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Bioluminescence of Tomopteridae species (Annelida): multidisciplinary approach

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ABSTRACT

Bioluminescence, *i.e.* the production of light by living organisms, is a widespread phenomenon in the Ocean. In the twilight of the mesopelagic realm (depth 200-1000 meters), animals have developed a high diversity of bioluminescence displays in order to respond to the three main challenges in life: avoiding predators, feeding and reproducing. Unsurprisingly, blue light is largely favoured: most pelagic animals are exclusively sensitive to this colour, whose wavelength offers an optimal propagation in sea water. However, the whims of evolution have also resulted in the emergence of unusual spectra. The yellow light of some tomopterid annelids arguably constitutes one of the most enigmatic examples. On the one hand, the capacity for a transparent worm to emit bright light seems paradoxical, while on the other hand, yellow light has a weak propagation coefficient and appears to be imperceptible to most species.

Yet, the bioluminescence of *Tomopteris* remains poorly documented. To date, among the twelve known luminous species, *Tomopteris nisseni* is the only one whose spectrum has been measured. The peculiarity of yellow light in the Ocean and the species-specific distribution pattern of the putative light organs (rosette and hyaline glands) have led most researchers to assume that light emission in *Tomopteris* is a means of intraspecific communication. However, this hypothesis has never been tested by experimental data. Here, we explore this functional hypothesis through a multidisciplinary approach of bioluminescence in *Tomopteris helgolandica* as a main model species. In addition, four related species (*T. carpenteri, T. pacifica, T. planktonis* and *T. septentrionalis*) allow comparative histology of light organs and physiology of emission patterns to be undertaken. *T. carpenteri* and *T. planktonis* are for the first time reported as bioluminescent species.

The histology of putative light organs is thus investigated in the parapodia of these five species. We evidence the structural homology between the rosette of the tail-bearing species (*Johnstonella* subgenus) and the hyaline glands of the tail-less species (*Tomopteris* subgenus) and suggest that both gland types probably evolved from a common light-emitting structure and differentiated along a functional and migrational axis extending from endocrine secretion close to the coelomic ramus to exocrine secretion close to the lateral margin of the pinna.

Our study of the emission control and pattern in *T. helgolandica* suggests that the light emission is triggered by the activation of nicotinic cholinergic receptors and a calcium-dependent intracellular mechanism involving L-type volgate-gated calcium channels. The latter possibly act in the membrane of sensory neurons to control the release of neurotransmitters or at the level of photocytes to

amplify and modulate their response to intense stimuli. Despite significant differences in their light emission patterns, cholinergic control is also suggested in its ally *T. planktonis*.

Indeed, an unexpected diversity in emission patterns appears at two levels: (1) at the biochemical level, *T. planktonis* constitutes the first documented example of blue emitter in Tomopteridae; (2) at the mechanistic level, we highlight the contrast between intra- and extraglandular emissions. Thus, we suggest the bioluminescent systems of tomopterids to have evolved from a common blue light emitting structure. The yellow light observed in most of them may have evolved by transfer of the luminescent energy produced in the blue range to an accessory yellow fluorescent protein. Although the bioluminescence of *Tomopteris* exhibits similarities with that of other marine polychaetes, these latter belong to distant taxa, which suggests that bioluminescence emerged on several occasions independently in annelids. The fact that tomopterids are deeply nested in a group where no other luminous species has been documented so far suggests that one of these emergences must have occurred during radiation of the Tomopteridae.

These observations also suggest that the ecological advantage provided by this bioluminescence is more diverse than originally suspected. The first behavioural approach of light perception by *T. helgolandica* supports the hypothesis of intraspecific communication, as the tested specimens significantly contrast their responses to various types of yellow stimuli. However, the long-shifted bioluminescent spectra produced by tomopterids seem to combine the benefits from their unique and conspicuous character at short distance on the one hand, and their propagation limits in sea water on the other hand. These benefits can make the bioluminescence of yellow emitting *Tomopteris* a defence mechanism that is both efficient, although not as efficient as blue light, and memorable by close predators, while limiting the chance of a coevolution of the visual systems of potential exploiters.

RÉSUMÉ

La bioluminescence, *i.e.* la production de lumière par des organismes vivants, est un phénomène répandu dans l'Océan. Dans la quasi obscurité du domaine mésopélagique (de 200 à 1000 mètres de profondeur), les animaux ont développé une grande diversité de patrons d'émission de lumière afin de répondre aux trois principaux défis du vivant : éviter les prédateurs, se nourrir et se reproduire. Sans surprise, la lumière bleue est largement favorisée : la plupart des animaux pélagiques sont exclusivement sensibles à cette couleur, dont la longueur d'onde offre une propagation optimale dans l'eau de mer. Toutefois, les caprices de l'évolution ont également donné lieu à l'émergence de spectres atypiques. La lumière jaune de certaines annélides Tomopteridae constitue sans conteste l'un des exemples les plus énigmatiques. D'une part, la capacité d'un ver transparent à émettre une lumière vive semble paradoxale ; d'autre part, la lumière jaune a un faible coefficient de propagation et semble imperceptible pour la plupart des espèces inféodées à ces profondeurs.

Pourtant, la bioluminescence des *Tomopteris* demeure peu documentée. Parmi les douze espèces lumineuses connues à ce jour, *Tomopteris nisseni* est la seule dont le spectre a été mesuré. La singularité de la lumière jaune dans l'Océan, ainsi que les schémas de distribution des organes lumineux supposés propres à l'espèce (glandes en rosette, glandes hyalines), ont mené la plupart des chercheurs à penser que l'émission de lumière des *Tomopteris* est un moyen de communication intraspécifique. Cette idée n'a toutefois jamais été testée expérimentalement. Dans le cadre de ce travail, nous explorons cette hypothèse fonctionnelle à travers une approche multidisciplinaire de la bioluminescence de *Tomopteris helgolandica*, principale espèce modèle. En outre, quatre espèces apparentées (*T. carpenteri*, *T. pacifica*, *T. planktonis* et *T. septentrionalis*) permettent d'étudier l'histologie comparée des organes lumineux et la physiologie des schémas d'émission. *T. carpenteri* et *T. planktonis* sont pour la première fois documentés en tant qu'espèces bioluminescentes.

L'histologie des organes lumineux supposés est ainsi investiguée dans les parapodes des cinq espèces. Nous mettons en évidence l'homologie structurelle entre la rosette des espèces du sous-genre *Johnstonella* et les glandes hyalines des espèces du sous-genre *Tomopteris*, suggérant que les deux types de glandes ont probablement évolué à partir d'une structure commune émettant de la lumière et se sont différenciés le long d'un axe fonctionnel et migratoire s'étendant de la sécrétion endocrine vers la branche parapodiale du coelome à la sécrétion exocrine proche de la marge latérale de la pinnule.

Notre étude du contrôle et du schéma de l'émission de *T. helgolandica* suggère que l'émission de lumière est déclenchée par l'activation de récepteurs nicotiniques cholinergiques et d'un mécanisme intracellulaire calcio-dépendant impliquant des canaux calciques voltage-dépendents de type L. Ces derniers pourraient agir dans la membrane des neurones sensoriels, afin de contrôler la libération de neurotransmetteurs, ou au niveau des photocytes, afin d'amplifier et moduler leur réponse aux stimuli intenses. Malgré des différences significatives dans leurs schémas d'émission de lumière, le contrôle cholinergique est également suggéré chez *T. planktonis*.

Une surprenante diversité de schémas d'émission apparaît en effet à deux niveaux : (1) au niveau biochimique, *T. planktonis* constitue le premier exemple documenté d'émetteur de couleur bleue au sein des Tomopteridae ; (2) au niveau mécanique, nous mettons en lumière le contraste entre les émissions intra- et extraglandulaires. Ainsi, nous suggérons que les systèmes bioluminescents des Tomopteridae ont évolué à partir d'une structure commune émettrice de bleu. La lumière jaune observée dans la plupart d'entre eux pourrait avoir évolué *via* un transfert de l'énergie luminescente produite dans la gamme du bleu vers une protéine fluorescente jaune auxiliaire. Bien que la bioluminescence de *Tomopteris* présente des similarités avec celle d'autres polychètes marins, ces derniers appartiennent à des taxons distants, ce qui suggère que la bioluminescence a pu apparaître indépendamment à plusieurs reprises parmi les annélides. Le fait que les Tomopteridae soient ancrés dans un groupe au sein duquel aucune autre espèce lumineuse n'a été documentée jusqu'à présent suggère que l'une de ces émergences a certainement eu lieu durant la radiation de cette famille.

Ces observations suggèrent également que l'avantage écologique procuré par cette bioluminescence est plus diversifié qu'il est communément soupçonné. La première approche comportementale de la perception lumineuse de *T. helgolandica* soutient l'hypothèse d'une communication intraspécifique, les spécimens testés contrastant significativement leurs réponses à différents types de stimuli jaunes. Toutefois, le décalage du spectre vers de grandes longueurs d'onde produit par les Tomopteridae semble combiner les avantages de leur caractère unique et visible à courte distance d'une part, et leurs limites de propagation dans l'eau de mer d'autre part. Ces avantages feraient de la bioluminescence jaune des *Tomopteris* un mécanisme de défense à la fois efficace et mémorisable par les prédateurs proches, tout en limitant les chances de coévolution du système visuel des exploitants potentiels.

APPENDICES

LIST OF PAPERS

This thesis is based on the following papers (reproduced with permission from the publishers) and manuscripts at various stages of the edition process. They are referred to in the text by their Roman numerals. The most important results are summarised and discussed in section 4, while the papers provide further details.

I. **Gouveneaux, A. and Mallefet, J.** (2013). Physiological control of bioluminescence in a deep-sea planktonic worm, *Tomopteris helgolandica*. *Journal of Experimental Biology* **216**, 4285-4289.
doi: 10.1242/jeb.090852

II. **Gouveneaux, A., Flood, P.R. and Mallefet, J.** (2016). Unexpected diversity of bioluminescence in planktonic worms (accepted in *Luminescence*).

III. **Gouveneaux, A., Flood, P.R., Erichsen, E.S., C. Olsson, J. Lindström and Mallefet, J.** (2016). Morphology and fluorescence of light organs in *Tomopteris helgolandica* and allies (Polychaeta: Annelida) (submitted).

IV. **Gouveneaux, A., Gielen, M.-C. and Mallefet, J.** (2016). Behavioural responses of the planktonic worm *Tomopteris helgolandica* (Polychaeta: Annelida) to photic stimuli (in preparation).

ABBREVIATIONS

ACh	AcetylCholine
ANCOVA	ANalysis of COVAriance
BRET	Bioluminescence Resonance Energy Transfer
ChAT	Choline AcetylTransferase
CP	ChromoProtein
DAPI	4',6-DiAmidino-2-PhenylIndole
DSC	Deep Sound Channel
DSLR	Digital Standard-Lens Reflex
FITC	Fluorescein IsoThioCyanate
FP	Fluorescent Protein
FWHM	Full Width at Half Maximum intensity
GFP	Green Fluorescent Protein
IR	Infra-Red
IRED	Infra-Red light Emitting Diode
KCl	potassium chloride
LED	Light Emitting Diode
L_{max}	maximal light intensity
L_{tot}	total emitted light
$MgCl_2$	magnesium chloride
Mq	Megaquanta
nAChR	nicotinic AcetylCholine Receptor
OBIS	Ocean Biogeographic Information System
P	Power
P	P-values
RFP	Red Fluorescent Protein
RGB	Red, Green, Blue
RLU	Relative Light Unit
ROV	Remotely Operated Vehicle
R^2	coefficient of determination
s.e.m.	standard error of the mean
SOFAR	Sound Fixing And Ranging channel
WT	Wild-Type
YFP	Yellow Fluorescent Protein
λ_{max}	maximal emission wavelength

1. Introduction

1.1. General context

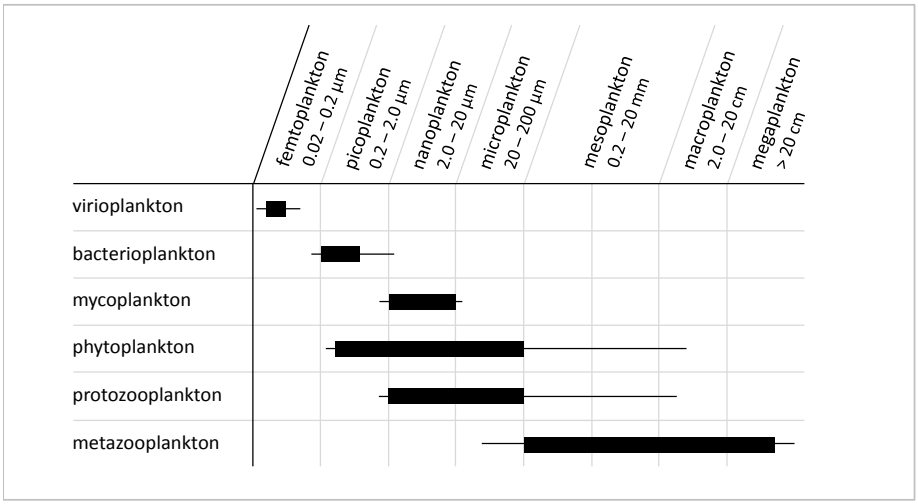
The pelagic domain is a three-dimensional and "featureless" space where it is virtually impossible for organisms to hide from visual predators (HAMMER 1996, WIDDER 2002, JOHNSEN 2014). These particular conditions determine to a large extent the structure of epi- and mesopelagic communities and have resulted in convergent evolution of a small number of adaptive strategies for survival. Amongst these are three mechanisms that are rare or nonexistent elsewhere: transparency, mirrors, and counterillumination: by transmitting, reflecting or imitating the ambient light, the cryptic organisms significantly reduce their visibility and the risk of predation (MCFALL-NGAI 1990, JOHNSEN 2014).

The mesopelagic annelid *Tomopteris helgolandica* Greeff 1879 is highly adapted to this environment: small size, two elongated setigerous cirri acting as balancing and floating organs, locomotion by undulating movements, dorsoventral flattening and, last but not least, high transparency (ÅKESSON 1962, JOHNSEN AND WIDDER 1998). However paradoxical it may appear, it also emits a bright yellow light (DALES 1971). Given that most mesopelagic bioluminescent organisms produce blue light (HERRING 1983), this unusual spectral characteristic has long been suggested to act as an intraspecific communication signal. However, the current knowledge of this bioluminescence is merely based on hypotheses and anecdotic observations. Thus, although it is well known, this intriguing yellow bioluminescence remains poorly documented.

