. 2002, A biological basis for management of glow-worm populations of ecotourism significance, Wildlife tourism research report (Cooperative Research Centre for Sustainable Tourism), No. 21.
20. Pugsley, C.W. 1984, J. Roy. Soc. N.Z., 14, 387-407.
21. Ohba, N., and Meyer-Rochow, V.B. 2005, Luminescent behaviour in the New Zealand Glowworm, *Arachnocampa luminosa* (Insecta; Diptera; Mycetophilidae). In Bioluminescence & Chemiluminescence, A. Tsuji, M. Matsumoto, M. Maedo, L.J. Kricka, and P.E. Stanley, (Eds.), World Scientific Publishing Co. Pte. Ltd, Singapore, 23-26.
22. Koilraj, A.J., Sharma, V.K., Marimuthu, G., and Chandrashekaran, M.K. 2000, Chronobiol. Int., 17, 757-765.
23. Hoenen, S. 2005, Eur. J. Entomol., 102, 663-668.
24. Biswas, J., and Ramteke, A.K. 2007, Biol. Rhythm Res., DOI: 10.1080/09291010701589978, http://dx.doi.org/10.1080/09291010701589978
25. Merritt, D.J., and Aotani, S. 2008, J. Biol. Rhythms, 23, 319-329.
26. Hudson, G.V. 1926, Ann. Mag. Nat. Hist., 9, 228-235.
27. Heiling, A.M. 1999, Behav. Ecol. Sociobiol., 46, 43-49.
28. Adams, M.R. 2000, J. Insect Behav., 13, 299-305.
29. Mansbridge, G.H. 1933, Trans. Roy. Ent. Soc. Lond., 81, 75-90.

Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Bioluminescence in Focus - A Collection of Illuminating Essays, 2009: 357-385
ISBN: 978-81-308-0357-9 Editor: Victor Benno Meyer-Rochow

19 Biophotons: A clue to unravel the mystery of "life"?

R. P. Bajpai
Sophisticated Analytical Instruments Facility, North Eastern Hill University
Shillong 793022, India

Abstract

This chapter summarises the evidence of ultraweak emissions of so-called biophotons in connection with living tissues irrespective of plant or animal origin and shows that biophoton signalling between living organisms can occur. Details are provided on how to measure and analyse biophoton emissions. The nature as well as salient features of the photon emissions are being discussed. Applications of ultraweak photon emissions are possible in the medical field and it is concluded that ultra-weak emissions of biophotons are representing one of the chief characteristics of "life". However, to what extent

Correspondence/Reprint request: Dr. R. P. Bajpai, Sophisticated Analytical Instruments Facility, North Eastern Hill University, Shillong 793022, India. E-mail: rpbajpai@nehu.ac.in

such signals can be controlled by an emitter and what machinery exists in the receiver to detect them, for the moment, remain unanswered questions. The essay ends with some speculation on the possibility of biophotons affecting the thinking, moods, and behaviours of human beings, linking philosophical visions of life with the physical world.

1. Historical perspective

Alexander Gurwitsch[1] was the first to try to understand the cause of coordinated nature of cell division in a developing organism and wondered if coordination is achieved by some form of radiation issuing from the developing organism[2]. He called this form of radiation mitogenic or cell division inducing and thought that the radiation should reveal itself by speeding up the rate of increase of cell division in a growing sample placed near a developing organism. Gurwitsch soon discovered a sensor of mitogenic radiation in the form of growing onion root tip where cells divide with higher frequency[3]. The tip of one root, the emitting source, was directed perpendicularly to a point close to the tip of second root, the detector. The rate of cell division was assessed under a microscope and was found to be perceptibly greater in the exposed region than on the side far away from the source. The effect vanished on insertion of a glass plate between the two roots but not on insertion of a quartz plate. Since glass absorbs ultra violet (UV) radiation while quartz is transparent to it, he suggested that mitogenic radiation probably contained UV radiation only. Gurwitsch found another detector – a growing yeast culture, which increased in turbidity as cells multiplied. The turbidity was measured by counting the number of cells in a block of yeast culture embedded in agar gel. UV radiation of weak intensity is also detected by the onset of growth in a bacterial culture. Gurwitsch determined the spectrum of mitogenic radiation with a quartz spectrometer and found links of different UV components with specific biological reactions. Gurwitsch also sought and found secondary mitogenic radiation, whose emission was stimulated by irradiation of a tissue with the primary emanation. These were remarkable results that failed to reproduce many times, perhaps because of the capricious nature of biological specimens and detectors. Hollaender and Claus[4] refuted the results and the refutation caused mitogenic radiation to become an undergrowth of science [2]. The interest in the subject continued to decline until the accidental discovery of weak emission of light from germinating plants by Colli and Fachini[5] using a photomultiplier tube. The intensity of emitted light was more than that in black body radiation but less than the intensity expected in forbidden transitions. The emission was therefore, called ultra weak photon emission. A photo multiplier tube is a non- biological detector, it is more

reliable and it gives reproducible results. Photomultiplier tubes made up of different materials are sensitive to electromagnetic radiation in different regions. There exist tubes capable of detecting UV or visible radiation of nearly similar intensities. This is in contrast to the biological detectors discovered by Gurwitsch, which were probably more sensitive to UV radiation than photomultiplier tubes but were too insensitive to visible radiation to detect the emission of light from living systems. Photomultiplier detectors made the detection of weak emission of light by biological samples a routine affair and rekindled the interest in the mitogenic radiation. Light emission was detected many times in many varieties of plants and in diverse species like yeast, helianthus, frog spawn, earthworms, wheat seedlings, and garlic (*Allium cepa*). Quickenden in Australia[6], Popp in Germany[7] and Inaba in Japan[8] fabricated highly sensitive dedicated photon counting systems with extremely low noise. The measurements with the dedicated systems established beyond doubt the phenomenon of ultra weak photon emission in almost all living systems from bacteria to human beings.

Ultra weak emission has intriguing features. The main intriguing features discovered in above measurements were universality, incessant emission, ultra weak intensity, unchanging average intensity and broadband spectrum mainly in the visible region. Universality, incessant emission and ultra weak intensity imply that the processes responsible for photon emission occur in every living system all the time but are either rare or involve macroscopic structures. The rare processes may originate from some imperfections in metabolic activities while the existence of macroscopic structures participating in metabolic activities is a new assumption. The two possibilities gave rise to imperfection and coherence theories of ultra weak photon emission respectively. The imperfection theory had many protagonists, the notable among them were Inaba[9], Quickenden[10] and Slawinski[11]. The lone notable protagonist of the coherence theory was Popp[12] but he never clearly specified the connection between coherence and macroscopic structure. The unchanging intensity of the signal is attributed to ambient amount of imperfection in imperfection theory and to long and almost non-decaying tail of hyperbolic decay in coherence theory. The emission in the visible range requires a mechanism to upgrade the radiation in infra red region obtainable from biochemical energy derived from (ATP→ ADP) and its variant reactions. The imperfection theory assigns the job of up gradation to radicals that initiate chain reactions based on reactive oxygen species. The coherence theory assigns it to a macroscopic structure whose parts coordinate biochemical energy reactions occurring at different space time locations. No specific scheme for the coordination has been formulated so far. It is pointed out that the coherence theory does not

preclude up gradation of energy with the help of radical reactions. The coordination needed in the successful implementation of any scheme may arise from the dynamical behaviour of macroscopic structure. It is obvious that above noted intriguing features cannot choose between imperfection and coherence theories. Popp therefore, investigated the behaviour of fluctuations in these signals by measuring photo cont distribution, the set of probabilities of detecting different numbers of photons in a small measuring interval called bin. Photo count distribution is expected to be normal in the imperfection theory but not in the coherence theory. Popp observed mostly Poisson and a few sub and super Poisson distributions. Popp considered these distributions only indicative of quantum nature because background noise was comparable to signals.