1.2. Plankton

1.2.1. General definitions

The term plankton was introduced in 1887 by the German zoologist Victor Hensen. Derived from the Greek Πλαγκτός/*plagtos* meaning drifting or errant, it practically brings together a large variety of organisms drifting in water masses. Their locomotion abilities, sometimes well-developed but insufficient to withstand most marine currents, distinguish them from the community of active swimmers forming the nekton (HARRIS *ET AL*. 2000, JOHNSON AND ALLEN 2012). However, the criteria defining the planktonic life habit remain difficult to establish, especially in fossils (RIGBY AND MILSOM 2000). The basic subdivisions of this heterogeneous group involve different criteria such as the systematic position of the organisms or their size [**Tab. 1.1**]. By associating these terminologies, we talk about nanophyto-planktonic or mesozooplanktonic fractions for example (SIEBURTH 1978). In 1893, Hæckel also distinguished holoplanktonic species spending their whole life cycle in the pelagic realm, from meroplanktonic species drifting in the ocean only for a part – generally the early stages – of their life. In the mid-1800's, many newly described



Tab. 1.1. Distribution of taxonomic-trophic compartments of plankton in a spectrum of size fractions (Modified from SIEBURTH *ET AL.* 1978 with permission).
Within metazooplankton, 5 orders of magnitude separate the smallest organisms (rotiferans and cladocerans) from the Scyphozoa *Cyanea capillata* reaching a bell diameter of over 2 m and tentacles of 30 m in length, as well as the siphonophore colonies *Praya dubia* measuring up to 40 m in length.

life-forms were not recognised as larvae but assigned to new phyla (JOHNSON AND ALLEN 2012). However, it is now estimated that the vast majority of marine animals has an indirect development and that 2/3 of the inventoried zooplanktonic species are larval forms (HARRIS *ET AL*. 2000, YOUNG *ET AL.* 2002). The other third is dominated by two groups: the crustaceans, represented by eight orders (cladocerans, ostracods, copepods, cirripeds, mysids, amphipods, euphausiids and decapods) and the gelatinous plankton, characterised by a fragile, generally transparent body constituted of up to 99% water (MILLER 2005). The most commonly encountered gelatinous organisms are cnidarians (including hydromedusae, siphonophores and scyphomedusae), ctenophores, chaetognaths and pelagic tunicates such as doliolids, salps and appendicularians (HARRIS *ET AL*. 2000, HADDOCK 2004). However, almost all marine phyla, including Annelida, Mollusca and Arthropoda, contain gelatinous species. The exceptional elegance and beauty of these planktonic organisms have been drawn and photographed for many years, combining scientific and artistic visions [**Fig. 1.1**].

The plankton is a complex assemblage that directly or indirectly mediates all aspects of ecology in the Ocean (RAYMONT 1976). According to certain estimations, phytoplankton contributes between 50 to 85% of the oxygen in Earth's

atmosphere and more than 90% of the total flux of particulate matter to the seabed is mediated by zooplankton (RIGBY AND MILSOM 2000).

1.2.2. Zonation of the open ocean and plankton distribution

Based on our very incomplete knowledge of marine biodiversity, we currently estimate that only 2% of the 235,000 known marine species live in the pelagic domain (derived from the greek πέλαγος/*pélagos* meaning open sea). The other 98% are benthic species living on or in the ocean floor (THURMAN 1997). In a general approach to the pelagic environment, the vertical zonation depending on depth is commonly used [**Fig. 1.2a**]. However, an important factor determining in many different ways the distribution of life in the Ocean is the availability of light, which depends on the intensity of natural incident light ($2x10^{35}$ photons enter the sea each second) and its penetration through the sea water [**Fig. 1.2b-e**]. The availability and the quality of light directly affect the primary production and limit the phytoplankton distribution to the euphotic zone [**Fig. 1.2c**] (RAYMONT 1976).

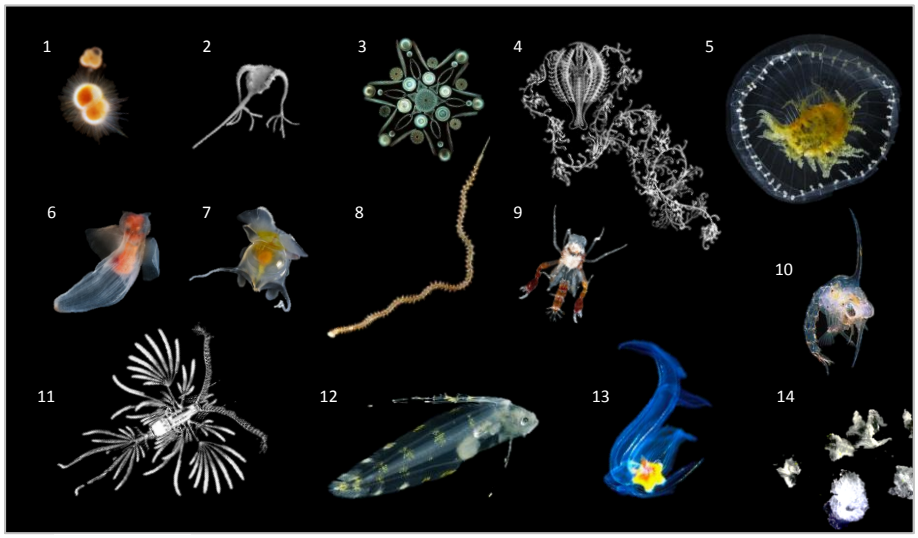


Fig. 1.1. Illustrations and photographs of planktonic organisms.
(1) *Globigerina sp.* (Foraminifera); **(2)** *Ceratium sp.* (Dinoflagellata) SEM imaging; **(3)** Geometric arrangement of diatoms by Watson and Sons (*ca.* 1885); **(4)** *Hormiphora sp.* (Ctenophora) by E. HAECKEL (1904); **(5)** *Orchistoma pileus* (Hydrozoa); **(6)** *Clione limacina* (Pteropoda); **(7)** *Cavolinia sp.* (Pteropoda); **(8)** Alciopid Polychaeta; **(9)** *Phronima sp.* (Amphipoda); **(10)** *Atelecyclus sp.* zoe larva (Decapoda); **(11)** *Euaugaptilus filigerus* (Copepoda) by E. HAECKEL (1904); **(12)** Pleuronectiform larva (Actinopterygii); **(13)** *Luidia sarsi* larva (Echinodermata); **(14)** Marine snow formed by abandoned gelatinous casing of Appendicularia. Relative size between species is not respected.
Photos are courtesy of J. DOLAN AND M.-D. PIZAY (2), P.R. FLOOD , Bathybiologica A/S (10, 13), S. JOHNSEN (5-9, 12), H. LYNK, http://www.victorianmicroscopeslides.com/ (3), S. MIRSHAK (14), C. SARDET (1, 14).

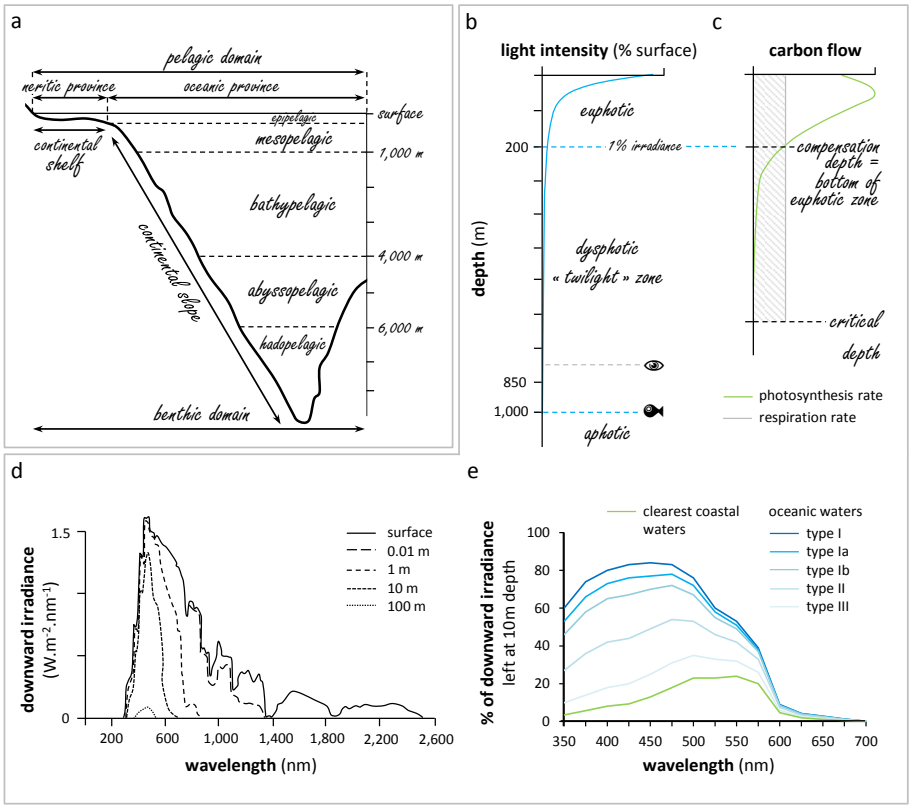


Fig. 1.2. Zonation of marine environment.
(a) Depth zones; **(b)** Light zones: exponential decline in light intensity with increasing depth (called attenuation) due to absorbance of light by the water itself, photosynthetic organisms, particulate matter and dissolved organic matter. The euphotic zone extends from the surface down to a depth where the residual light intensity falls to 1% of the solar irradiance at the surface (around 200 m depth on the clearest oceanic waters). At 850 m, there is no longer enough light for a human eye to see by (👁) but the most sensitive deep-sea visual systems can catch photons down to 1000 m depth, the extreme limit of light penetration (🐟) (WARRANT AND LOCKET 2004, JOHNSEN 2014); **(c)** Photoautotrophic activity in the euphotic zone: the compensation depth is the depth at which the carbone assimilation by phytoplankton equals the respiratory carbon losses or when net photosynthesis is 0. The critical depth is the depth where the integrated daily photosynthetic carbon assimilation is balanced by the integrated daily respiratory carbon losses. So the phytoplankton population can only proliferate if mixing (nutrients input) is shallower than the critical depth (TETT 1990); **(d)** Complete spectrum of downward irradiance (watt per square meter) reaching the sea surface and four depths: even in the clearest sea water, wavelength selective attenuation phenomena isolates a narrow spectra range of mostly blue light. The largest part of the total irradiance is converted into heat which has substantial thermodynamic implications; **(e)** Transmittance of downward irradiance for optical water types of Jerlov (modified after JOHNSEN 2012 with permission). Oceanic waters are classified from the clearest (type I) to the murkiest (type III).

Zooplankton distribution mainly depends on the trophic state of the area (*i.e.* the availability of biologically useful nutrients and the associated growth potential of the various trophic levels) but is also influenced by both temperature – directly affecting growth and reproduction – and water depth (HARRIS *ET AL*. 2000). First, the shelf break marks a distinct change in composition of neritic and oceanic planktonic communities. In fact, the neritic plankton is composed of a large proportion of meroplankton due to the proximity of the sea bottom. On the other side, oceanic zooplankton is characterised by the presence of many vertical migrants – mainly mesozooplankton feeding on phytoplankton and microzooplankton – moving between the epipelagic and the mesopelagic zones. During their diel migrations, they bring down higher trophic levels including nektonic organisms in a massive displacement detectable by remote sensing (HAYS 2003, MILLER 2005). The high density of organisms forms one or several layers, known as deep scattering layers, reflecting and scattering the sonar signal to create what were called phantom bottoms. Situated at a depth of 100 to 200 m during the night, they can sink down to 900 m during the day. In the North Atlantic waters, the largest migration amplitudes were observed in clear waters favouring the penetration of light (attenuation coefficient of 0.025, while it usually remains within the interval 0.030-0.040) (CLARKE 1933, DICKSON 1972, JERLOV 1976). Consequently, the most likely ultimate reason for zooplankton diel vertical migration is to reduce the light-dependent mortality risk due to ultraviolet exposure, but most importantly, predation from visually orientating planktivores (RHODE *ET AL.* 2001, WILLIAMSON *ET AL.* 2011). However, the trophic state of deep waters has been observed to influence the pattern of migration so there must be a trade-off between maximum energy input and maximum protection (LAMPERT 1989, HAYS 2003). Other major vertical movements are seasonal or intimately related to life history traits of the animals, sometimes involving profound metabolic changes. In most of the species investigated, the young stages live in upper layers or even near the surface (BANSE 1966). Adjusting their vertical positioning in the water column, migrants can take advantage of favourable currents. However, these horizontal transports, named selective tidal transports in coastal regions (HILL 1998, FORWARD AND TANKERSLEY 2001), would most often be consequences of vertical migration and do not seem to imply voluntary migrations (HAYS 2003). Going down to the bathy and abyssopelagic zones, the environment becomes purely aphotic and the gelatinous plankton scarce. In fact, transparency making them invisible in the dysphoric zone is of lesser relevance at these depths (JOHNSEN 2014). Characterised by a food supply greatly reduced, the deep-sea ecosystems are based on aggregated organic particles and microorganisms exported from the upper layers as marine snow (term coined by the American naturalist and explorer William Beebe). Discovered in the late 1970s, the hydrothermal vents provide through the bacterial chemosynthesis

another substantial source of organic matter. The primary beneficiaries of this production are benthic organisms, but the prevalence of some holoplanktonic species in areas exposed to the hydrothermal fluids suggests that they could be closely associated and adapted to these extreme conditions (KIM AND MULLINEAUX 1998). Finally, planktonic organisms are by definition highly sensitive to and dependent on the dynamics of water masses as well as its variability. For example, the changing pattern of circulation of the North Atlantic Ocean driven by the North Atlantic Oscillation is well-known to influence the planktonic biological processes (MANN AND LAZIER 2006).

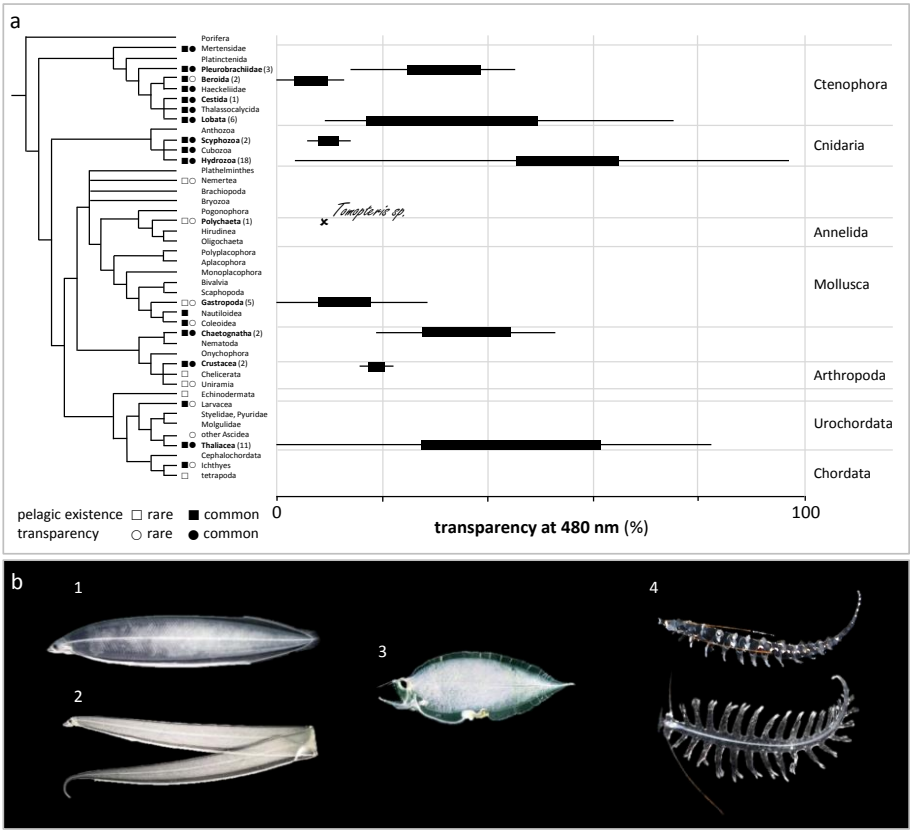


Fig. 1.3. Transparency
(a) Degrees of transparency in various pelagic groups (after JOHNSEN 2001, JOHNSEN AND WIDDER 1998, 2001). Transparency probably evolved several times and in close association with pelagic existence. In brackets is mentioned the number of species analysed to establish the ranges of transparency plotted on the right; **(b)** Laterally (1-3) and dorso-ventrally (4) flattened transparent organisms: (1-3) Anguiliform leptocephali larvae of Anguillidae, Nemichthyidae and Eurypharyngidae (Actinopterygii), (4) Lateral and dorsal views of *Tomopteris helgolandica* (Polychaeta: Tomopteridae). Sizes are not proportional.
Photos are courtesy of A. GOUVENEAUX (b4), Terrapub (b1-3, reprinted from MILLER 2009 with permission).

1.2.3. Cryptic adaptations

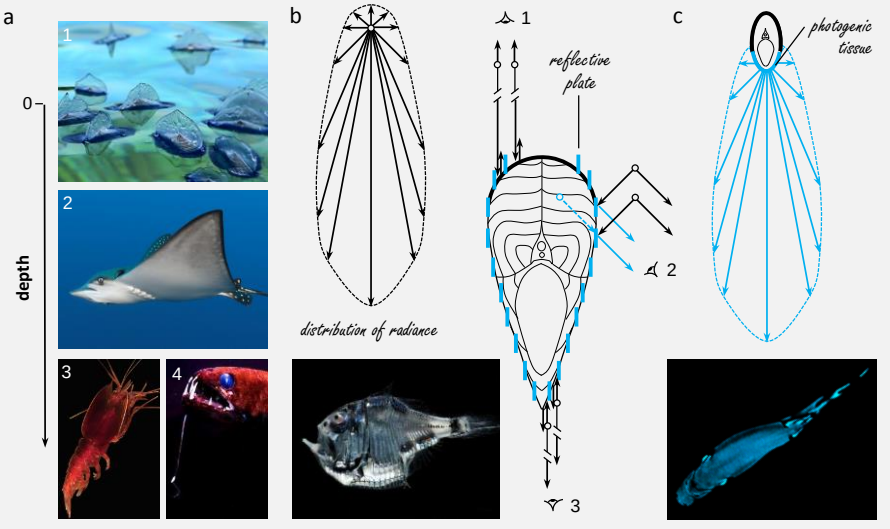
In the field of pelagic visual ecology, scientists usually consider that the organisms spend their whole life playing hide-and-seek (WIDDER 2002, JOHNSEN 2014). The three-dimensional and "featureless" environment offered by the pelagic realm makes this a huge challenge. These particular conditions resulted in the convergent evolution of three main cryptic strategies: transparency, mirrors and counter-illumination [**Box 1.1, Fig. 1.3**] (reviewed by JOHNSEN 2014). The various morphologies and behaviours involved in these mechanisms are rare or nonexistent in other habitats (MCFALL-NGAI 1990, JOHNSEN 2014). Pigment-based camouflages also exist in the pelagic environment but they operate on the same principles and use many of the same pigments as those encountered in freshwater or terrestrial habitats (JOHNSEN 2002, 2005, 2014). However, the selective advantage provided by countershading [**Box 1.1**] would be less important in terrestrial and aerial environments than in oceanic light conditions (RUXTON *ET AL*. 2004). Some organisms can combine different forms of camouflage (*e.g.* upper mesopelagic fishes using a three-fold strategy including dark pigments along the back, silvery sides and photophores on the ventral surface (LAND AND OSORIO 2011)) or switch from one to another under changing light conditions (*e.g.* cephalopods are able to vary their appearance between transparency and pigmentation *via* the expansion and contraction of red chromatophores (ZYLINSKY AND JOHNSEN 2011)). In the present section, particular attention is paid to transparency as the most prevalent, and yet least understood, of these mechanisms (JOHNSEN 2014).

As previously mentioned, transparency is the primary characteristic of gelatinous plankton. It mostly evolved amongst invertebrates larger than 1 cm living in the top 500 m of the Ocean (JOHNSEN 2014). The underlying principle of this camouflage is quite intuitive: to reduce its visibility by minimising absorption and scattering of light. Most of the organic molecules do not absorb light. However, due to their composite nature creating many refractive index discontinuities, organic tissues are highly susceptible to scatter light. Basically, the internal constitution of transparent organisms seems simply less sophisticated than those of opaque related taxa, but that cannot be the whole explanation. Some organisms have a flattened body shape to reduce the length of the light path through their tissues [**Fig. 1.3b**]. Some other organisms with more massive body have a highly hydrated extracellular matrix (*e.g.* mesoglea of Cnidaria). However, these adaptations could have first evolved to increase buoyancy or reduce the metabolic cost of a large body (TSUKAMOTO 2009, JOHNSEN 2014). Regarding other taxa such as crustaceans or molluscs, which have a more complex organisation, the mechanisms achieving transparency remain unclear. Amongst the hypotheses, Johnsen and Widder (1999) predicted that the ultrastructural constitution – especially the size, shape and

Oceanic pelagic **pigmentation** seems primarily mediated by depth **(a)** although there are many exceptions (JOHNSEN 2002). Pleustonic species (plancton living at the air-water interface) tend to be entirely blue **(a1)** (HERRING 1967). In near-surface waters, most pigmented organisms are countershaded showing dark-blue dorsal surfaces and lighter ventral surfaces **(a2)** (Johnsen and Sosik 2003). This gradation of color is supposed, through the mechanism of self-shadow concealment, to reduce the capacity of predators to recognise the animal as a three-dimensional object (RUXTON *ET AL.* 2004, ROWLAND 2011). Finally, as depth increases, red and black pigmentations become more prominent in invertebrates and vertebrates respectively **(a3)** (Herring 1973, Herring and Roe 1988). These blue-absorbing pigments have been suggested to act against the bioluminescent searchlights [Fig. 1.10b] of certain predators (JOHNSEN 2005).

The term **mirrors** defines the silvery structures covering fish or cephalopods' flanks, formed of stacks of thin crystals mainly composed of guanine. The vertical orientation of these reflective surfaces strongly suggests that they evolved to function as camouflage **(b)**. However, we can find similar structures, with different orientation and spectral properties, as components of visual and photogenic organs [Fig. 1.7a-b] (DENTON 1970).

The organisms using **counterillumination** emit ventrally a bioluminescence which perfectly match the intensity, the spectral and the angular distributions of the downwelling light **(c)** (DENTON 1972, HADDOCK *ET AL.* 2010). This camouflage is found in many midwater organisms including crustaceans, squids, sharks and bony fishes able to control their light intensity to match the variations of the environment (YOUNG AND ROPER 1977).



Box 1.1. Cryptic adaptations of the external surface of oceanic pelagic species
(a) Depth-mediated patterns of pigmentation: (1) *Velella velella* (Hydrozoa: Velellidae); (2) *Aetobatus narinari* (Chondrichthyes: Myliobatidae); (3) *Notostomus sp.* (Malacostraca: Oplophoridae); (4) *Echostoma sp.* (Actinopterygii: Stomiidae); **(b)** Cross-section of a fish body showing the typical vertical orientation of reflective guanine plates and the resulting camouflaging effect (modified after DENTON 1970 with permission): the predator eyes at the side receive the reflected light by the mirrors that matches the light behind the animal (2). The inefficiency of mirrors for an observer situated above (1) or below (3) is balanced by the laterally-flattening of body and the countershading effect. Photo: *Argyropelecus sp.* →

(Actinopterygii: Sternoptychidae); **(c)** Cross-section of a fish using bioluminescence as counterillumination signal. Photo: *Etmopterus spinax* (Chondrichthyes: Etmopteridae) (CLAES *ET AL.* 2010, CLAES AND MALLEFET 2010, 2011).
Photos are courtesy of D. FENOLIO (a4, b), S. JOHNSEN (a3), J. MALLEFET (c), S. MIRSHAK (a1), T. WU (a2).

packing of organelles – strongly influence the light scattering per unit tissue volume. Finally, the variations of the refractive index could be balanced by soluble clearing agents raising the index of intra- and extracellular fluids. This was suggested in transparent amphipods by Sweeney (JOHNSEN 2014).

Transparency is the only form of camouflage that is efficient from all viewing angles and at all depths (JOHNSEN 2014). It is also one of the few adaptations involving an organism's entire volume. Indeed, to achieve a maximal efficiency, dramatic anatomical and physiological modifications are usually required. Consequently, trade-offs may occur between, for instance, size, complexity, and locomotor performances. Often delicate and less agile, the success of transparent organisms in prey-predator interactions critically depends on their visibility and in particular on their sighting distance (the maximum distance at which they are detectable by an animal relying on visual cues) (JOHNSEN 2001). Again, this highlights the tight connection between transparency and the planktonic mode of life [**Fig. 1.3a**]. Conversely, mirrors, counterillumination and countershading pigmentation are all superficial alterations encountered in larger mobile organisms (*e.g.* fishes, sharks, cephalopods) that must be able to maintain a constant posture in the water column for the success of their camouflage [**Box 1.1**] (WEBB 2002).

However, Johnsen (2003) demonstrated that each of these cryptic strategies – the most perfect transparency included – can be altered, especially in shallow waters. Various sophisticated visual systems such as ultraviolet vision and polarisation sensitivity take advantage of these weaknesses and would be able to "break" the camouflage of the species they interact with (FRANK AND WIDDER 1996, LOSEY *ET AL.* 1999, WARRANT AND LOCKET 2004, JOHNSEN 2014). These co-evolved adaptations thus make the hide-and-seek game far more challenging than initially expected.

1.2.4. Reproduction, dispersal and speciation

The Ocean is a fluid and dynamic environment forming a spatial continuum of approximately 1.3 billion km³. Thus, when it comes to ensuring sexual reproduction (the taxa are diploid and sexual species), the planktonic mode of life can have both advantages and disadvantages. Indeed, whereas the difference in scale between

smallest drifters and hydrodynamic processes does not offer favourable conditions for male and female gametes to encounter, the mixing and dispersal of non-swimming early life stages (*e.g.* eggs, spores, larvae and juveniles representing, as a reminder, 2/3 of planktonic diversity) is of primary importance, especially for meroplanktonic species, which are sometimes sessile during the rest of their life cycle (COWEN AND SPONAUGLE 2009).

Most planktonic invertebrates have an indirect development, including one or several larval stages. For instance, copepods transition from six nauplii to five copepodite stages before moulting into adults. However, direct development exists in ctenophores, ostracods and chaetognaths (JOHNSON AND ALLEN 2012). Fertilisation is mainly external but its success can be increased by sexual and courtship behaviours, which coordinate the release of reproductive substances into the water. In species with internal fertilisation, these behaviours are sometimes highly complex. They increase mate encounter rates and mediate partner choices. For example, female copepods are able to signal their presence to males in various ways: pheromones can be deposited as a trail in the wake of cruising females, or spread out as a diffuse plume or cloud by hovering females (BAGØIEN AND KIØRBOE 2005a, KIØRBOE *ET AL.* 2005). The courtship patterns are usually species-specific and typically composed of behaviours such as "hop and sink", search swimming, chase swimming, escape or dance (TSUDA AND MILLER 1998). In the smallest species, synchronised hops seem to be the expression of hydromechanical communication involving similar detection roles played by males and females (BAGØIEN AND KIØRBOE 2005b). Bioluminescence is also a sex recognition signal and an important component of courtship displays in numerous species (HERRING 2000, 2007). This will be discussed in more detail in the next chapter. Following a successful encounter, some holoplanktonic species use specialised appendages that allow partners to grasp each other without drifting away during the copulation.

Being highly exposed to predation, free-living propagules can have armed bodies (*e.g.* spined decapod zoeae [**Fig. 1.1(10)**]) or produce chemical deterrents (*e.g.* sponges, bryozoans, echinoderms) (MORGAN 1989, LINDQUIST AND HAY 1996). There are only a few known cases of parental care in zooplankton including the females phronimid (Hyperiidae, Amphipoda) which cohabited with their youngs in a barrel-like gelatinous structure recycled from salps. Aoki and associates (2013) identified up to 5 different growth stages in the same barrel.

By definition, marine currents affect many aspects of the life of strictly planktonic organisms. However, the planktonic phase of benthic and coastal organisms is a critical stage of their life cycle ensuring by itself connectivity between distant populations (VANCE 1973, COWEN AND SPONAUGLE 2009). As the success of larval recruitment affects many commercially valuable coastal species

(*e.g.* mussels, oysters, shrimps), their dispersal patterns were particularly well described in comparison with open ocean populations (PEIJNENBURG AND GOETZE, 2013). Dispersal distances of coastal species larvae range from a few metres to thousands of kilometres, whereas the dispersal duration may be a few minutes, a tidal cycle, many months or even a year (SCHELTEMA 1986, LARGIER 2003, KINLAN AND GAINES 2003, SHANKS 2009). The long-term transports require larvae to be planktotrophic. However, there is evidence of dissolved organic matter or phytoplankton uptakes (facultative planktotrophy) by lecithotrophic larvae extending the duration of their survivorship (VANCE 1973, KEMPF AND HADFIELD 1985, JAECKLE AND MANAHAN 1989). Although a short pelagic larval duration contributes to limiting the dispersal distance, more than 90% of the data collected by Shanks (2009) surprisingly exhibit (up to 100,000 times) shorter realised dispersal distances than predicted by passive larval drift models, both in a steady current and in a more realistic Lagrangian flow field (SIEGEL *ET AL.* 2003, SHANKS *ET AL.* 2003, SHANKS 2009). These results clearly demonstrate that planktonic organisms are not transported as passive particles. It is known today that the species-specific and habitat-specific dispersal patterns commonly observed result from complex interactions between multiple-scale physical processes and biological traits (LARGIER 2003, COWEN AND SPONAUGLE 2009, JOHNSON AND ALLEN 2012).

Coming back to the holoplankton, early research on the genetic structure of these populations often supported the concept of nearly unlimited dispersal. However, these studies mainly focused on very abundant species. Recent molecular data from a broader array of species suggested a more complex and nuanced pattern of dispersal and connectivity amongst oceanic populations (PEIJNENBURG AND GOETZE, 2013). Thus, we now have evidence for open ocean barriers, especially thermic in nature, that restricted genes flow and forced allopatric speciation over evolutionary times (THORNHILL *ET AL.* 2008, GOETZE 2011). Given the fluctuations between extinction and radiation periods since the Precambrian, it is currently assumed that the cooling of high latitude seas during global glaciations, by increasing temperature gradients, created ecological opportunities for speciation through horizontal and vertical isolation of populations (LIPPS 1970, MILLER 2005). Although the majority of plankton fail to fossilise, we observe a general diversification of worldwide plankton through the Phanerozoic eon (RIGBY AND MILSOM 2000).