The convincing support to coherency theory came from the study of what Gurwitsch called secondary emanations. Strehler and Arnold[13] were the first to observe secondary emanations using a non-biological detector as afterglow in photosynthetic tissues of green plants after light illumination. The afterglow is observable for a long time and the phenomenon is called delayed luminescence. Delayed luminescence is not restricted to photosynthetic tissues but is a universal phenomenon of living systems though the strength of emitted signal is higher in photo synthetic systems. The phenomenon has also been observed in a few complex non-living systems[14]. The distinguishing feature of a delayed luminescence signal is the peculiar shape with two regions, decaying and non-decaying. The photon flux in the decaying region decreases by 2 to 3 orders of magnitude in a short time. The decaying region is followed by a long tail region in which the photon flux is fluctuating but remains almost constant on an average. The decaying region is easy to measure due to higher flux and further the background noise is negligible in this region[15]. This region has been measured in numerous systems by a large number of investigators. A decaying region of a photon signal is usually analysed to determine the decay constant and strength of its different exponentially decaying components. The analysis fails and yields inconsistent and unsatisfactory values of decay constants and strengths in delayed luminescence signals. A delayed luminescence signal is not separable into different component decays. The signal has a definite but peculiar shape that lacks exponential decay character. Any definite shape other than the exponential decay means that numbers of photons emitted at different time intervals are correlated. Some additional mechanism must operate in living system to ensure correlation in photon number during decay- a macroscopic time interval. The peculiar and definite shape rules out imperfection theory. The delayed luminescence signal of a living system is sensitive to many physiological and environmental factors

and can pick up minute changes in these factors. The sensitivity of the signal requires a linkage between metabolic activities and the additional mechanism. The sensitivity suggests many potential applications of the phenomenon of delayed luminescence. These applications have not been actualised because of our inability to extract relevant parameters of a signal lacking exponential decay character. We need a framework to describe the shape of such a signal. Popp proposed a phenomenological model, in which shape arises from dynamical evolution of photon field associated with a living system. The classical solution of the dynamics predicts hyperbolic shape of the photon signal. The model reproduces broad features of delayed luminescence phenomenon and assigns the asymptotic region of hyperbolic shape to ultra weak photon emission. The model integrates delayed luminescence and ultra weak photon emission. The integration is formally expressed by using a common word biophoton emission for the two photon emissions. The two photon emissions are identified by the adjectives light induced and spontaneous. Delay luminescence signal is light induced biophoton signal and ultra weak photon signal is spontaneous biophoton signal. The name "biophoton" emphasizes peculiar features and biological relevance of signals. The model made a paradigm shift of far reaching consequences. It was strongly resisted and its acceptance has been requiring more and more evidence. Popp has responded by measuring the delayed luminescence signal of many systems. He fine tuned the model to fit the measured data. The model correctly reproduces the initial decaying portion of a delayed luminescence signal and extracts four parameters from it. The sensitivity of parameters, particularly the one related to the strength of signal, has been put to use in actual applications with reasonable success. The success, however, has not given widespread acceptance to the model because of the ad hoc fine tuning.

The dynamical model proposed by Popp is solvable in quantum field theory. The solution of the photon field is a squeezed state with its specifying parameters[16] time dependent. The shape of the signal[17] has a simple expression containing four unknown parameters. The unknown parameters take real positive values depending on the initial conditions and emitting system. Different values of parameters give rise to different shapes. The model correctly reproduces the shapes of biophoton signals without any fine tuning. The model maps the shape of biophoton signal in its parameter space. Quite often, one combination of parameter plays the dominant role in the mapping. This combination of parameters not only measures the shape of a signal but provides an ordering of shapes. The success in explaining the shape demands investigation to justify the basic assumption of the model. The basic assumption is that a decaying biophoton signal is in a pure

quantum state. The validity of the assumption was demonstrated by measuring the probability of no subsequent photon detection of biophoton signal in a small interval[18]. The probability for various intervals in the range (10 μs -100 μs) was measured at different portions of the delayed luminescence signal emitted by a leaf. The measurements demonstrated the quantum nature of the signal. The tail region of the signal that corresponds to spontaneous biophoton emission was also included in the measurements. This region permits the measurement of the probabilities of detecting different number of photons in a measuring interval, called bin size. The measured probabilities give valuable information about the quantum state of the signal. The expressions of various probabilities in the squeezed state with time dependent parameters are too complicated to calculate. We therefore, approximate the exact squeezed state solution in the region of spontaneous emission by an effective squeezed state specified by time independent parameters. The approximation simplifies the calculations and permits the estimation of the time independent parameters of squeezed state from the photo count distribution at any bin size. The photo count distributions in a signal measured at 14 bin sizes in the range (50ms-500ms) yielded same estimates of squeezed state parameters[19]. The result justifies the approximation and provides an irrefutable proof of biophoton signal in a pure quantum state. The pure quantum state of biophoton signal implies the existence of a quantum structure in the living system emitting the signal because a pure quantum signal can emanate only from a quantum structure. Further, the parameters extracted from of a biophoton signal- four from the decaying region and four from the spontaneous emission region- are attributes of the living system and its quantum structure. These are holistic attributes that open up new dimensions of living systems to study and investigate.

2. Frameworks of analysis

A biophoton signal is experimentally determined by counting the number of photons detected in contiguous bins of sizeΔ. Let the number of detected photons in a bin around the time t be n (t). The set {n (t)} of measurements at times separated by Δ is the digitised signal, whose shape gives the dependence of n (t) on t. A theoretical model prescribes the functional dependence of n (t) on t in terms of a few unknown signal specific parameters and provides a framework to analyse digitised signals. The analysis consists of estimating the parameters of a digitised signal. The estimation of parameters is easier in the region in which n(t) varies with time e.g. the decay region of a biophoton signal. The decay region is used for determining decay

parameters of signals. The region in which n(t) does not vary with time can estimate only one combination of parameters. However, if n(t) fluctuates in this region and its fluctuations have definite structure, then the fluctuations provide some additional information about the signal. The statistical moments characterize the structure inherent in fluctuations and variance, the second moment, is the most revealing moment. A quantity Q equal to (variance/mean -1) was earlier used for indicating the presence of structure in fluctuations[20] and ascertaining its nature. The set of probabilities of detecting different number of photons in a bin, called photo count distribution, can also characterize the structure inherent in fluctuations. This characterization is more helpful in extracting information from a quantum signal, in which various probabilities of detecting photons are theoretically calculable. Photo count distributions can be measured for many bin sizes and all distributions should yield the same estimates of the parameters of the signal because bin size is a kinematical quantity and should not affect the estimates of the parameters of a signal. Bin size should not affect the estimates of parameters in decaying region as well. Robustness of estimation to change in bin size is the test of the validity of model and the correctness of the framework of description. Three frameworks have been used in the analysis of biophoton signals. The important features of description in these frameworks are given.

2.1. The conventional framework

A photon signal from an isolated system arises from the probabilistic decay of many independent units in some excited state. The depletion of the number of units in excited state with time confers shape to the signal. Shape is a statistical feature, whose character has to exponential and decaying. A living system may have more than one type of decaying units. The shape of biophoton signal originating from exponential decay of m types of units is given by

$$n(t) = \sum_1^m C_i e^{-\lambda_i t} + C_0 \qquad (1)$$

, where C_0 is the strength of background contribution and C_i the strength of i^{th} decay mode with decay constant λ_i. A mode corresponds to the decay of one type of units and has a definite frequency given by the energy difference between the two states of the units. The decay constant of a mode is related to the lifetime and width of the decaying state. The lifetime determines the duration of decaying region. The durations of light induced biophoton signals suggests that the contributing modes have life times of the order of 1s, which implies sharp decaying states and discrete emission spectra. Continuous

spectrum with broad structures requires large number of decays, whose strengths if adjusted can mimic a signal with hyperbolic decay character. The adjustment will break down if some components are filtered out. The remnant signal obtained after filtering out some components will have to show exponential decay character in this framework. The framework cannot describe non-decaying spontaneous biophoton signals for a non-decaying signal can arise only if decaying states of every mode are continuously replenished. The photo count distribution in the framework is expected to be normal or Bose Einstein.

2.2. The framework of Popp

Popp[21] suggested that the shape of a light induced biophoton signal is the consequence of dynamical evolution of a photon field given by the Hamiltonian

$$H = \frac{p^2}{(1+\lambda t)^2} + \frac{1}{2}(1+\lambda t)^2\omega^2 q^2 \qquad (2)$$

, where p and q are usual canonically conjugate momentum and position variables of photon field of frequency ω and the constant λ determines the strength of damping. It is the Hamiltonian of a damped harmonic oscillator with time dependent mass and frequency terms. The solution of the classical equation of motion is

$$q = \frac{q_0}{(1+\lambda t)}\sin(\omega t + \theta) \qquad (3)$$

, where q_0 and θ are integrating constants. The integrating constants are signal specific parameters. The solution has a stable frequency but its amplitude hyperbolically damped. The energy of the oscillator is proportional to the square of amplitude. The energy is equated to the number of photons multiplied by Planck's constant and frequency and it gives $n(t)=N_0/(1+\lambda t)^2$ as the shape of signal. The analytical expression does not quite reproduce the shape of observed signals and hence the expression is arbitrarily modified to

$$n(t) = N_0(1+\lambda t)^m + A_0 \qquad (4)$$

, where N_0, λ and m are signal specific parameters giving respectively the strength, damping, and shape of the signal and A_0 gives background contribution and should by measuring system specific but is not. The new expression correctly reproduces small initial portion of the decay region. The value of m in signals of different living systems is in the range $1 \leq m \leq 2$. The

model has been many successful applications based mainly on the sensitivity of N_0, the strength of signal, to various factors. NB1, the number of counts detected in the first bin, is also a measure of signal strength. It is directly measurable and is equally effective in various applications. Popp suggested photo count distribution to be Poisson and quantum state to be a coherent state.

2.3. The framework of Bajpai

It is a quantum field theory framework[23] that implements the proposal of Popp. The framework describes an electromagnetic field interacting with living system in the interaction picture. The description has two elements, interacting photon field operator and state vector. The Hamiltonian of eq.(2) determines the dynamical evolution. The dynamic evolution of interacting photon field is equivalent to the evolution of a free quasi photon field. The creation and annihilation operators of quasi photon field are unitarily related to creation and annihilation operators of free photon field by time dependent coefficients. The sate vector of the interacting field is and remains a coherent state of quasi photon field or equivalently a squeezed state of free photon field with time dependent specifying parameters. The time dependence of the expectation value of number of photons in the state is given by the following expression

$$n(t) = B_0 + \frac{B_1}{(t_0+t)} + \frac{B_2}{(t_0+t)^2} \qquad (5)$$

, where $t_0=\lambda^{-1}$ and B_i's are three algebraic expressions of the parameters defining a squeezed state, mode frequency ω and λ. B_i's are independent and take positive values only. Eq.(5) is a description of biophoton signal with four signal specific parameters. The description contains a decaying and a non-decaying component. The decaying component is identified with delayed luminescence and non-decaying component with spontaneous emission. The estimate of B_0 from a digitised signal is the sum of background noise and contribution of spontaneous biophoton emission and is expected to be signal specific. It is further pointed out that there are many damped harmonic oscillators that have frequency stable classical solutions. The generic frequency stable solution is

$$q = \frac{q_0}{f(t)}\sin(\omega t + \theta) \qquad (6)$$

, with any well behaved function f(t) non zero for positive t. A quantum field theory framework can be constructed around the generic solution. The Hamiltonian of the generic solution permits recasting into the Hamiltonian of

free quasi photon, whose coherent state is and remains a squeezed state of photon. The shape of photon signal obtained in the dynamic evolution has following form:

$$n(t) = B_0 + B_1\left(\frac{f(t)'}{f(t)}\right) + B_2\left(\frac{f(t)'}{f(t)}\right)^2 \quad (7)$$

Eq.(7) permits a large variety of shapes. The choice $f(t)=\lambda^{-1}+t$ gives the earlier form of eq.(5). The probabilities of detecting different number of photons are not easily calculable in the squeezed state with time dependent parameters. The problem of calculation is circumvented by assuming that the time dependencies of parameters become very weak and ignorable in the non-decaying. The assumption makes the probabilities of detecting different number of photons calculable.

3. Materials and methods

The measurement of both light induced and spontaneous biophoton signals is non invasive. The apparatus required in the measurement are a measuring chamber, a source of stimulating radiation and a detector. The measuring chamber is light proof with provision for the entry of stimulating radiation and the exit of emitted photons. The stimulating radiation enters through a window or fibre cable and its duration is controlled by a shutter. The exit is usually through a quartz window, which is transparent to visible radiation but opaque to UV radiation. Sample is placed in the chamber in a sample holder made of quartz or metal that does not emit visible range photons after exposure to stimulating radiation. The stimulating radiation is obtained from an ordinary 100-250 watt lamp, a monochromator, or a UV lamp. UV lamp is used mainly for stimulating cultured cells, which do not get stimulated by visible radiation. The time of stimulation is adjustable and varies from 5-10s. The detector is a photo multiplier tube capable of detecting electromagnetic field of energy around 10^{-16} watt. The photomultiplier tube has a large single scintillation crystal sensitive over a broad range. The actual range depends on the material of crystal. The photomultiplier tube sensitive in the range (350-800nm) appears most appropriate to measure biophoton signals of botanical samples while the tube sensitive in the range (300-600nm) is for biophoton signals of human subjects. The detector operates in single photon mode and counts the number of photons in large number of contiguous bins. Both, bin size and number of bins are adjustable. Spectral decompositions of a biophoton signal are obtained by inserting band pass or interference filters.

The signal emitted by a living system just after stimulation is very intense and can blind a photo multiplier tube. The measurement of light induced biophoton signal is, therefore, made after a delay of 10ms. The delay eliminates contributions of fluorescence signals of different materials. The mechanical shutters controlling the entry and exit makes the number of counts of a few initial bins erroneous in some measuring systems. The erroneous counts in these systems are ignored and it introduces a little more delay in measurements. One usually measures the decaying part of a signal for 3-5 minutes using bins of sizes in the range 10-200ms. The copies of the decaying part for measurements with higher bin sizes are obtained by merging the observed counts in appropriate number of contiguous bins of lower sizes. We use the copies of the signal obtained by merging up to 10 bins in the estimation of decay parameters. The calculated values in a copy are obtained by integrating the expression over the bin size i.e. $\int n(t)dt$. Estimation is done by least square minimisation giving equal weight to all copies. The decay parameters should not depend on the bin size and we use it as a criterion to check the correctness of a framework. The framework (2c) nearly fulfils this criterion while the other two frameworks show large variations in estimated parameters with bin size. The framework (2c) will be used in subsequent discussions. It is pointed out that many successful applications of framework (2b) are based on the sensitivity of overall strength of the signal to physiological and environmental factors. There are many measures of overall strength; the total number of counts in a portion of the signal of any length provides a measure. All measures are not equally efficacious in bringing out the sensitivity of overall strength. The most efficacious measure is the number of counts in the first bin, NB1. The rapid decrease in the signal with time implies that smaller the bin size, greater the sensitivity of NB1. The measurement of the decaying portion can be repeated every 3 minutes and of NB1 every minute.

The measurement of spontaneous biophoton signal of a sample is made after eliminating the effect of its stimulation by laboratory illumination. The sample is therefore, kept in the dark chamber for at least half an hour before the start of measurements. Photons are counted in a large number of bins of same size. The number of bins and their size depend upon the stability of a system. The measurements in 30000 bins of 50ms size take 25 min. The sample should remain stable and unchanging in this duration, which is true for samples of lichens but not for human subjects. The measurements with bin size 50ms are used for determining digitised copies of the signal for measurements with bin sizes varying from 50ms to 500ms in steps of 50ms. Human subjects become restless and tense after a few minutes. The duration of 3 min appears optimal for measurements with human subjects and

measurements in 3600 bins of 50ms can be made in this duration. The measurements for background noise are made with the same protocol but without a sample. The probabilities of detecting different numbers of photons in a bin are obtained from every digitised spontaneous biophoton signal.