1.3. Bioluminescence

Bioluminescence (gr. *bios*, life; lat. *lumen*, light) is the production of visible light by living organisms resulting from a chemical reaction (HARVEY 1952, SHIMOMURA 2006, HADDOCK *ET AL.* 2010). It must be distinguished from other natural optical

phenomena such as fluorescence, phosphorescence or iridescence, with which it was and is still sometimes confused [**Box 1.2**]. The first written reference to a luminous organism is probably a Chinese poem from the Book of Odes dating back to 800-700 B.C. (HARVEY 1957, DOBSON 1964, LEE 2008). The "night travellers" to which it refers are most probably fireflies, known to be common in South China. Later, Aristotle (384-322 B.C.) and Pliny the Elder (23-79 A.D.), in his *Naturalis Historiæ*, were the first to record detailed observations of terrestrial and marine self-luminescent organisms. The expression "cold light" was introduced by Aristotle himself who noticed that, unlike other known light sources such as the sun and the fire, this light does not generate heat (HARVEY 1957, LEE 2008). Until the 17th century, this natural phenomenon was reported by intellectuals and natural historians, but it contributed to nourishing more myths and superstitions than scientific curiosity. In 1667 came the first experimental data with R. Boyle who demonstrated that the removal of air dimmed or extinguished the light. Amongst others, the German priest A. Kircher (1602-1680) compiled many reports of living light and suggested it performs a biological function, especially to communicate.

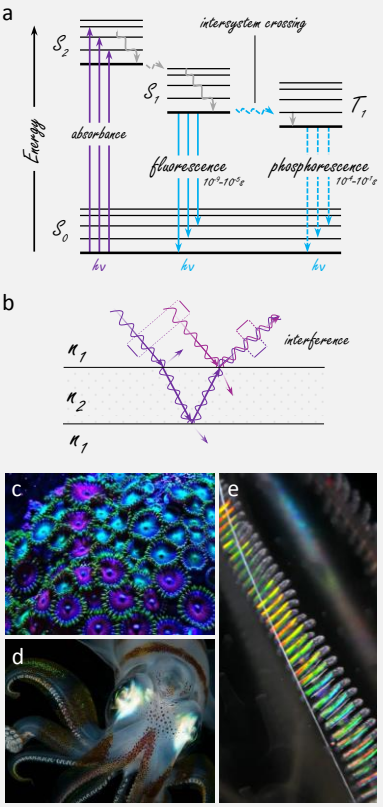
Ranging from bacteria to fishes, we currently know that the organisms able to produce their own light are sporadically distributed amongst numerous taxa, apparently without following any phylogenetic constraint (HERRING 1987, HARVEY 1952, HASTINGS AND MORIN 1991, HADDOCK *ET AL.* 2010). However, the optical properties of the marine environment, as well as its large-scale stability would have been favourable for the evolution of bioluminescence, since around 80% of the 700 genera known to contain bioluminescent species are found in the oceans. Lastly, the emergence of life in the Ocean occured more than 3 billion years before terrestrial habitats were colonised. Although represented at all depths and all latitudes, marine luminescence is more prevalent in open and deep environments than in benthic and shallow waters (HADDOCK *ET AL.* 2010, WIDDER 2010). In contrast, bioluminescence remains rare in terrestrial environments, with only a few representatives in bacteria, fungi, snails, earthworms and several arthropod lineages (*e.g.* fireflies, beetles, centipedes, millipedes) (WIDDER 2001, VIVIANI 2001, DESJARDIN *ET AL.* 2008, VIVIANI 2009, LEWIS AND CRASTLEY 2008, OBA *ET AL.* 2011). In freshwater ecosystems, bioluminescence is nearly absent, with the exception of some insect larvae and the gastropod *Latia neritoides* (MEYER-ROCHOW AND MOORE 1988).

When one considers all living organisms, every aspect of bioluminescence shows a remarkable diversity: biochemistry, morphology, physiology and function (HASTINGS AND MORIN 1991, HADDOCK *ET AL.* 2010). A general overview is proposed in the following sections.

Fluorescence and **phosphorescence** are both light emissions initiated by photoexcitation. As the molecules are not stable in this excited state, the absorbed energy can be dissipated through various non-radiative relaxation processes or in the case of fluorescence, re-emitted in the form of a longer wavelength photon **(a)**. Another possible pathway is called intersystem crossing which is a change of spin multiplicity from an excited singlet state to an excited triplet state. Phosphorescence is the direct transition back to the singlet ground state. That is the slowest process, several orders of magnitude slower than fluorescence, resulting in a dim glow.

Iridescence (gr. *îris* , rainbow) defines a form of structural coloration displayed by certain surfaces which appear to change color according to the angle of observation or illumination. This optical phenomenon is based on light scattering from nanoscale variation of refractive index in periodic structures (thin layer, multiple layers, photonic crystals). The constructive and destructive interferences among scattered light waves modulate the incident light, by amplifying or attenuating selected wavelengths **(b)**.

After KINOSHITA 2008, JOHNSEN 2012



Box. 1.2. Light-emitting phenomena in living organisms.
(a) Jablonski energy diagram: fluorescence is most often observed between the first excited electron state (S_1) and the ground state (S_0) because at higher energies it is more likely that energy will be dissipated through vibrational relaxation, between vibrational levels (curved grey arrow) and internal conversion, between two vibrational levels in different electronic states (curved dashed grey arrow). The intersystem crossing can compete with fluorescence only from the zeroth vibrational level of an excited singlet state; **(b)** Interference of light beams reflected from the front and back surfaces of a thin film, K_1 and K_2 being the refraction coefficients of each medium; **(c)** Coral fluorescence under UV lighting (zoanthid button polyps); **(d)** Dynamically tuneable structural coloration produced by iridophores (*i.e.* aggregations of iridescent cells in the skin) in a squid (TAO *ET AL.* 2010, WARDILL *ET AL.* 2012); **(e)** Iridescent rainbow pattern of a ctenophore formed by the selective reflection from a two-dimensional photonic crystal of its swimming plates (WELCH *ET AL.* 2006).
Photos are courtesy of www.orphek.com (c), C. TANG www.atlasomega.com (d) and C. SARDET (e).

1.3.1. Chemical principles and evolution

The light-producing chemical mechanism was one of the first aspect of bioluminescence addressed by scientists. The first example of the luciferin-luciferase system was demonstrated in 1885 by the French pharmacologist R.

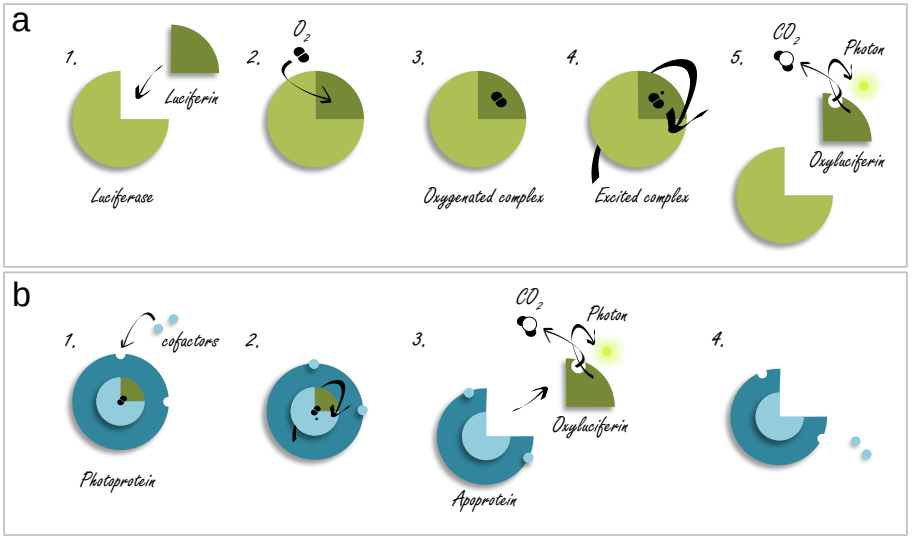


Fig. 1.4. Chemical principles of bioluminescence
(a) Luciferin/luciferase system: the enzyme binds to the substrate [(1)] forming a luciferin/luciferase complex [(2)] which reaches an electronically excited and instable state in the presence of molecular oxygen [(3-4)]. As it goes back to the ground state, the complex dissociates and releases one photon of light and one molecule of carbon dioxide. The product formed by the oxidation of the luciferin is called the oxyluciferin [(5)]; **(b)** Photoprotein system: the photoprotein is an enzyme-substrate complex more stable than its dissociated components [(1)]. The activation of this complex by various cofactors (*e.g.* Ca^{2+}, Mg^{2+}, ATP) [(2)] results in a conformational change of the photoprotein and the release of the oxyluciferin subunit, one photon and carbon dioxide [(3-4)].

Dubois in *Pyrophorus noctilucus* (Arthropoda: Elateridae). Subsequently described as a general principle, this system produces light from the oxidation of an organic substrate – referred to by the generic term luciferin (lat. *lūx*, light; *ferre*, to carry), catalysed by an enzyme – the luciferase [**Fig. 1.4a**]. In some cases, this oxidative reaction first requires the activation of a substrate storage form (*e.g. Renilla* enol-sulfate luciferin) (SHIMOMURA 2006).

In the 1960's, the major contribution of Professor O. Shimomura opened new perspectives for both fundamental and applied research: the discovery of an unusual protein that requires neither oxygen nor enzyme to catalyse the light emission in the hydrozoan jellyfish *Aequorea victoria* disrupts the classical concept of luciferin-luciferase reaction (SHIMOMURA *ET AL.* 1962, SHIMOMURA 2005). This molecule, called aequorin, was the first isolated photoprotein: a stable complex including a luciferase and an oxygenated form of luciferin [**Fig. 1.4b**]. Many similar systems were then described, such as Ca^{2+}-sensitive photoproteins from coelenterates (berovin, clytin, halistaurin, mitrocomin, mnemiopsin, obelin, phialidin) and radiolarians (thalassicolin), superoxide-activated photoproteins from

scale-worms (polynoidin) and the bivalve *Pholas dactylus* (pholasin). Finally, the photoprotein of the millipede *Luminodesmus sequoiae*, the only known example of terrestrial origin, requires ATP and Mg^{2+} to emit light (SHIMOMURA 1981, 1985, NICOLAS *ET AL.* 1982, DAUNERT AND DEO 2006, SHIMOMURA 2006).

The story does not end there. Shimomura also isolated from *A. victoria* tissues a molecule involved in an energy transfer mechanism (called BRET for Bioluminescence Resonance Energy Transfer) responsible for a shift from the primary emitted blue light resulting from the chemiluminescent reaction to the green light emitted *in vivo* by the jellyfish, the green fluorescent protein or GFP (GOROKHOVATSKY *ET AL.* 2004, CHALFIE AND KAIN 2005, SHIMOMURA 2005). The high potential of this discovery was revealed later by M. Chalfie and R.Y. Tsien who respectively developed the GFP as a marker for gene expression and improved its spectral characteristics (CHALFIE *ET AL.* 1994, TSIEN 1998). The works of these three scientists were awarded the Nobel Price in Chemistry in 2008.

Given the molecular diversity involved in known light-producing systems and the numerous uncharacterised systems which do not cross-react with known luciferases, it is undoubted that many other mechanisms remain to be discovered (HASTINGS 1983, CAMPBELL 2012). Therefore, we currently estimate that bioluminescence has evolved independently between 40 and 50 times (HASTINGS 1983, HADDOCK *ET AL.* 2010). This highlights not only the important ecological value of this adaptation but also its surprisingly easy development. Pointed out by Darwin in this first writing of the "Origin of Species" and more recently by the astrophysicist F. Hoyle, the multiple evolution of features as sophisticated as light organs in distant species by independent graduated evolutionary steps can be difficult to admit in terms of probability (CAMPBELL 2012). While luciferins appear to be highly conserved across phyla with only seven recognised chemical families of substrates shared by phylogenetically distant species [**Fig. 1.5**], luciferases derive from distinct evolutionary lineages as shown by poor sequence similarities between phyla (VASSEL *ET AL.* 2012). Coelenterazine, for example, is known to be widely distributed in bioluminescent marine organisms, occurring in eight phyla, both as a luciferin and as the functional group of Ca^{2+}-sensitive photoproteins (SHIMOMURA *ET AL.* 1980, HADDOCK *ET AL.* 2010, VASSEL *ET AL.* 2012). This molecule is predicted to originate from the cyclisation of three amino acids (Phe, Tyr, Tyr) (MCCAPRA 1990) but only two crustaceans – *Systellaspis debilis* and *Metridia pacifica* – have so far been found to achieve its biosynthesis (THOMSON *ET AL.* 1995, OBA *ET AL.* 2009). Indeed, many species using this compound appear to be unable to synthesise it *de novo*, and must, therefore, obtain it through their diet (TSUJI AND HANEDA 1966, WIDDER 2010). It was experimentally proven for the mysid shrimp *Gnathophausia ingens* (FRANK *ET AL*. 1984) and the jellyfish *A.victoria*, one of the species in which the chemical