The probability of detecting n photons in a bin is represented by P^n for $n = 0,1, \ldots n_{max}$, where n_{max} is the maximum number of photons detected in any bin. The photo count distribution P is the set of probabilities $\{P^n\}$. Photons detected in a bin come from two independent sources, biophoton signal and background noise. As a result, the observed probabilities and signal strength k given by the average counts in a bin are different from the probabilities and signal strength of biophoton signal. A subscript obs, bg, or sig is added to probabilities and signal strength to indicate whether probabilities and signal strength are of observed signal, background noise or biophoton signal. The properties with the subscripts obs and bg are measurable and with the subscript sig are calculable. The three sets of properties are related. The observed signal strength is the sum of other two signal strengths

$$k_{obs} = k_{sig} + k_{bg} . \tag{8}$$

Similarly, the observed probabilities are convolution of signal and background probabilities

$$P_{obs} = P_{sig} \otimes P_{bg} , \tag{9a}$$

which expresses the following algebraic equations:

$$P^n_{obs} = \sum_{j=0}^{n} P^j_{sig} P^{n-j}_{bg} . \tag{9b}$$

The algebraic equations can be solved recursively to obtain any P^n_{sig} starting with n=0. The procedure fails quickly due to compounding of errors of P^n_{obs} and P^n_{bg}. The error in any P^n_{sig} is higher than in P^n_{obs} and their difference increases with n. The probabilities and signal strength of a biophoton signal are calculated by assuming the signal to be in a squeezed state. A squeezed state $|\alpha, \xi\rangle$ is specified by two complex parameters α and ξ or equivalently by four real parameters, the magnitudes and phases of the two complex parameters, i.e. $\alpha = |\alpha| \exp(i\phi)$ and $\xi = r \exp(i\theta)$. Every property calculated in the squeezed state $|\alpha, \xi\rangle$ is expressible by a function of four parameters. The calculated expression of signal strength k_{sig}(cal) is:

$$k_{sig}(cal) = |\alpha|^2 + \sinh^2 r \tag{10}$$

$k_{sig}(cal)$ is equated to k_{sig} that is well determined and has very small error in a signal of constant average intensity. Eq.(10) then becomes a constraint relation and it reduces the independent squeezed state parameters to three by expressing $|\alpha|$ as a function of r and k_{sig}. The independent parameters are taken to be r, θ and ϕ. The calculated expression of probability P^n_{sig} (cal) of detecting n photons in a bin in the squeezed state is given by

$$P^n_{sig}(cal) = \left|\langle n|\alpha,\xi\rangle\right|^2 \tag{11}$$

, where $|n\rangle$ is an eigen state of the number operator with eigen value n. The scalar product of number and squeezed states for a single mode photon field is given by[22]

$$\langle n|\alpha,\xi\rangle = \frac{1}{\sqrt{n!\cosh r}}\left[\frac{1}{2}\exp(i\theta)\tanh r\right]^{\frac{n}{2}} \exp\left[-\frac{1}{2}\left(|\alpha|^2 + \alpha^{*2}\exp(i\theta)\tanh r\right)\right] \times H_n\left[\frac{\alpha+\alpha^*\exp(i\theta)\tanh r}{(2\exp(i\theta)\tanh r)^{\frac{1}{2}}}\right] \tag{12}$$

,where α^* is the complex conjugate of α and H_n is the Hermite polynomial of degree n. The least square fitting of P^n_{sig} 's to $P^n_{sig}(cal)$'s can in principle estimate three unknown parameters but high errors in P^n_{sig}'s make the estimation unsatisfactory. The problem of high errors is reduced[23] in the estimation based on the least square fitting of P^n_{obs}'s to $P^n_{obs}(cal)$'s because the error in $P^n_{obs}(cal)$ is much smaller than in P^n_{sig}. $P^n_{obs}(cal)$ is calculated by convoluting $P^n_{sig}(cal)$ and P^n_{obs}

$$P^n_{obs}(cal) = \sum_{i=0}^{n-i} P^{n-i}_{sig}(cal) P^i_{bg} \tag{13}$$

$P^n_{sig}(cal)$ is an exact expression and is without any error. Convolution in eq.(13) compounds only the small errors of P^i_{bg}. The three parameters are estimated by minimizing the function

$$F = \sum_{\text{bin size}} \sum_{i=0}^{n_{max}} \left(P^i_{obs}(cal) - P^i_{obs} \right)^2 \qquad (14)$$

The summation over bin size ensures that parameters common to all copies of signal obtained by merging the counts of contiguous bins are estimated. The minimum value F_{min} obtained is an indicator of the quality of estimation.

4. Salient features of biophoton signals

Fig.1 pictorially summarizes the universal features of light induced and spontaneous biophoton signals. The figure depicts a hypothetical biophoton signal, whose fluctuations have been smoothed out for the sake of clarity. Fluctuations are observed in measurements with any bin size. The smoothed out signal is a robust curve and nearly same curve is obtained in repeating the measurements. The figure has four distinct regions- pre stimulation, during stimulation, decaying and tail. The flux of emitted photons is almost unchanging in the pre-stimulation and tail regions but it changes rapidly during stimulation and decaying regions. The regions of unchanging flux represent spontaneous biophoton signal and the decaying region light induced biophoton signal or delayed luminescence. The flux decreases continuously in the decaying region. The duration of decaying region is a characteristic of

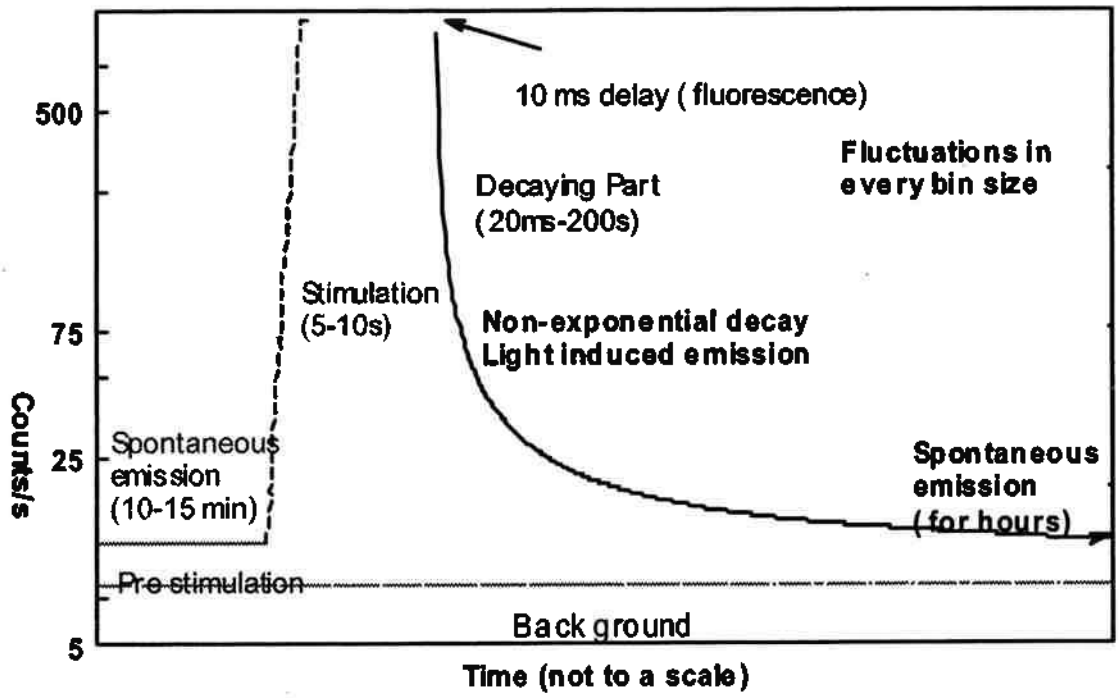


Figure 1. Typical shape of a smoothed out biophoton signal: A hypothetical signal summarising the main features of the different regions of biophoton signals is drawn not to a scale.

the emitter. It ranges from 200ms to 200s in different living systems. The figure mentions 10-15 min as the duration of pre stimulation region but it can be much larger in quasi stable systems that have very slow growth and decay rates. The depicted flux of spontaneous biophoton signal is 12.2counts/s and of background noise 8.5counts/s. The emitted photon flux is undetectable during stimulation as it is difficult to distinguish between photons emitted by a sample and photons stimulating the sample. The emitted flux is, therefore, depicted by broken lines in the figure. The expected flux likely to be emitted increases rapidly during stimulation and becomes too large in a short time. Too large flux is depicted by a gap in the figure. The gap extends for 10ms in the post stimulation region. The distinguishing feature of a biophoton is the lack of exponential decay character in both, decaying and non-decaying regions of the signal. Different living systems emit similar biophoton signals. The signals however, differ in strength and shape. The shape and strength seem to identify a living system. The shape and strength are sensitive to many factors, physical, physiological, genetic, emergent and holistic.

The digitised shape of a signal N (Δt, t) is determined by repeatedly detecting the number of photons in a fixed interval. The number N of photons detected in the interval depends on its duration Δt and the time of its commencement t measured after the stimulation of the sample by light. The number of photon detected in the first interval N (Δt, 10ms) is given a special name NB1, where 10ms indicates the delay in the measurement. NB1 is substantially higher than background noise and shows saturation effect with intensity of stimulating light I and the duration of exposure τ. NB1 initially increases with I and τ but attains its saturation value in less than one second of exposure to normal laboratory illumination. The saturation value is observed over wide ranges of I andτ. The other values in the digitised shape N (Δt, t) do not follow NB1 before saturation but do attain stable values after saturation. Repeated measurements on a sample yield same stable values of NB1 and N (Δt, t). Only stable values will, henceforth, be considered. The excitations of a sample by light of different wavelengths yield different values of NB1. The dependence of NB1 on the wavelength of excitation λ_{exc} is given by a smooth curve and that has broad structures[24]. The curve is called excitation curve. Different samples have different excitation curves. NB1 in a monochromatic stimulation is smaller than in the white light stimulation. The sum of the values of NB1 obtained in monochromatic stimulations of two or more wavelengths is greater than the value of NB1 obtained in the stimulation containing those wavelengths.

Spectral decompositions of the signal are obtained by inserting filters prior to detection. The spectral decompositions indicate broadband emission

spectrum. The typical percentages of red, green and blue spectral components in NB1 of a sample of young *Actebularia* obtained with the help of band pass filters are 91%, 7% and 2%. The relative percentages change with the age of the sample and are also different for different species. The influence of the wavelength of excitation λ_{exc} on emission spectra is weak. In particular, the delayed luminescence signal emitted by a sample excited with red light has blue component as well. The relative percentages in the spectral components of N (Δt, t) also have similar behaviour. Various spectral decompositions lack exponential decay character.

The temperature of the sample affects NB1 and N (Δt, t) of its biophoton signal. The effects on NB1 and on the counts in various regions of N (Δt, t) are different. NB1 is maximally affected by temperature. The variation of NB1 with temperature from 1°C to 40°C was studied in samples of a lichen species *Parmelia.tinctorum* using white light stimulation. NB1 decreased monotonically and nonlinearly with the temperature of the sample in the range 1°C to 22°C. NB1 at 22°C was nearly one fifth of its value at 1°C. The value of NB1 was specific to the temperature of the sample in the range (1°C - 22°C). NB1 seems capable of sensing the temperature in this range with an accuracy of 0.1°C. NB1 increased with temperature beyond 22°C, peaked at 25°C and then decreased slowly till 40°C. The variation of temperature in this range affected NB1 in a hysteresis like manner. Temperatures beyond 40°C inflicted fatal damage to the sample. NB1 of a damaged sample was much smaller and cooling a damaged sample to lower temperatures did not restored earlier values.

NB1 and N (Δt, t) depend on many other factors as well and sense changes in those factors. NB1 is the most discriminating parameter but it uses only the information contained at a single point of the decay curve. The decay curve has mainly been used for estimating the smoothed out value of NB1. The estimation procedure basically utilises only a small portion of the decay curve because of the rapid initial decay and yields an inferior estimate of NB1 to that obtained by averaging the results of its repeated measurements. The decay curve has been measured in many systems but the data are not in public domain, only the results analysed in the framework of Popp are. The results establish the capabilities of NB1 and N (Δt, t) to identify different physiological states of a living system. The capabilities have many potential applications. The information contained in the entire decay curve is utilised in the framework of Bajpai. The analysis in this framework has been done in few systems only and it reveals that NB1 and $\log(B_1/B_2)$ are very sensitive while B_0 and t_0 are less sensitive indicators of various factors determining a physiological state. Both NB1 and $\log(B_1/B_2)$ have higher values in signals of healthy living systems; sickness, stress and deprivation seem to reduce their values.

Some non-living complex systems also exhibit delayed luminescence and emit signals lacking exponential decay. The decay curve of these systems can also be analysed similarly. The parameters extracted from the decay curve can identify different states of complex systems. An important application worth mentioning is the capability of the analysis to differentiate between sugar globuli medicated with high potency homeopathic remedies and placebo sugar globuli [14]. The parameters extracted from N (Δt, t) in the two types of samples are different. The parameters of medicated globuli and not of placebo globuli change in presence of specific frequency magnetic field. The observed changes were reversible and repeatable.

The study of fluctuations provides information about the quantum entity present in a living system. This type of study can be made only in the region of spontaneous biophoton emission. The study shows that the photons emitted in different modes (or frequencies) are strongly coupled[25]. Strong coupling implies that the probabilities of detecting different number of photons of any mode in a bin depend only on the signal strength of the mode and the probabilities of different modes are equal to those of composite signal with same strength. The measured probabilities in a photon signal provide information about its quantum state, which in case of a squeezed state is completely determined by the signal strength and three other parameters r, θ and ϕ. The four parameters have been determined in photon signals spontaneously emitted at different anatomical locations of human subjects[26]. The parameters show interesting patterns. The signal strength is different at different anatomical locations in a healthy human subject but other three parameters have same values. These parameters appear to have same values in every healthy human subject. All three parameters are affected by local injury, inflammation or sickness[27]. In contrast, practicing meditation over years affect r but not θ andϕ[28]. Fluctuations in the decaying region have been studied using bins of size 10 μs. The measurements in 1000 contiguous bins take only 10ms, in which duration the decay of light induced biophoton signals of photo synthetic systems can be ignored. The measurements determine the probability of detecting no photon in 10 μs reasonably well. These measurements have been repeated continuously over the entire decay region to obtain the dependence of probability of no photon detection on signal strength. The measured dependence demonstrates quantum nature of photon signal [18].

5. Implications and speculations

The experimental data summarized in the previous section provide ample evidence of the lack of the exponential decay character in biophoton signals emitted by isolated living systems. Only the framework of section 2.3 can

explain the smoothed out shapes of these signals. The framework envisages a dynamic origin of the shape. The observed shape manifests an evolving quantum squeezed state. The squeezed state in the spontaneous emission region is specified by four real parameters, which are estimated from the fluctuations of the signal in the region. The parameters estimated from the smoothed out signal and from fluctuations in the spontaneous emission region are new characteristics of a living system. The nature of new characteristics is holistic. They open up new planes of investigation and understanding. Let us dwell upon some obvious implications of the spontaneous emission of photons in squeezed state. As all living systems spontaneously emit fluctuating photon signals and fluctuations of the signal measured in whichever living system indicate a quantum squeezed state of the signal, one suspect that the emission of photon signal in a squeezed state is a unique feature of a living system. Non-living systems including non-living counterparts of living systems do not have this feature. A living system thus, differs from its non-living counterpart in two properties, "life" and biophoton signal. The two differing properties offer a chance to remove the basic objection against treating living system as physical system. The objection stems from the fact that two physical systems cannot differ in one property alone and there has to be at least another distinguishing property law like related to the first property in all aspects. The law like relation in all aspects of two properties is called isomorphism. If the biophoton signal turns out to be isomorphic to "life", then both, a living system and its non-living counterpart, can be physical systems. We envisage that biophoton signal is indeed isomorphic to "life". Isomorphism makes biophoton signal and "life" equally mysterious. The unusual properties of biophoton signals indicate and are law like related to the unusual features of the "life". The isomorphism makes the study of the biophoton signal a powerful method for unraveling the mysteries of "life". The study should provide complete information about the properties of "life" and living systems. The isomorphism shifts the emphasis of investigations from living systems to non-living photons, which kindles the hope of measuring of every feature of "life" because every feature of the isomorphic photon signal is measurable. Quantum nature of biophoton signals makes "life" a quantum phenomenon. The restriction of quantum state of photon signal to squeezed states considerably reduces the complexity of "life" for all features of "life" have to be expressible by the values of four real parameters. The observation of quantum biophoton signal for macroscopic time implies that quantum phenomenon responsible for "life" remains stable for macroscopic time. These are broad implications that emanate from the following four ingredients of the envisaged isomorphism:

1. Biophoton signal emitted by a living system is a quantum photon signal.
2. The quantum photon signal is in a squeezed state.
3. The squeezed state is specified by four measurable parameters that take continuous values.
4. The quantum photon signal exists for macroscopic time.