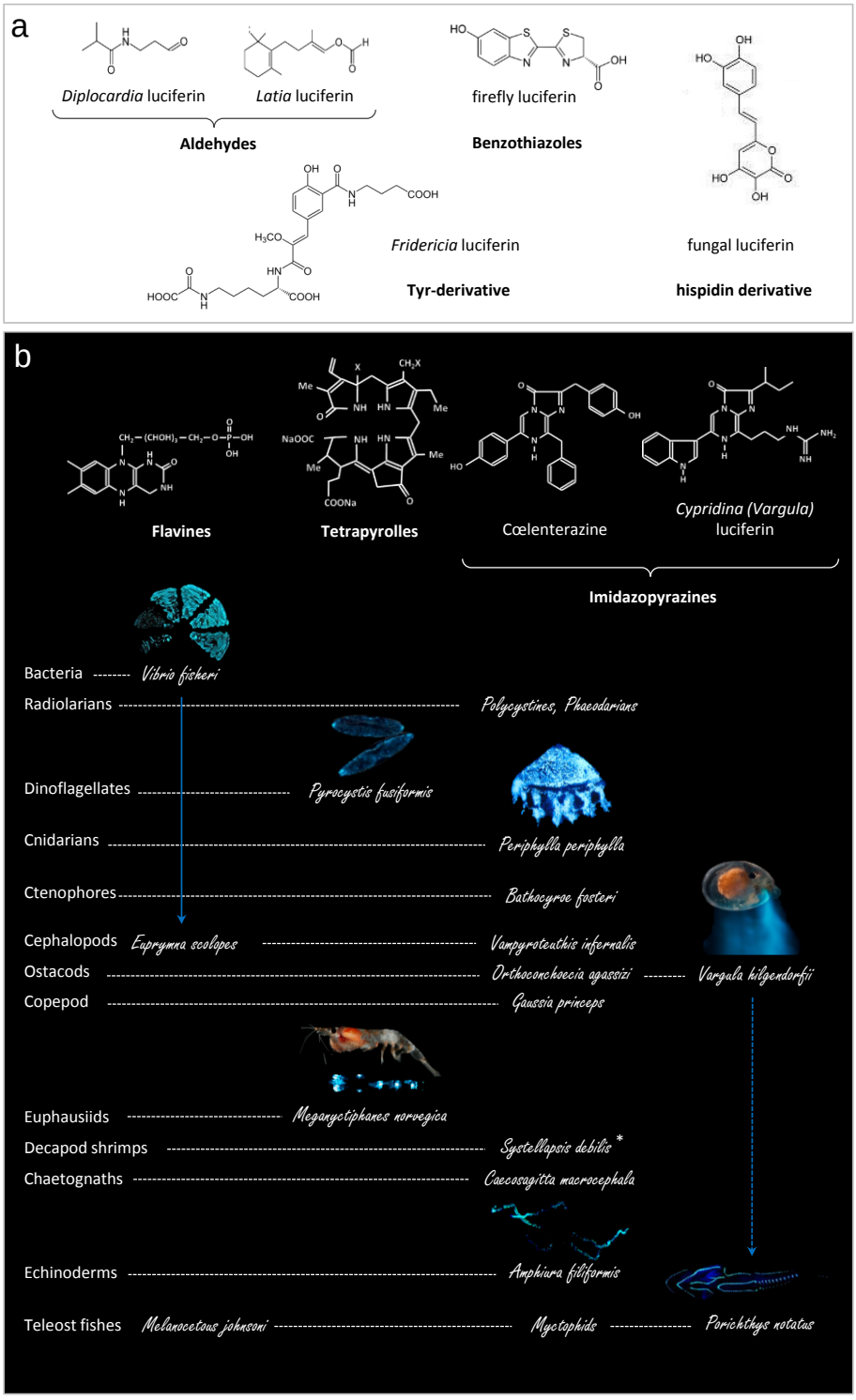

a
Diplocardia luciferin
Latia luciferin
Aldehydes
firefly luciferin
Benzothiazoles
Fridericia luciferin
Tyr-derivative
fungal luciferin
hispidin derivative
b
Flavines
Tetrapyrolles
Cœlenterazine
Cypridina (Vargula) luciferin
Imidazopyrazines
Bacteria
Vibrio fisheri
Radiolarians
Polycystines, Phaeodarians
Dinoflagellates
Pyrocystis fusiformis
Cnidarians
Periphylla periphylla
Ctenophores
Bathocyroe fosteri
Cephalopods
Euprymna scolopes
Vampyroteuthis infernalis
Ostacods
Orthoconchoecia agassizi
Vargula hilgendorfii
Copepod
Gaussia princeps
Euphausiids
Meganyctiphanes norvegica
Decapod shrimps
Systellapsis debilis *
Chaetognaths
Caecosagitta macrocephala
Echinoderms
Amphiura filiformis
Teleost fishes
Melanocetous johnsoni
Myctophids
Porichthys notatus

Fig. 1.5. (previous page) Structural diversity of luciferins from terrestrial (a) and marine (b) organisms.
Examples of symbiosis with bioluminescent bacteria and trophic transfert are represented by a solid blue arrow and a dashed blue arrow respectively. The asterisc indicates *de novo* synthesis of cœlenterazine. (After WIDDER 2001, 2010 and HADDOCK *ET AL.* 2010, PETUSHKOV *ET AL.* 2014).
Photos are courtesy of E. LOWNDES (P. fusiformis, V. hilgendorfii, elliot@mrlowndes.com*), J. MALLEFET (M. norvegica, O. aranea, P. periphylla, P. notatus).*

structure was first described (SHIMOMURA AND JOHNSON 1975, HADDOCK *ET AL.* 2001). A similar trophic transfer was evidenced from cypridinid ostracods to the fish *Porichthys notatus* (BARNES *ET AL.* 1973) [**Fig. 1.5b**]. Moreover, non-luminescent organisms have also been found to contain and store significant amounts of coelenterazine from preys they have ingested (SHIMOMURA 1987, THOMSON *ET AL.* 1997). Assuming the efficiency and regularity of these trophic transfers, the acquisition of light emitting capabilities theoretically only requires the development of an endogenous enzyme. According to a recent study, four amino acids engaged in the substrate-enzyme binding with coelenterazine would be needed to create a solvent cage providing the appropriate electrochemical environment to catalyse a chemiluminescent protoreaction (CAMPBELL 2012, VASSEL *ET AL.* 2012). These results bring the first important element to explain why such a variety of enzymes has acquired the same catalytic properties. This diversity also suggests that it has been relatively common for bioluminescence to evolve from preexisting metabolic pathways (TIMMINS *ET AL.* 2001). The current hypothesis for the origin of bioluminescence systems is that prototypical light emitting oxidative reactions initially developed as preventive defence against deleterious reactions of oxygen through the “futile” consumption of oxygen – *i.e.* without generating useful product although the reaction achieves a biological effect (MCELROY AND SELIGER 1962, TIMMINS *ET AL.* 2001). Following the increase of oxygen concentration in the atmosphere and ocean surfaces approximately 2.5 to 1 billion years ago, and then in the deep-sea environments *ca.* 580 million years ago, bioluminescence amplified until reaching a sufficient intensity to be perceived by light-sensitive organisms. The chemiluminescent properties of luciferins or their precursors then became subject to selective pressure independently of their oxygen-consuming role (TIMMINS *ET AL.* 2001). The fossil records being rare, we cannot precisely date the first appearance of bioluminescence in evolution. Apart from a *ca.* 100 million years old myctophid specimen showing photogenic structures on the ventral surface, most attempts at dating are based on general morphological similarities or phylogenetic separations between bioluminescent and non-bioluminescent lineages (HADDOCK *ET AL.* 2010, CAMPBELL 2012).

1.3.2. Photogenic structures

In bioluminescent metazoans – which will be the main subject of this section – the light emitted reaction generally occurs in specialised cells (photocytes), epitheliums and organs (photophores) developed by the organism itself (in this context, reference is made to intrinsic or endogeneous bioluminescence) (HASTINGS AND MORIN 1991). Only cephalopods and fishes owe their bioluminescent properties to pure cultures of bacterial symbionts hosted within light glands (in this case, reference is made to extrinsic or symbiotic bioluminescence) (NEALSON AND HASTINGS 1979, 1992, RUBY AND MORIN 1979, DUNLAP AND KITA-TSUKAMOTO 2006). Some pyrosomes (colonial thaliaceans, Tunicata) also appear to produce light from photocytes containing bacteroid intracellular organelles believed to have an endosymbiotic origin (MACKIE AND BONE 1978), but this is still a matter of debate. Finally, bacteria are also found as free planktonic forms like other bioluminescent unicellular organisms (dinoflagellates and radiolarians) (RUBY *ET AL.* 1980, LAPOTA *ET AL.* 1988, LATZ *ET AL.* 1991).

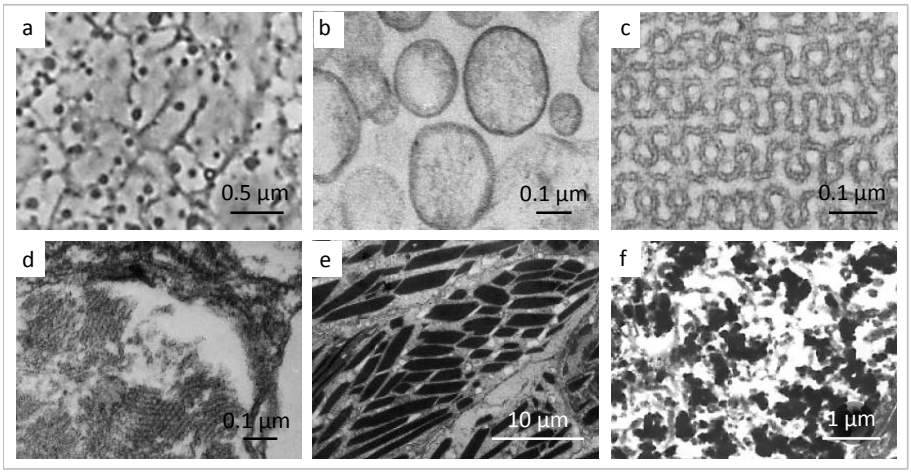


Fig. 1.6. Structural diversity of microsources.
(a) Scintillons : membrane-bond bodies from *Noctiluca miliaris* (Dinoflagellata) (retrieved from ECKERT AND REYNOLDS 1967 with permission, ©1967 Roger and George Journal of General Physiology); **(b)** Lumisomes: membrane-enclosed particles from *Renilla reniformis* photocytes (Cnidaria) (retrieved from ANDERSON AND CORMIER 1973 with permission); **(c)** Photosomes: paracrystalline membranous structures from *Acholoe astericola* photocytes (Polychaeta) (retrieved from BASSOT AND NICOLAS 1978 with permission); **(d)** Post-emission vesicles from *Amphipholis squamata* dissociated photocytes (Echinodermata) (retrieved from DEHEYN *ET AL.* 2000 with permission); **(e)** Crystalloids from *Theuthowenia megalops* photocytes (Cephalopoda) (retrieved from HERRING *ET AL.* 2002 with permission); **(f)** Glowons: membrane-free granular inclusions from *Etmopterus spinax* photocytes (Chondrichthyes) (retrieved from RENWART *ET AL.* 2014 with permission).

The light emitting molecules usually do not react directly in the cytoplasm, but are synthesised and compartmentalised in particular organelles of great morphological and functional diversity [**Fig. 1.6**]. These subcellular sources of luminescence, called microsources, are differently named according to taxa: "scintillons" in dinoflagellates, "lumisomes" in cnidarians, "photosomes" in annelids, "glowons" in sharks. Since their identification requires extensive electron microscope examination, these structures have been described in few organisms (reviewed by SWEENEY 1980). Some authors noticed morphological similarities between photogenic and photoreceptive microstructures from the same organisms, evoking the possibility of similar physiological and subcellular mechanisms (BUCK 1961, ARNOLD AND YOUNG 1974, ARNOLD *ET AL.* 1974, BASSOT AND NICOLAS 1978). However, although a dual role for the photocytes in both sensing and producing light was reinforced by recent studies (next page), the morphology of the microsources discovered since then does not corroborate this idea and suggests a more nuanced interpretation.

1.3.2.1. Simple photocyte systems

Photocytes are the smallest functional unit of light emission[1] that can be located singly, in clusters or widely scattered. They typically lie on superficial tissues, more precisely in or close to the ectoderm. However, they can be more deeply embedded in translucent tissues – the mesoglea in cnidarians, the spines in ophiuroids – ensuring the transmission of light (HASTINGS AND MORIN 1991).

The bioluminescent activity of photocytes can be intracellular, involving intrinsic microsources, or extracellular. Secretion may be direct, *via* exocytosis of cellular products, or indirect, involving the action of contractile elements, either within the photocytes or in adjacent muscular tissues (MARTIN AND ANCTIL 1984, KRÖNSTRÖM *ET AL.* 2009). In some cases, different types of glandular cells can produce the light emitting reactants separately, allowing these compounds to combine and produce the chemiluminescent reaction only once released into the external environment (HARVEY 1952, BASSOT 1966, HASTINGS AND MORIN 1991). This mechanism is known in the ostracod *Cypridina (Vargula) hilgendorfii* (MORIN 1986, SHIMOMURA 2006). Alternatively, the reactants can be activated during their expulsion by various cofactors naturally present in the environment (*e.g.* oxygen, cations). Some secreting species such as hemichordates and polychaetes appear to produce both intracellular and secreted bioluminescence depending on biological

[1] Some authors consider that the smallest functional units of light emission are the microsources. The term functional unit must be understood here as including all the basic elements of the cellular machinery needed to produce the bioluminescent chemicals.

(life stage, maturity…) or environmental factors (season, nature of the excitation…) (BAXTER AND PICKENS 1964, HERRING 1985a, WIDDER 2002, DEHEYN AND LATZ 2009).

The organisms bearing simple photocytes (mainly cnidarians, ctenophores, echinoderms and nemerteans) are believed to use bioluminescence as a reflexive behaviour against predation because of supposedly limited photosensitive capabilities (HADDOCK AND CASE 1999). However, Schnitzler and associates (2012) have evidenced the co-expression of opsin and photoprotein genes in photocytes of *Mnemiopsis Leidyi* (Ctenophora) suggesting a dual role for these cells in both sense and produce light. An unexpectedly high opsin diversity has also been found in the bioluminescent brittle-star *Amphiura filiformis* (DELROISSE *ET AL.* 2014). Additionally, the co-occurrence of luciferase and opsins in the spine inner tissues of this brittle-star supports the hypothesis of a feedback control of luminescence throught extraocular receptors (DELROISSE 2015).

Slightly more complex arrangements occur in molluscs, annelids, crustaceans and fishes showing clustered photocytes (*e.g.* in the distal parts of amphipods' appendages, HERRING 1981). Apart from pigmented cells restricting the angle of light emission, these simple light organs lack accessory structures as observed in photophores (HASTINGS AND MORIN 1991).

1.3.2.2. Photophores

Photophores are complexly organised and controlled light organs (HASTINGS AND MORIN 1991). Metazoans can bear from several to hundreds of these organs, each of which measuring up to several millimetres in diameter. They are particularly well-developed in midwater crustaceans, cephalopods and fishes that exhibit efficient visual systems and complex behaviours. Their size may then vary for a given species proportionally with that of the animal and depending on the sex and their location on the body (HERRING 2007). In counterilluminating species, photophores are mainly distributed in the ventral and lateral regions, and are oriented downward (CLARKE 1963, HERRING AND MORIN 1978). In some Dalatiidae (Chondrichthyes), their density decreases regularly from ventral to dorsal area (REIF 1985) [**Fig. 1.7c**].

Structurally, a single photophore consists of a central photogenic region composed of photocytes usually arranged in a rosette, surrounded by various structural elements including pigmented cells, reflectors, lenses and filters [**Fig. 1.7a,b**] (HASTINGS AND MORIN 1991). Combined, these dioptric devices form organs whose level of sophistication suggests how their biological function can be important.