The ingredients are expressed as observable features of biophoton signals. The observing of the features establishes and tests the validity of envisaged isomorphism. The features were observed in signals emitted by many samples of the three species of lichens and in signals emitted by human subjects at various anatomical locations. It is ample evidence and experimental support of isomorphism. Each feature on its own has profound implications. The first feature is the most crucial ingredient that necessitates a radical departure from the conventional picture of photon emission. The conventional picture visualises photon emission of an isolated system in the transition from higher energy state to lower energy state of a large number of independent units-atoms, molecules or more complex structures. The transitions of different units are probabilistic, which causes photon signal to decay exponentially due to depletion of units in the higher energy state. The independence of units rules out correlation among photons of the emitted signal. A biophoton signal does not have exponential decay character and its photo count distribution exhibit specific type of correlation. The conventional picture cannot be valid in biophoton emission. Biophoton emission must occur through a holistic mechanism that operates during the lifetime of a living system. The material constituents participating in the holistic mechanism must make up a quantum entity because only a quantum entity emits a quantum photon signal. The first feature therefore implies the existence a composite quantum structure of participating constituents. It will be called quantum entity.

Molecular biology stipulates all biological properties to originate from and be expressible by biomolecules. The quantum entity should therefore, be a composite quantum structure of bio-molecules. A composite quantum structure can have two types of properties, local and holistic. A local property depends on individual constituents but not on their specific states in the composite structure. A local property is therefore, expressible in terms of the properties of its microscopic constituents and is called microscopic property. Quantum framework is not necessary for its description. The classical reductionist framework of molecular biology can correctly describe all local properties of the quantum entity. Local properties do not reveal the presence of quantum entity. A holistic property depends on individual constituents and also on the state of the composite structure. The dependence of a holistic

property on the state of composite structure is correctly understood only in the quantum framework. The classical framework has to invoke ad hoc correlations among constituents to describe this dependence, which makes the description macroscopic. Holistic properties are therefore, called macroscopic properties. There is no origin of invoked correlations but it appears knowable in some properties and unknowable in others. The perception of origin divides the holistic properties in two classes, psyche and consciousness. The correlations invoked to describe the properties of psyche class can arise from exchange of information through a physical signal and hence the origin of these correlations appears knowable. Such a signal has not been discovered so far. Perhaps, it is a figment of imagination and exchange of information does not occur. The classical framework will always encounter an unbridgeable gap between perception and reality of the properties in the psyche class. The gap was visualised earlier and called psychosomatic gap. The correlations invoked to describe the properties of consciousness class can arise from exchange of information in a mode that either violates a fundamental law or requires superluminal communication and hence the origin of these correlations appears unknowable. The properties of consciousness class were, therefore, thought to belong to a reality beyond science. The elusive origin of invoked correlations is the biggest problem in understanding holistic properties of a living system. The problem arises from the use of classical framework. The quantum framework resolves the problem. Holistic properties are properties of a composite quantum entity and the correlations invoked in the classical framework contain information about the quantum state of the composite entity. Incidentally, local properties are holistic properties not requiring any correlation. The first feature thus implies three classes -microscopic, psyche and consciousness- of properties in living systems. Some living systems show another class of properties that are incomprehensible in the classical framework and require additional inputs besides biomolecules and correlations. These were also included in the consciousness class. The use of the quantum framework necessitates the division of the erstwhile consciousness class into two sub classes, soft and hard. The properties of the soft consciousness class are comprehensible in the quantum framework and but not of the hard consciousness class.

Mood of a living system is another ramification of the quantum entity. Mood is an attribute ascribed in the classical framework to a living system whose properties are different at different times or situations without an obvious reason. The quantum framework ascribes different properties to different states of the quantum entity. Many quantum states are available to a quantum entity and these states have different properties. A quantum state

can be stable or fickle to external noise. The quantum entity in the stable state will have same properties at different times or situations but not in the fickle state. The classical framework does not differentiate among various quantum states and is forced to introduce an extraneous concept of mood to account for changes in the properties of quantum entity in a fickle state due to external noise. The envisaged isomorphism permits the detection of changing states of quantum entity by changes in squeezed state parameters of biophoton signal. It makes the mood measureable in living systems.

A quantum entity will participate in biological processes. Some processes may take quantum route, in which every step in a process is a quantum transition. Quantum route implies massive parallel processing, which means quantum processes are faster and efficient. A biophoton signal has to emanate from biological processes taking quantum route because of its quantum nature. The ubiquitous presence of biophoton signal requires these biological processes to occur at all times in every living system. The fundamental biological processes of transcription, replication and protein synthesis occur at all times in every living system and we suspect them to be responsible for biophoton emission. The suspicion requires them to take quantum route. Quantum route for fundamental biological processes was speculated earlier for explaining the basic facts of genetic code, namely occurrence of four types of nucleotide bases, codons made up of three nucleotides and twenty amino acids. The explanation hinges on quantum selections made by nucleotides and codons. A nucleotide makes quantum selection in one transition and is able to select the desired nucleotide from four nucleotides and not two nucleotides allowed in the classical selection. Similarly, a codon makes quantum selection in three transitions and is able select the desired amino acid from among twenty possible amino acids and not eight amino acids allowed in the classical selection. The basic facts of genetic code merely reflect optimal utilisation of resources using the most efficient selection machinery. A necessary condition for operating the quantum selection machinery is the existence of objects participating in selection processes in pure quantum states. Nucleotides, codons and amino acids should be either in pure quantum states or the constituents of a composite structure in a pure quantum state. The latter possibility probably occurs as it leads to the existence of quantum entity. All constituents of the composite structure need not show quantum character all the times, only the constituents involved in selections at an instant need show it. The relaxed requirement permits to build a model of quantum entity in the classical framework. The constituents of the quantum entity acquire and loose quantum character depending on the dynamical requirement in the model. The model assumes two states of different characters of nucleotides, amino acids and codons; one

state has classical character and the other has quantum character. The state with classical character has lower energy and a constituent makes a transition to higher energy state of quantum character after extracting requisite energy from the usual biochemical machinery. The biochemical machinery increases the number of constituents in states showing quantum character to such an extent that they form a macroscopic object called quantum patch. A living system has many quantum patches distributed throughout its body. Many constituents of a quantum patch simultaneously make transition to their classical states by emitting photon. It is a possible mechanism to up convert biochemical energy. A quantum patch makes the transition to classical states of its constituents after quantum selections and also because of de-cohering interactions with local environment. Both factors restrict the growth of number and size of quantum patches. The state of quantum entity determines the distribution and sizes of quantum patches. The assembly of the quantum patches makes up the quantum entity. The distribution and sizes of quantum patches determine the spectral composition of its biophoton signal. Similar spectral composition of biophoton signals suggests similar distributions of number and sizes of quantum patches. Similar distributions occur because of similar local environments in different living systems. Biophotons in the model originate mainly in the regions where transcription, replication and protein synthesis occur.