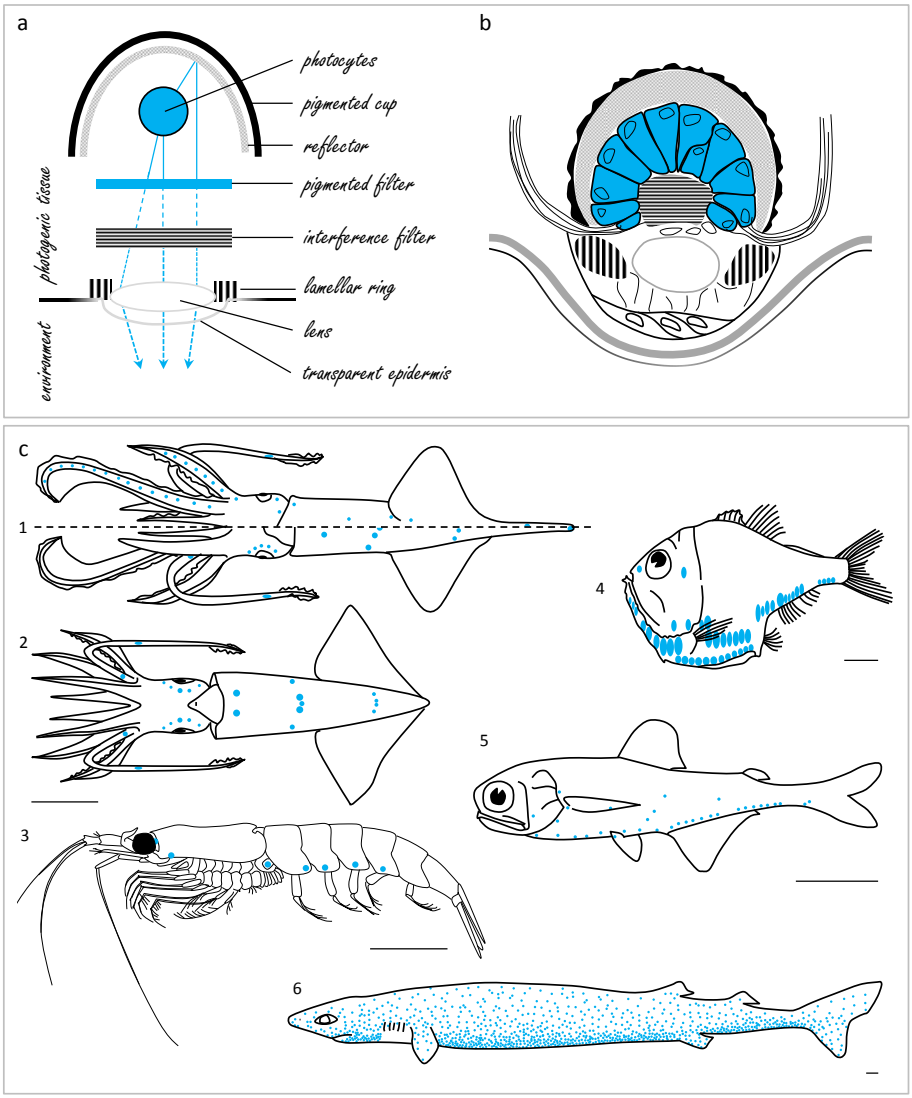


Fig. 1.7. Intrinsic light organs.
(a) Diagram of photophore general organisation including some common optical accessory structures (*e.g.* a pigmented cup protects the underlying tissues from light undesirable effects, a reflector reflects the light in the correct direction, filters tune the light emission wavelength and intensity, a lens focusses the light) (after HASTINGS AND MORIN 1991 with permission); **(b)** Composite representation of thoracic photophores of schizopod shrimps (similar graphic patterns highlight analogous structures) (after BUCK 1961 with permission); **(c)** Photophores (blue colored) distribution patterns in mesopelagic species: (1-2) *Lycoteuthis diadema* (Cephalopoda) ♂ in dorsal and ventral views (1) and ♀ in ventral view (2), (3) *Meganyctiphanes norvegica* (Crustacea), (4) *Argyropelecus sp.* (Actinopterygii), (5) *Myctophum spinosum* (Actinopterygii) and (6) *Isistius brasiliensis* (Chondrichthyes) (redrawn from HERRING AND MORIN 1978, REIF 1985 and HERRING 2007 with permission). Scale bar 10 mm.

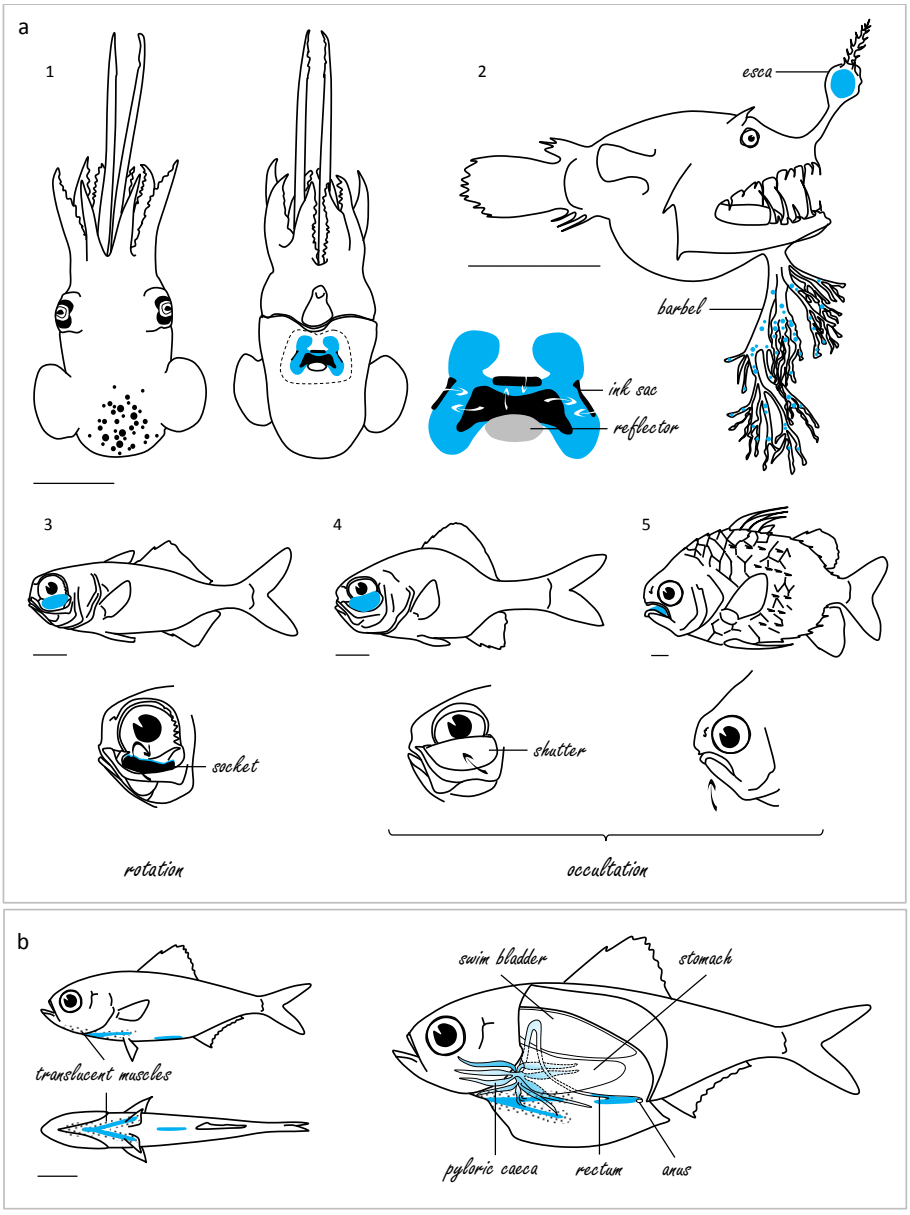


Fig. 1.8. Glandular symbiotic (a) and non symbiotic (b) light organs.
(a) Bacterial symbiosis: (1) *Euprymna scolopes* (Cephalopoda) in dorsal and ventral views: diverticula of the ink sac expand and contract over the bacteria-containing tissue (blue colored) to control the expression of light (after MONTGOMERY AND MCFALL-NGAI 1998) (2) *Linophryne arborifera* female (Actinopterygii) producing light from both the esca and the barbel (redrawn from HANSEN AND HERRING 1977, HERRING AND MORIN 1978 with permission), (3-5) *Anomalops katoptron*, *Photoblepharon palpebratus* and *Cleidopus gloria-maris* (Actinopterygii): the continuous light from symbiotic bacteria can be blocked by rotation or occultation of the light organ (redrawn from HERRING AND MORIN 1978 with permission); →

(b) Non symbiotic organs: *Parapriacanthus ransonneti* (Actinopterygii) has a Y-shaped thoracic luminous duct and a linear anal luminous duct which are connected to the pyloric caeca and to the rectum respectively. The luciferin stored in the pyloric caeca might be obtained by feeding on luminous preys such as *Cypridina* (redrawn from HERRING AND MORIN 1978, HANEDA *ET AL.* 1966 with permission). Scale bar 10 mm.

1.3.2.3. Glandular light organs

Morin (1983) suggested a clear distinction between discrete photophores and glandular light organs that he defined as gland-like outpocketings of the gut or body surface containing intrinsic or bacterial light emitting material [**Fig. 1.8**]. They have the same optical accessory structures than the photophores but are generally larger and limited in number to one or two bodies per individual. Here again, only teleost fishes, some squids and shrimps are concerned (HERRING AND MORIN 1978).

Bacterial light organs are the dominant type of glandular light organs, especially in demersal organisms [**Fig. 1.8a**]. The association between hosts and symbionts (*e.g. Vibrio*, *Photobacterium*) is species-specific but many symbiotic bacterial strains appear to transit through different ecological niches (NEALSON AND HASTINGS 1992, DUNLAP AND KITA-TSUKAMOTO 2006). The host can usually control the light emission by indirect mechanisms that will subsequently be discussed (NEALSON AND HASTINGS 1979).

Non-bacterial light organs intrinsically producing light appear in some benthic pempherid and apogonid fishes. The light is emitted ventrally from thoracic and anal ducts embedded in translucent muscles and anatomically connected to the digestive tract [**Fig. 1.8b**]. All or part of their biochemical system is presumably derived from that of cypridinid ostracods absorbed through their diet (HANEDA *ET AL.* 1966, TSUJI AND HANEDA 1966).

1.3.3. Control mechanisms

Some bioluminescent organisms – especially the procaryotes – produce a continuous glow. However, most luminescent displays are transient events involving control mechanisms (NICOL 1960, CASE AND STRAUSE 1978). Two control levels are commonly distinguished: intrinsic control including the photogenic reaction and the related intracellular signalisation pathways, and extrinsic control represented by peripheral extracellular control pathways (CASE AND STRAUSE 1978).

1.3.3.1. Symbiotic systems

Basically, the luminescence of bacteria is controlled by a mechanism called quorum sensing. It was discovered in the genus *Vibrio* producing light at high population density in response to the accumulation of secreted signalling molecules called "autoinducers"; the detection of a minimal threshold stimulatory concentration of this autoinducer leads to the activation of the luciferase structural operon *luxCDABE* (NEALSON AND HASTINGS 1979, HASTINGS AND GREENBERG 1999, MILLER AND BASSLER 2001). However, it is currently known that organisms bearing bacterial light organs can directly modulate the light emitted by their symbionts, offering them more or less favourable growth conditions (*e.g.* blood supply regulation). Such mechanisms, which are mediated by physiological, biochemical and genetic factors, may be combined with or replaced by mechanical control systems. For example, the light organ can be instantly occulted by a kind of shutter, the rotation of the organ [**Fig. 1.8b**] or by chromatophore insinuation modulating the degree of tissue translucence (NICOL 1960, HERRING 1978, NEALSON AND HASTINGS 1979, HASTINGS AND MORIN 1991).

1.3.3.2. Endogenous systems

In self-luminescent metazoans, the emission is controlled by coupling mechanisms between photocytes and various excitable cells. Although bioluminescence can be partly mediated by nerve-free epithelial propagation of the excitability state, especially in lower invertebrates (*e.g.* in Cnidaria: BASSOT *ET AL.* 1978, ANCTIL 1979, DUNLAP *ET AL.* 1987, GERMAIN AND ANCTIL 1996), the emission process remains mostly controlled by neural pathways (NICOL 1960, CASE AND STRAUSE 1978, ANCTIL 1987). Hormonal control has only been demonstrated in sharks so far (*Etmopterus spinax*, *E. splendidus* and *Squaliolus aliae*, CLAES AND MALLEFET 2009, 2011, CLAES *ET AL.* 2011, 2012).

Numerous studies have been devoted to the control mechanisms for endogenous light production, combining electrophysiological, pharmacological and immunohistochemical approaches (reviewed by CASE AND STRAUSE 1978, ANCTIL 1987). A variety of neural structures can be involved in the control of bioluminescence, ranging from subepidermal nerve nets and plexuses in invertebrates endowed with simple nervous systems, to centralised systems (ganglia) in higher taxa. The photocytes are not always directly innervated. Instead, the nerve fibres can reach adjacent cells relaying the excitatory signal to photocytes by another pathway. For instance, in the Euphausiid *Meganyctiphanes norvegica*, bioluminescence control might be achieved through the action of

sphincter-like elements regulating the haemolymph (*i.e.* oxygen) supply to the photocytes (KRÖNSTRÖM *ET AL.* 2009).

From a pharmacological point of view, the nervous control of bioluminescence involves various classical neurotransmitters and neuromediators including adrenaline, noradrenaline, acetylcholine (ACh), serotonin, GABA and nitric oxide (ANCTIL 1979, ANCTIL 1987). However, their implication and mode of action cannot be generalised, even within a given taxa (*e.g.* in ophiuroid echinoderms: DEWAEL AND MALLEFET 2002a, MALLEFET 2009). The recurring contribution of cellular and molecular calcium-dependent mechanisms has been discussed by Case and Strause (1978).

1.3.3.3. Visual feedback control

The perception of their own light production by bioluminescent organisms seems to be a key step in the control process. For instance, it is not uncommon that counterilluminating species also possess suborbital or ocular photophores synchronised with their ventral photophores (HERRING AND LOCKET 1978, HERRING AND MORIN 1978). Moreover, extraocular photoreception capabilities observed in midwater cephalopods and fishes (YOUNG *ET AL.* 1979, TONG *ET AL.* 2009) could possibly exist in lower, non-visual bioluminescent invertebrates (e.g. in coelenterates, echinoderms: SCHNITZLER *ET AL.* 2012, DELROISSE *ET AL.* 2014).

1.3.4. Bioluminescence displays

From the emission of bright green flashes along the arms of the brittle-star *Ophiopsila aranea*, to the release of a blue glowing slime by the tube worm *Chaetopterus variopedatus*, bioluminescent organisms exhibit a wide variety of signals controlled upstream and downstream of the luminescent reaction. They are commonly characterised by different parameters such as spectral distribution, intensity, periodicity and angular distribution.

1.3.4.1. Spectral distribution

One essential feature of bioluminescence having an important ecological significance is its spectral composition or colour (HASTINGS AND MORIN 1991). It is basically determined by the molecular structures involved in the chemiluminescent reaction, but it may be affected by the reaction environment (*e.g.* pH) or by other factors such as accessory fluorescent proteins or optical biological filters (HASTINGS 1996, WILSON AND HASTINGS 1998).

1.3.4.1.1. Chemically-based diversity

In the simplest case, the bioluminescent emission spectrum matches the fluorescence spectrum of an excited luciferase-bound product or intermediate of the reaction (ELEY *ET AL.* 1970, SHIMOMURA AND JOHNSON 1970). The luciferase structure can itself alter the colour, like in the fireflies where substitution of a single residue in the luciferase is known to result in significant shifts in the emission spectrum (WOOD 1990, WILSON AND HASTINGS 1998, NAKATSU *ET AL.* 2006, HOSSEINKHANI 2011). In the same way, mutations in photoprotein sequences can change the wavelength of light emission (REMINGTON 2000). However, the bioluminescence spectrum may not match the fluorescence of the luciferase reaction product due to the presence *in vivo* of secondary emitters. Apart from the well-known coelenterate GFP, wild-type blue, yellow and red fluorescent proteins (FPs) were isolated from the bacteria *Photobacterium phosphoreum*, *Vibrio fischeri* and the dragon-fish *Malacosteus niger* respectively (LEE AND KOKA 1978, KOKA AND LEE 1979, DAUBNER *ET AL.* 1987, CAMPBELL AND HERRING 1987). Since the YPF of *V. fischeri* Y-1 alters both the emission spectrum and reactional kinetic, it is believed to interact with one of the intermediates prior to the formation of the excited state (RUBY AND NEALSON 1977, DAUBNER *ET AL.* 1987, ECKSTEIN *ET AL.* 1990). The currently known GFP-like constitute a superfamily of fluorescent, non-fluorescent (chromoproteins or CPs) or colourless proteins which can be involved or not in a bioluminescence process. According to Shagin *et al.* (2004), the evolution history of cnidarian GFP-like would have been punctuated by 15 colour diversification events. Despite a strong interest in discovering new FPs/CPs for biotechnological applications, the possible biological functions of these molecules in non-bioluminescent lineages, such as the colourful reef-building corals, remain largely unexplored (VERKHUSHA AND LUKYANOV 2004, ALIEVA *ET AL.* 2008, PALMER *ET AL.* 2009).