The second feature, namely squeezed state of quantum biophoton signal, is established from the photo count distributions measured in a biophoton signal at many bin sizes. The photo count distributions with different bin sizes yield nearly same estimates of the parameters specifying a squeezed state. The photo count distributions with various bin sizes in the range (50ms-6s) were measured in the biophoton signal of a sample of lichen over period of more than 5h. All of them suggested the same squeezed state of the signal. Another reason for the squeezed state is the non-exponential decay of two to three orders of magnitude in the intensity of a light induced biophoton signal. The large decay is obtainable in the evolution of squeezed state but not of a coherent state in the quantum framework. This the only model that successfully reproduces all aspects of light induced and spontaneous biophoton signals in a unified scheme. A squeezed state is a minimum uncertainty state, propagates with very little expense of energy and is detectable even if its energy is below the noise level. The emission of quantum photon signal in a squeezed state raises many questions. How does a living system generate such a photon signal and why? What is the role of the signal in establishing coherence, long range order and stability in living systems? Are living systems aware of the possibility of almost lossless information transfer to long distances by biophoton signals? Do living systems

extract and decipher information contained in biophoton signals? The answers of these questions will have many implications. Even the questions suggest clues for understanding "life" in its myriad manifestations. The first two questions point out the need of more theoretical investigations for building a coherent and stable quantum entity around electromagnetic field in a squeezed state. The other two questions suggest the need of more phenomenological investigations at this juncture particularly of the responses of living systems to biophoton signals. A change in the rate of cell division in response to specific biophoton signal has been observed in onion roots, yeast culture and amphibian eggs[29]. The other living systems may respond differently and there could be better ways of detecting responses. The technique of delayed luminescence appears promising for it can detect minute changes in a living system. If the response of a living system persists for a few minutes after its interaction with a biophoton signal, then the technique can measure this response. The technique shows the positive influence of a psychic healer on a water starved sample of lichens from a distance. The healer probably beamed specific biophoton signal that alleviated the problem of water starvation.

The third feature state the fact that squeezed state needs only four parameters for its specification. It reduces the complexity of living system to four measurable attributes. The continuous values allow the parameters to faithfully capture the immense diversity of living systems and their moods in the envisaged isomorphism. Mood is used in generic sense and includes all holistic properties e.g. health, vivacity, germination capacity, etc. One expects to find species specific patterns in quantum attributes; the attributes of all members of a species may lie in definite ranges. One also expects to calibrate attributes of a living system for measuring any holistic property. The information in a squeezed state can be coded in its four parameters, which means that the information carrying capacity of a signal in a squeezed state is four fold to that of a signal in a coherent state. The estimates of squeezed state parameters are different for different bin sizes in some biophoton signals. These signals are not in squeezed states. They probably indicate ill health.

The fourth feature- the stability of quantum photon signal for macroscopic time- is difficult to comprehend and implement in the classical framework. The feature allows the determination of photo count distribution and estimation of squeezed state parameters. Photo count distribution has no meaning if the signal changes during measurement. The living system emitting a stable signal or rather the quantum entity responsible for emission has to be stable as well. Two stabilities- of biophoton signal and of quantum entity-are associated with every living system. Only the framework of quantum field theory can implement both stabilities, still the cause and effect

linkage between the stabilities is visualized in the classical framework. There are three possibilities of linkage: 1. The stability of biophoton signal is primary and the instructions/information transmitted by it to spatially separated quantum patches stabilizes the quantum entity. 2. The stability of quantum entity is primary while biophoton signal arises from its spontaneous acts and reflects its stability. 3. The stabilities of photon signal and of quantum entity are at the same footing, which happens if biophoton signal and quantum entity are in an entangled states. The mechanism for implementing any possibility is neither known nor speculated but the linkage in every possibility shifts the emphasis of investigations from living system to non-living photons. The last possibility is philosophically more appealing. The possibility implies two equivalent descriptions of a living system, one based on the properties of quantum entity i.e. the properties of matter and the other based on the properties of photons i.e. the properties of field. The state of a living system can be ascertained either by observing its matter content as is done in various pathological and diagnostic tests or equivalently by determining the squeezed state parameters of its biophoton signal. Further, the state of a living system can be changed from a sick to normal state by manipulating either matter or entangled field or a combination of the two. The corrective measures in modern medicine are based only on the manipulation of matter but corrective measures based on the manipulation of field should be equally effective. The optimum strategy for managing a sick state may involve manipulation of both types. The reason for the success of alternative therapies in some sick subject may lie in inadvertent manipulations of biophoton fields. There is a need to study the relation of quantum parameters with the state of health and to find ways of altering quantum parameters of the biophoton signal of a sick subject.

The living systems play only a passive role in above implications. They are treated like a black box emitting biophoton signal in squeezed state. But living systems play active roles as well. The active roles have many more implications and permit new uses of biophoton signals. A living system playing active roles needs to have the capability to detect a biophoton signal, to measure its properties, to decipher the information contained in the properties and to beam biophoton signals with desired properties if needed. These are permissible physical capabilities but the evidence of the existence of these capabilities in living systems is scanty and anecdotal. The implications of capabilities are quite often considered speculative. The capability to detect biophoton signal has been demonstrated in onion roots, yeast cells and amphibian eggs. These system show measurable response to some but not all biophoton signals. The response only to selective signals implies that a living system responds only to biophoton signals whose

squeezed state parameters lie in specific ranges and the ranges of squeezed state parameters determine the type of a biophoton signal. There is enough experimental evidence to support that above quoted living systems have the capability to detect biophoton signals of the type a system emits and also of a few more types. The evidence is extrapolated to all living systems. Every living system detects biophoton signals similar to the one it emits but may not show measurable response of detection. The lack of measurable response is attributed to poor sensitivity of the detector, bad technique used in its measurement and inappropriate properties used for its measurement. The lack of measurable response does not preclude the existence of biophoton channel of information transfer. Perhaps, such a channel does exist and living systems emitting same or similar types of biophoton signals communicate among through this channel. It is then possible to identify morphogenetic field (or its many variants) with biophoton field of a living system. Many laboratories routinely detect biophoton fields using non-living detectors. The information content of the field will hopefully, be deciphered in near future. It will then clarify many aspects of morphogenetic field.

The capability of human beings to communicate via biophoton channel needs more careful examination because a human subject will know if it has detected a biophoton signal and will be able to tell so to other human beings. A human subject getting information via biophoton channel does not seem to exist. There is a need to understand why human subjects are ignorant of their capability to detect biophoton signals. We suggest that a new born child senses biophoton signals emitted by other human beings but does not know how to decipher information contained in the signals. The child also senses photon signals received from her sensory channels and does not know how to decipher information contained in these signals as well. She has to learn the art of deciphering information from signals and communicating her experiences. The signals from sensory channels are strong, classical and easy to interpret. The society assists her in deciphering information from signals of sensory channels and teaches her the art of communicating experiences. In contrast, the signals of biophoton channel are weak, quantum in nature and difficult to interpret. The society does not teach her the technique of extracting information from a quantum signal. She starts filtering out biophoton signals and concentrates her attention only on classical sensory signals due to societal intervention. Perhaps after a period of bewilderment, she associates meaning only to classical signals. The society encourages her to ignore the obstructions caused by biophoton signals. She soon starts treating biophoton signals as noise to be ignored. She brushes aside the innate ability to detect biophoton signals. The innate ability, however, remains intact and can be used in future if she learns to decipher information from quantum

biophoton signals. The learning will enable her to access information about other objects via biophoton channel and to see invisible objects. She can be in communion with the entire world via biophoton channel. Many religious traditions envisage such a capability acquirable. One may not always relish the acquiring of this capability. Imagine the horror of a person who acquires it by chance and then starts knowing the guarded secrets of acquaintances. Even a true narration of splendour and beauty of nature learnt via biophoton channel will fetch him the epithet paranormal. The knowledge gained through the additional capability will make him nonconformist. The society packs nonconformists to solitary confinement either in jail or in jungle.

The resources required in determining the classical state of a biophoton signal are only a small fraction of the resources required in determining its quantum state. Classical state is characterised by one parameter-the intensity of signal- and its determination requires the measurement of photon number in a few large size bins. The quantum state is characterised by many parameters and its determination requires the measurement of photon number in many thousand bins, an assumption about the quantum state and a procedure for estimating the parameters. Even one determination of quantum state is a big drain on resources and many such determinations strain a living system to the point of breakdown. Living systems therefore, avoid determining quantum state and resort to inferences based on classical states as often as possible. It is a survival strategy. The determination of quantum state becomes imperative in some situations e.g. in a noisy environment, in clogged or obstructed classical channels. The detection of some combinations of parameters of quantum state of the signal is unaffected by noise and clogged or obstructed classical channels hardly affect the determination of these combinations of quantum parameters.