1.3.4.1.2. Intraspecific variation

A single bioluminescent individual typically emits only one colour, but in rare cases, we can observe dual emissions *in vivo* (HERRING 1983, WIDDER *ET AL.* 1983, LATZ *ET AL.* 1988, HADDOCK AND CASE 1999, JOHNSEN *ET AL.* 2012). The greatest spectral contrast between two bioluminescent signals is probably to be found in three genera of stomiatoid fishes – *Pachystomias*, *Aristostomias* and *Malacosteus* – which have ventral and postorbital light organs producing a bluish emission (460-490 nm) and an infraorbital organ producing light with much longer wavelengths: 600-700 nm (WIDDER *ET AL.* 1984, SCHWAB AND MARSHALL 2004, HERRING AND COPE 2005). This red bioluminescence would result from the combination of filter effects and energy transfer mechanisms to a fluorescent protein (DENTON *ET AL.* 1970, 1985). In that case, bioluminescence is usually produced from distinct light emitting systems

(although probably derived from each other) and anatomically separate structures, but this is seldom as clear. For example, the emission spectrum of *Abylopsis tetragona* (Siphonophora) shows a major peak at 489 nm, and a secondary peak at 450 nm of increasing intensity towards the anterior part of the animal (HADDOCK AND CASE 1999). The sea pen *Umbellula magniflora* also displays a colour gradient ranging from bright green (505 nm) at the base of the stalk to blue (495 nm) on the plume (JOHNSEN *ET AL.* 2012). The emission spectra of pennatulids are also bimodal, typically characterised by a narrow peak in the blue range and a long wavelength shoulder. Their emission wavelength would be affected by a different degree of association with GFP along the length of the rachis (WAMPLER *ET AL.* 1973, HERRING 1983, HERRING 1983, WIDDER *ET AL.* 1983, JOHNSEN *ET AL.* 2012).

The spectral distribution can also exhibit changes over time, during the ontogenic development – the blue-green gradient from the juvenile light organ of the pinecone fish *Cleidopus gloriamaris* is absent in the adults (WIDDER *ET AL.* 1983); in the Japanese firefly *Luciola lateralis*, one luciferase (maximal emission wavelength, λ_{max} = 539 nm) is predominantly expressed in eggs and pupae, while its homologous (λ_{max} = 550 nm) is expressed in larval and adult lanterns (OBA *ET AL.* 2014) – or following environmental conditions – the midwater squids *Abraliopsis sp.* and *Abralia trigonura* can adjust their luminescence colour under the influence of temperatures of day/surface and night/deep habitats to match the qualitative variations of downwelling sunlight (YOUNG AND MENCHER 1980). According to Latz and colleagues (1988), another squid, *Leachia lemur*, can actively control the emission of at least six colours from deep blue to green. Spectral changes could result from the recruitment of different light-emitting structures, or by associated interference filters and reflectors (*infra*, p. 29).

1.3.4.1.3. Between-habitat variation

In contrast to terrestrial and freshwater bioluminescent species, which emit green-yellow light, the emission maxima of most marine organisms fall within the range 440-520 nm, corresponding to a blue-green light (HERRING 1983, WIDDER *ET AL.* 1983, HASTINGS 1996). These wavelengths, in addition to reaching amongst the maximal transmission coefficients in the sea water [**Fig. 1.2d,e**], typically coincide with the spectral absorption peaks of visual pigments from most marine animals (WARRANT AND LOCKET 2004). It even appears that the visual pigments of deep-sea fishes are better adapted to improving sensitivity to bioluminescence than to the down-welling daylight at mesopelagic depths, which is dominated by slightly shorter wavelengths (YOUNG 1981, WARRANT AND LOCKET 2004). This once again emphasises the omnipresence of living lights in the Ocean as well as the way they influence and structure interactions between species. Within the mainstream community of blue-

green emitters, spectral differences have long been observed between species from different marine habitats [**Fig. 1.9**]. The predominance of green emitters in neritic and benthic environments (490-520 nm), compared to oceanic and pelagic organisms, which are mostly blue emitters (440-490 nm), would merely be driven by the optical characteristics of marine waters [**Fig. 1.2e**] (YOUNG 1981, HERRING 1983, MORIN 1983, WIDDER *ET AL.* 1983, LATZ *ET AL.* 1988, HASTINGS AND MORIN 1991, HASTINGS 1996, HADDOCK AND CASE 1999, JOHNSEN *ET AL.* 2012). Because of suspended sediments due to terrigenous supply and bottom currents, the coastal and benthic habitats are probably more turbid than those found in the open ocean and possibly have a longer wavelength of maximal transmission (JERLOV 1976).

However, aside from environmental constraints, phylogenetic effects are also possible and could explain numerous outliers [**Fig. 1.9b**]. Indeed, some species have escaped the evolution forces determining the near homochromatic nature of bioluminescence and produced "exotic" colours (YOUNG 1981). The red colour was already mentioned in the well-studied stomiatoid fishes (*supra*, p. 26) and is also to be found in the railroad-worms (λ_{max} = 600-638 nm) (VIVIANI AND BECHARA 1997). Yellow and orange are mostly produced by insects, remaining exceptional in marine organisms. Amongst them are the tomopterid annelids, whose maximal emission approximate 565 nm (FRANCIS *ET AL*. 2014, LATZ *ET AL.* 1988).

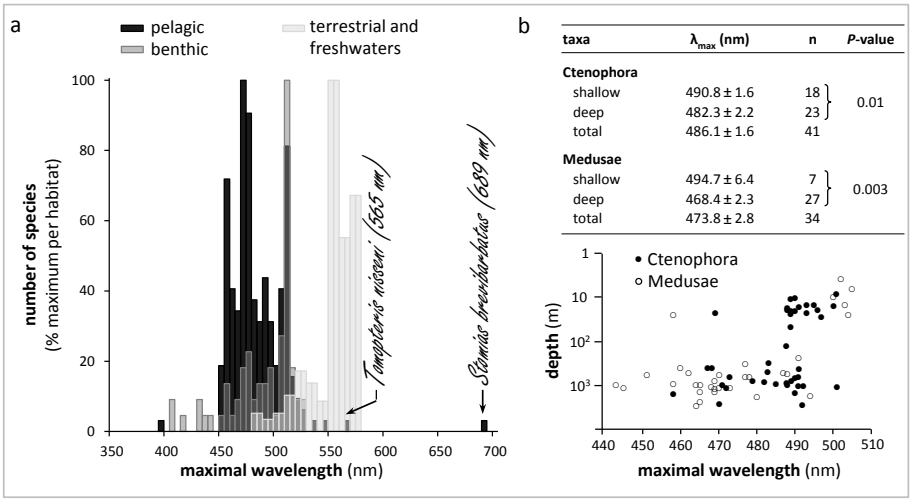


Fig. 1.9. Distribution of bioluminescence emission maxima (λ_{max}) in various habitats.
(a) Comparison of emission maxima of pelagic, benthic and terrestrial organisms (modified from HERRING 1983, HASTINGS AND MORIN 1991 with permission). Two unusual wavelengths are represented by *Tomopteris nisseni* (Polychaeta) and *Stomias brevibarbatus* (Actinopterygii) (LATZ *ET AL.* 1988); **(b)** Depth effect on emission maxima in bioluminescent deep-sea gelatinous plankton (modified from HADDOCK AND CASE 1999 with permission).

1.3.4.1.4. Optical structures

Some bioluminescent fishes, cephalopods and crustaceans showing sophisticated photophores have developed various accessory structures such as absorption or interference filters with spectrally selective properties (DENTON *ET AL.* 1985) [**Fig. 1.7a,b**].

Pigmented material is found in many photogenic tissues, restricting the emitted light spectra to a relatively narrow bandwidth according to the nature and the density of pigments. They seem particularly prominent in the mesopelagic fishes with large ventral photophores (DENTON *ET AL.* 1985).

The photophores from *Pterygioteuthis microlampas* (Cephalopoda) ventral surface are associated with iridophores which act as a structural colour filter transmitting blue light while reflecting yellow-green light back into the photophores (ARNOLD *ET AL.* 1974). In the bioluminescent squid *Abralia trigonura*, a variety of interference structures contribute to regulating at the same time the spectra, the intensity and the angular distribution of the emitted light by altering spaces between the platelets and therefore their optical characteristics (YOUNG AND ARNOLD 1982).

On a different scale, Deheyn and Wilson (2010) have demonstrated the wavelength-specific diffusion of bioluminescence through the shell of a marine snail (*Hinea brasiliana*), suggesting evolutionary co-option of light production and biomineralisation of diffuser material. Similarly, the dorsal spines of the velvet belly lanternshark *E. spinax* could hypothetically transmit about 10% of the luminescence produced by the underlying photophores (CLAES *ET AL.* 2013). The transmitted light which would be detectable in the shark's natural environment by most of its potential predators could function as an aposematic (warning) signal.

1.3.4.2. Other parameters

1.3.4.2.1. Kinetics and intensity

A light emission is a kinetic event. In the pelagic realm, the patterns of light emission range from flashes (< 2 s) to pulsing or continuous glows (> 5 s) (MORIN 1983). The pattern mainly depends on the species, the type of luminous tissue, the method, the strength and the duration of the stimulus, but in some circumstances, it can reflect the physiological condition of the organism (HERRING 1985b). Some species also have a repertoire of different responses that they can use for different purposes (*e.g.* in Leiognathid fishes: MCFALL-NGAI AND DUNLAP 1983). In intracellular systems, the light emission can be observed at the level or individual microsources. Their activity can be synchronous or not: in polynoid worms, macroflashes are shaped by a process that combines intracellular recruitment of the microsources

with the spatial extension of the photogenic area at tissue level (BASSOT 1987), whereas in dinoflagellates, the variable amplitude of the macroflashes induces changes in the amplitudes of each microflashes, and all microsources appear to participate in the formation of each flash (ECKERT AND REYNOLDS 1967).

The light intensity produced by an organism is basically proportional to the quantity of reactive material involved and to the nature or the intensity of the stimulus. However, as previously mentioned, various accessory dioptric structures can create divergent, collimated or focused beams perceived as being more or less intense.

Young and Roper (1977) have experimentally shown that a number of midwater cephalopods, fishes and shrimps are able to adjust their luminosity in a matter of minutes to match the alterations of overhead illumination.

1.3.4.2.2. Angular distribution

In the same way as for the wavelength and the intensity, the angular distribution of bioluminescence is of primary importance in organisms using light as a camouflage (counterillumination), since it must approximate that of downwelling light. This match is thought to be achieved by the distribution (*e.g.* REIF 1985), the orientation (*e.g.* DENTON *ET AL.* 1972) or even the mobility (*e.g.* HARDY 1964) of their photophores. Consequently, camouflage is assumed effective from a wide range of angles of viewing, both ventrally and laterally.

In a more general sense, the presence of oriented photogenic structures possibly involving dioptric structures, reflectors or light-guides usually channel the light into specific directions: outwards, forwards (searchlights) or towards the eyes (feedback control).

1.3.5. Ecological value

Where bioluminescence has evolved, it is generally ecologically important and highly effective, although it has been regarded as physiologically non-essential [2] for survival (HASTINGS AND MORIN 1991, CAMPBELL 2012). For organisms living in complete darkness or in dimly lit environments, bioluminescence is the primary source of light and a major form of communication (HERRING 1990, 2000, WIDDER 2002, 2010, HADDOCK *ET AL.* 2010).

[2] Campbell (2012) stated this as clear evidence, because normally bioluminescent organisms deprived of dietary intake containing the luminescent substrate (*supra*, p. 15) survive either in aquarium or in their natural habitat. However, this could be qualified since the biochemical luminescence system of some bacteria was shown to stimulate the DNA repair upon UV radiations, while they are unable to compete with dark mutant bacteria in the absence of UV irradiation (CRYŻ *ET AL.* 2003).

All organisms share three main challenges in life: avoiding predators, feeding and reproducing. All of these can involve bioluminescence. Thus, an organism can use it in many different ways, either during its whole life or only during critical life stages. However, most of the suggested functions remain untested because of the difficulties raised by *in situ* observations or the

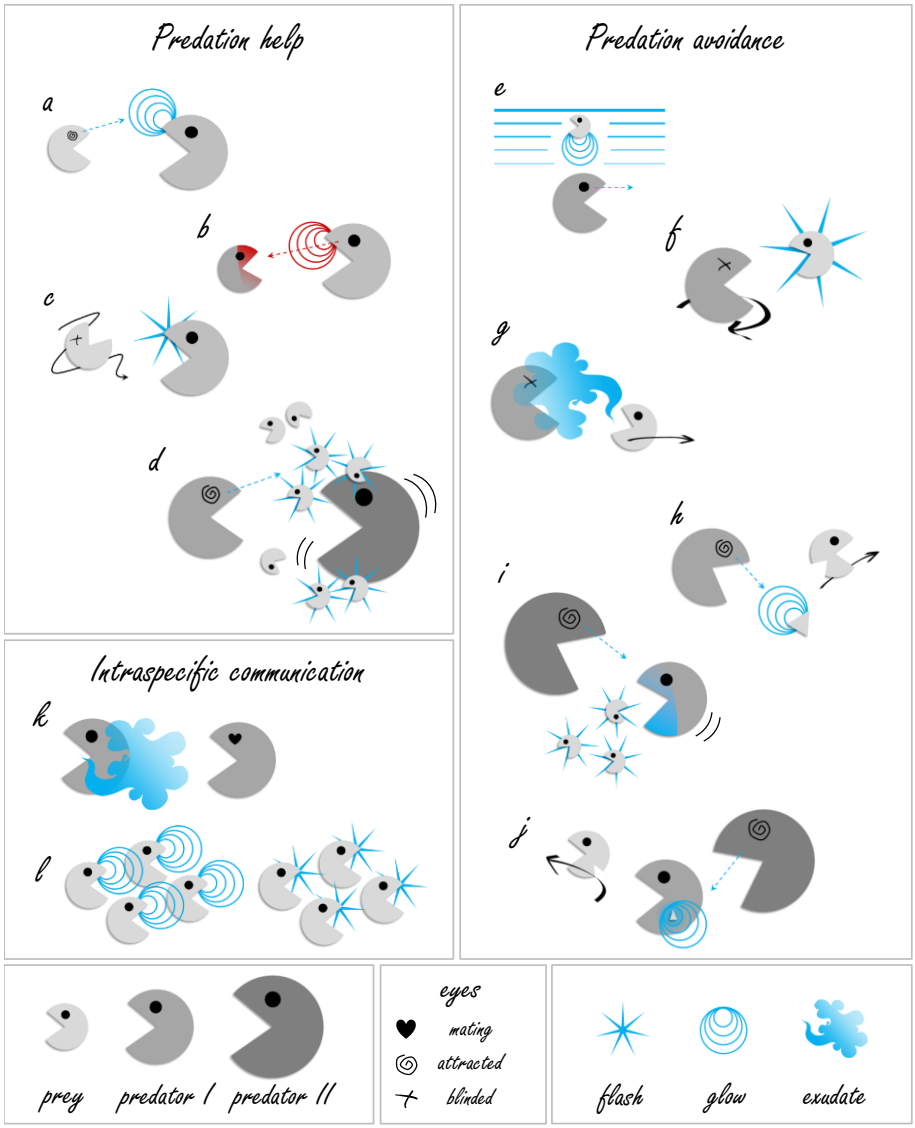


Fig. 1.10. Schematic illustration of the functions of marine bioluminescence.
(a) Lure prey or attract host; **(b)** Illuminate prey (searchlight), break camouflage; **(c)** Stun of confuse prey; **(d)** Attract prey using external, stimulable light as a lure; **(e)** Counterilluminate; **(f)** Startle; **(g)** Misdirection, smoke screen; **(h)** Distractive body part; **(i)** attract a secondary predator by making the first predator visible (burglar alarm); **(j)** Sacrificial tag; **(k)** Mate attraction; **(l)** Congregation.

experimental recreation of natural interactions. Current hypotheses are generally based on a general knowledge of the biology of species, their bioluminescent pattern and their environment (reviewed by BUCK 1978, HADDOCK *ET AL.* 2010).