The capability of a living system to determine quantum state of biophoton signals is evolutionary advantageous. The system gets access to information of other living systems not available otherwise. The system will know about various events and processes affecting biophoton signals of other systems. The system will appear to have the power of remote sensing. Perhaps, clairvoyance and extra sensory perception arise from the use of information obtained from biophoton signals. A living system can use biophoton channel for remote intervention if it has an additional capability to beam coded biophoton signals that influence other systems. One wonders if wishful thinking and blessings generate coded biophoton signals. It is feasible but it needs experimental verification. The power of remote intervention is achievable more easily if living system is entangled with its biophoton field. The living system intending to intervene has to set its biophoton detecting machinery to some desired state and wait for the detection of biophoton field

of targeted living system in the desired state. The act of detection accomplishes the desired intervention. The targeted living system attains the state entangled with the detected photon state. There are many interesting questions connected with this mode of remote intervention. Which living systems have biophoton detection machinery? How does a living system adjust its photon detecting machinery to a desired state? How much time does a system wait for detection? Can a human subject acquire the capability to detect quantum state of a biophoton signal? Do prayer, meditation, breathing exercises and drugs help in acquiring this capability? We do not know the answers of these questions but we suspect that answers will provide physical basis of the phenomena like memory transcendence, paranormal perception, remote healing and some alternative therapies.

Finally, it is conceivable that a living system capable of determining quantum state of a biophoton signal may also have the capability to determine quantum state of its own biophoton field. The capability will confer the living system ability to self introspect and make mid course correction. The implications of the ability are easy to contemplate in a system with entangled biophoton field. The entangled biophoton field is a true and instantaneous image of the quantum entity of the system. The system can monitor its quantum entity by observing its biophoton field. The monitoring provides a feedback loop to take corrective measures. The possibility to observe and analyse oneself is the additional ingredient that can explain supervenience of the hard problems of consciousness. The additional ingredient integrates metaphysical and philosophical visions of life with physical sciences.

References

1. Gurwitsch, A. G. (1923). Die Natur des spezifischen Erregers der Zellteilung. *Arch. Entw. Mech. , 100*, 11-40.
2. Gratzer, W. B. (2001). *The Undergrowth of Science: Delusion, Self-deception, and Human Frailty.* Oxford University Press.
3. Gurwitsch, A. G., & Gurwitsch, L. D. (1943). Twenty Years of Mitogenetic Radiation: Emergence, Development, and Perspectives. *Uspekhi Sovremennoi Biologii (English translation: 21st Century Science and Technology.(1999) Fall, 12, No 3:) , 16*, 305-334.
4. Hollaender, A., & Claus, D. W. (1937). An experimental study of mitogenetic radiation. *Bulletin of the National Research Council, Washington , 100*, 3-96.
5. Colli, L., & Facchini, U. (1954). Light emission by germinating plants. *Nouvo Cimento , 12*, 150-156.
6. Quickenden, T. I., Que, H., & Shane, S. (1974). Weak luminescence from the yeast Saccharomyces cerevisiae and the existence of mitogenetic radiation. *Biochemical and Biophysical Research Communications , 60*, 764-770.

7. Ruth, B., & Popp, F. A. (1976). Experimentelle Untersuchungen zur ultraschwachen Photonenemission biologischer Systeme. *Zeitschrift für Naturforschung , 31C*, 741-745.
8. Inaba, H., Shimizu, Y., Tsuji, Y., & Yamagishi, A. (1979). Photon counting spectral analyzing system of extra-weak chemi- and bioluminescence for biochemical applications. *Photochemistry and Photobiology , 30*, 169-175.
9. Inaba, H. (1997). Photonic sensing technologay is opening new frontiers in biophotonics. *Optical Review , 4*, 1-10.
10. Quickenden, T. I. (1981). On the existence of mitogenetic radiation. *Speculations in Science and Technology , 4*, 453-464.
11. Slawinski, J. (1988). Luminescence research and its relation to ultraweak cell radiation. . *Experientia , 44*, 559-571.
12. Popp, F. A. (1992). Some Essential Questions of Biophoton Research and Probable Answers. In F. A. Popp, K. H. Li, & Q. Gu, *Recent Advances in Biophoton Research and its Applications* (pp. 1-46). Singapore: World Scientific.
13. Strehler, B. L., & Arnold, W. (1951). Light production by green plants. *J. Gen. Physiol. , 34*, 809-820.
14. Lenger, K., Bajpai, R. P., & Drexel, M. (2008). Delayed luminescence of high homeopathic potencies on sugar globuli. *Homeopathy , 97*, 134-140.
15. Bajpai, R. P. (2003). Quantum coherence of biophotons and living systems. *Indian J. Exp. Biol. , 41*, 514-527.
16. Yuen, H. P. (1976). Two-photon coherent states of the radiation field. *Phys. Rev. A , 13*, 2226-2243.
17. Bajpai, R. P., Kumar, S., & Sivadasan, V. (1998). Biophoton Emission in the Evolution of a Squeezed State of Frequency Stable Damped Oscillator. *Applied Mathematical Computation , 93*, 277-288.
18. Bajpai, R. P. (1999). Coherent Nature of the Radiation Emitted in Delayed Luminescence of Leaves. *J Theo Bio , 198*, 287-299.
19. Bajpai, R. P. (2004). Biophoton emission in a squeezed state from a sample of Parmelia tinctorum. *Phys. Letters A , 322*, 131-136.
20. Walls, D. F., & Milburn, G. J. (1995). *Quantum Optics.* Berlin Heidelberg: Spronger_Verlag.
21. Popp, F. A. (1989). Coherent Photon Storage in Biological Systems. In F. A. Popp, U. Warnke, H.L. Körnig & W.Peschka, *Electromagnetic Bioinformation* (pp. 144-167). München: Urban and Schwarzenberg.
22. Orszag, M. (2000). *Quantum Optics.* Berlin Heidelberg: Springer Verlag,.
23. Bajpai, R. P. (2008). Quantum nature of photon signal emitted by Xanthoria.parietina and its implications to biology. *Indian Journal of Experimental Biology , 46*, 420-432.
24. Bajpai, R. P. (2003). Biophoton emission of a lichen species Parmelia.tinctorum. *Indian Journal of Experimental Biology , 41*, 403-410.
25. Bajpai, R. P. (2005). Squeezed State Description of Spectral Decompositions of a Biophoton Signal. *Physics Letters A , 337*, 265-273.
26. Van Wijk, R., Van Wijk, E. P., & Bajpai, R. P. (2006). Photon count distribution of photons emitted from three sites of a human body. *J Photochemistry Photobiology , B84*, 46-52.

27. Bajpai, R. P., & Drexel, M. (2008). Effect of colorpuncture on Spontaneous Photon Emission in a Subject Suffering from Multiple Scelerosis. *J Acupunct Meridian Stud , 1*, 114-120.
28. Van Wijk, R., Van Wijk, E. P., & Bajpai, R. P. (2006). Photo count distribution of photons emitted from three sites of a human body. *J Photochem Photobio B Biology , 84*, 46-55.
29. Beloussov, L. V., Burlakov, A. B., & Louchinskaia, N. N. (2003). Biophotonic patterns of optical interactions between fish eggs and embryos. *Ind J Expt Bio, 41*, 424-430.

" The New Zealander V.B. MEYER-ROCHOW, who holds a PhD in Neurobiology as well as a DSc from the Australian National University in Canberra, currently works as a Full Professor in Biology at Jacobs University, Bremen (Germany) and Docent in Physiology at the Medical School, University of Oulu (Finland). His comparative research on the functional anatomy and ultrastructure of sense organs and his ethnobiological studies allowed him to visit and work in various (and sometimes remote) places of the world, to name but a few, from Antarctica to Greenland, Jamaica to Papua New Guinea, Japan to Brazil, Namibia to New Zealand, and North East India to Central Australia. "