1.3.5.1. Predation help

In the most general sense, bioluminescent flashes are thought to be repellent, whereas glows are usually described as attractive signals (NICOL 1959, MORIN 1983). The most illustrious representative of this second category is probably the anglerfish. Indeed, in 9 of the 11 families of deep-sea anglerfishes, females attract their prey using a lure containing self-luminous bacterial symbionts [**Fig. 1.10a**]. The light source is located near the mouth, at the end of a moveable fishing rod evolved from the first dorsal-fin spine or from a ventral barbel [**Fig. 1.8a2**] (HANSEN AND HERRING 1977, MUNK 1999). This more or less passive use of bioluminescence has been suggested for other taxa, especially siphonophores and cephalopods (JOHNSEN *ET AL.* 1999, ROBISON *ET AL.* 2003, HADDOCK ET AL. 2005).

In the context of active research of prey in the dark, a bright forwards pointing light can simply contribute to improving the visual acuity and the chances of capture [**Fig. 1.10b**]. The large suborbital organs of *Photoblepharon* and *Anomalops* (Actinopterygii) [**Fig. 1.8a4**] operate in this may (BUCK 1978, HERRING AND MORIN 1978). The red light produced by some Stomiidae would have the additional advantage of revealing the red colour of many deep-sea invertebrates [**Box 1.1a3,4**], which is invisible to blue-green searchlights (JOHNSEN 2005, 2014).

At the most offensive level, bright flashes can be used to stun or confuse preys [**Fig. 1.10c**]. It was suggested in the squid *Taningia danae* and in the myctophid *Diaphus*, but there is little to no experimental evidence for this (KUBODERA *ET AL.* 2007, HADDOCK *ET AL.* 2010).

Finally, some non-bioluminescent predators, such as marine mammals, are thought to use the mechanically stimulable bioluminescence produced by plankton to locate their prey [**Fig. 1.10d**] (ROHR *ET AL.* 1998, VACQUIÉ-GARCIA *ET AL.* 2012).

1.3.5.2. Predation avoidance

As already stated, the camouflage by counterillumination is one of the main functions of bioluminescence in the mesopelagic environment [**Box 1.2, Fig. 1.10e**]. In practice, this strategy enables to approach a prey more easily or to avoid a confrontation with a predator (*e.g.* WIDDER 1998, JONES AND NISHIGUCHI 2004, CLAES *ET AL.* 2010). When it is unable to avoid the interaction, a prey can use bioluminescence as a deterrent signal. Typically, the light emission is mechanically induced in order to produce a startle (bright flash) or a disorienting (luminous

cloud) effect [**Fig. 1.10f,g**]. This strategy is believed to be widely used amongst pelagic invertebrates (HADDOCK *ET AL.* 2010).

Aposematism is a particular defensive mechanism by which a predator, through repeated contacts, learns to associate the bad taste or the toxicity of a prey to its bioluminescence and thus to avoid it (*e.g.* MAREK *ET AL.* 2011, JONES AND MALLEFET 2012a, 2013). This strategy is particularly interesting in organisms able to autotomise and regenerate bioluminescent body parts, such as ophiuroids. If the prey is caught, this mechanism can still benefit the population.

Autotomy can also be used to create a diversion: the distractive body part is released at a distance from the cryptic non-luminescing potential prey, which thus can escape [**Fig. 1.10h**]. In annelids, the elytra of scale worms and the "bombs" released by the acrocirrid genus *Swima* are perfect illustrations of this phenomenon (MORIN 1983, OSBORN *ET AL.* 2009).

The burglar alarm strategy exploits an exterior participant: the light emitted by the prey makes the predator vulnerable to attack from higher order predators [**Fig. 1.10i**] (MESINGER AND CASE 1992, JONES AND MALLEFET 2012b). This can last until the end of the interaction or extend during several hours afterwards. Indeed, bioluminescence can persist as an external tag (*e.g.* the sticky glowing exudate produced by the deep-sea squid *Vampyrotheutis infernalis*, ROBISON *ET AL.* 2003) or within the stomach of a transparent animal [**Fig. 1.10j**].

1.3.5.3. Intraspecific communication

The use of bioluminescence for sexual recognition and mating [**Fig. 1.10k**], suggested in fireflies in 1647, is now also known in the marine environment (BUCK 1978). The cypridinid ostracods, which have extensively been studied, show species-specific displays of secreted luminescence involved in complex mate-finding behaviours (MORIN 1986, MORIN AND COHEN 2010). Similarly, epitokes of the syllid annelids have long been known for their bioluminescence displays that occur according to lunar periodicity (*infra*, p. 40).

In short, this function was frequently – and sometimes abusively – postulated on the basis of diverse observations: *e.g.* luminescent-based sexual dimorphism, unisexual distribution of photophores, synchronic maturation of gonads and light organs, or limitation of luminescence to the breading season (reviewed by HERRING 2000, 2007).

In motile organisms, specific bioluminescent patterns can facilitate social congregations [**Fig. 1.10l**] providing mutual benefits, especially for feeding or defence (BUCK 1978).

Light emission and visual sensitivity at unusual wavelengths suggest that bioluminescence is used as a private communication channel rather than in a predator-prey interaction context (HERRING 1990, HADDOCK *ET AL.* 2010).

1.3.6. Bioluminescence in plankton

The smallest planktonic organisms are certainly responsible for the most impressive natural bioluminescent phenomenon on Earth. Famous for their toxicity but also for their bioluminescence, red tides – so named after the reddish colour of the seawater in daylight – result from phytoplanktonic blooms dominated by various species of dinoflagellates (*e.g. Noctiluca*, *Pyrocystis*, *Pyrodinium*, *Ceratium*, *Lingulodinium*, *Protoperidinium*) (HERRING 2001). The bioluminescence of these organisms being triggered by mechanical disturbance, their abundance (up to 10^7 cells/l) reveals the seawater turbulences or can betray the presence of a swimming organism (HASTINGS AND MORIN 1991, LATZ *ET AL.* 1994, ROHR *ET AL.* 1998). Similar glowing or sparkling patches usually encountered in shallow waters are likely to be surface mating aggregations of luminous animals such as ostracods, euphausiids shrimps or syllid worms (HERRING 2001).

A less frequently observed large-scale bioluminescent display is called "milky sea", in reference to the descriptions reported by mariners since the 17^{th} century. Most agree that the sea surface turns into a uniform glowing white colour often extending as far as the horizon, as if their boat were sailing through milk or over a snow-covered field (NEALSON AND HASTINGS 2006). Indeed, luminescence can cover an extensive area ranging from tens to hundreds of kilometers, be detectable from space and last from several hours to several days (MILLER *ET AL.* 2005, 2006). Such phenomena were usually reported in calm seas, especially in the Northwest Indian Ocean in zones characterised by oceanic fronts. Although bacteria are the only known marine organisms producing light continuously, the concentration of these free-living forms in the open ocean is too low to achieve bioluminescence induction by quorum sensing (NEALSON AND HASTINGS 1979, 1992). The favourable conditions would be offered by the massive development of *Phaeocystis* microalgae acting as substratum and nutrient source for colonising luminous bacteria (*e.g. Vibrio harveyi*) (LAPOTA *ET AL.* 1988, NEALSON AND HASTINGS 2006). However, additional samplings and *in situ* measurements are needed to confirm this hypothesis.

Since several decades, *in situ* planktonic bioluminescence has become a useful tool to assess population structure and dynamics, thereby complementing bio-optical (fluorescence, absorption) and acoustic approaches. In fact, the intraspecific differences in terms of excitability and bioluminescence kinetics allow

researchers to quantify and discriminate specific functional groups, and even identify many light sources to the species level (LOSEE *ET AL.* 1985, LATZ *ET AL.* 1994, KOCAK *ET AL.* 1999, WIDDER AND JOHNSEN 2000, WIDDER 2002, MOLINE *ET AL.* 2009, HADDOCK *ET AL.* 2010). A given bitoluminescence signature can thus be related to the nature of the stimuli (hydrodynamic processes, swimming non-luminous organisms such as marine mammals or fish) inducing this bioluminescence (HARVEY 1952, LAPOTA *ET AL.* 1989, ROHR *ET AL.* 1998, VACQUIÉ-GARCIA *ET AL.* 2012). Today, numerous devices are equipped with bathyphotometric sensors combined, in some cases, with a turbulence excitation system (*e.g.* static, profiling and undulating systems, autonomous underwater vehicles, diving mammals carrying data recorder) to measure what is called "stimulable bioluminescence" or "bioluminescent potential" (CLARKE AND KELLY 1965, SELIGER *ET AL.* 1969, WIDDER *ET AL.* 1993).

Despite the significant contribution of unicellular organisms – forming what is called "background luminescence" – to oceanic bioluminescence (KELLY AND TETT 1978, LAPOTA *ET AL.* 1988, BATCHELDER AND SWIFT 1989), a large proportion of meso- and macroplanktonic phyla is known to contain luminous species (NICOL 1958, POUPIN *ET AL.* 1999, HADDOCK *ET AL.* 2010). This is hardly surprising, since the pelagic domain is favourable to the evolution of both planktonic life mode and bioluminescence. Furthermore, these evolutionary orientations seem to be correlated since numerous bioluminescent species are the only pelagic representatives amongst typically sedentary taxa (*e.g.* Acrocirridae, Flabelligeridae (Annelida), presented in the next section).

It may seem paradoxical that the gelatinous organisms, whose transparency tends to make them invisible, emit visible light. Yet the co-occurrence of these two characters is not exceptional. As a matter of fact, it appears to be very common in some taxa. For instance, amongst the highly transparent hydrozoans (JOHNSEN AND WIDDER 1998) [**Fig. 1.3a**], most siphonophores are bioluminescent, except for some Cystonectae, benthic rhodaliid species, and two physonect genera (HADDOCK *ET AL.* 2010). Actually, the gelatinous organisms could have developed bioluminescence as a countermeasure against various constraints created by transparency: to be invisible to predators gives a major competitive advantage, but it is crucial to remain visible to its conspecifics. Thus, bioluminescence can especially allow sexual partners to find each other in the oceanic space (HERRING 2000). Moreover, transparency usually involves a structural simplification of the body, which is often thin and fragile or even deprived of locomotor organs. In this case, bioluminescence can be repellent to avoid contact with others or take the form of lures that attract prey (HADDOCK *ET AL.* 2005). None of these functions has clearly been demonstrated. However, given the limited photosensing abilities of cnidarians and ctenophores, the evolution of bioluminescence as well as the

selection of specific wavelengths would be more probably driven by the photosensing systems of the fishes and crustaceans with which they interact (HADDOCK AND CASE 1999).

1.3.7. Bioluminescence in polychaetes

(Generalities about annelid polychaetes, including a glossary, are presented in appendix A.)

According to the last estimates, more than 80 families of polychaetes are currently recognised, including over 9000 valid species (FAUCHALD AND ROUSE 1997, STRUCK *ET AL.* 2007, 2011, WEIGERT *ET AL.* 2015). Since their appearance in the Precambrian, annelids have successfully colonised all habitats where water was available. Today, polychaetes are particularly abundant in the Ocean where they take advantage of a large diversity of life history strategies (BRUSCA AND BRUSCA 2003). Although they are thought of as benthic organisms, many polychaetes dwell in the water column for a part of their life, and at least nine families, which mostly belong within Phyllodocida, have acquired a holopelagic existence independently (ROUSE AND PLEIJEL 2001, 2003, HALANYCH ET AL. 2007, OSBORN AND ROUSE 2008).

To our knowledge, bioluminescence capabilities are clearly established in eight unrelated families in which bioluminescence is believed to have appeared independently [**Fig. 1.11**]. Most of these taxa include mero- and holoplanktonic species: Syllidae, Tomopteridae, Acrocirridae, Cirratulidae, Flabelligeridae (DAHLGREN 1916, HERRING 1987, HASTINGS AND MORIN 1991, POUPIN *ET AL.* 1999, HADDOCK *ET AL.* 2010, OSBORN *ET AL.* 2009, 2011). However, some strictly benthic representatives are known in Polynoidae, Terebellidae and Chaetopteridae (HARVEY 1952, HADDOCK *ET AL.* 2010). Finally, photogenic capabilities were suggested in Alciopidae (OKADA 1925, HARVEY 1952, CLARK 1970, DALES 1971, POUPIN *ET AL.* 1999) as well as in some nephtyid, nereid, onuphid and opheliid specimens, but that remains most often speculative (HARVEY 1952, HERRING 1987).

1.3.7.1. Polynoidae

The polynoid worms are purely benthic animals whose major morphological characteristic is a double row of scales or elytra covering their dorsal surface. These neuroectodermal structures are made of a double layered epithelium protected with a cuticle (PAVANS DE CECCATTY *ET AL.* 1977). They can autotomise easily at their site of insertion (elytrophore) and regenerate in 10-15 days (NICOLAS 1977).

Bioluminescent species are known from numerous genera including *Lagisca*, *Acholoë*, *Polynoë*, *Gattyana*, *Harmothoë*, *Malmgrenia*, *Lepidonotus*, *Polyennoa*, *Eunoë* and *Hesperonoë* (HERRING 1978). Photocytes lie in the lower epithelium of the elytra in a photogenic area concentric to the elytrophore. The site

of flashing typically appears near this central point and extends towards the periphery as successive waves. All species appear to emit green light (λ_{max} = 510-520 nm) (HERRING 1983). The main interspecific differences were noticed in photocytes distribution and in the light propagation pattern within the photogenic area (BASSOT AND BILBAUT 1977a, BASSOT AND NICOLAS 1995). Herrera (1979) has demonstrated that at least some photocytes received direct innervation, the excitation being propagated by conduction through the epithelium and achieved by calcium entry into photocytes (reviewed by ANCTIL 1987).

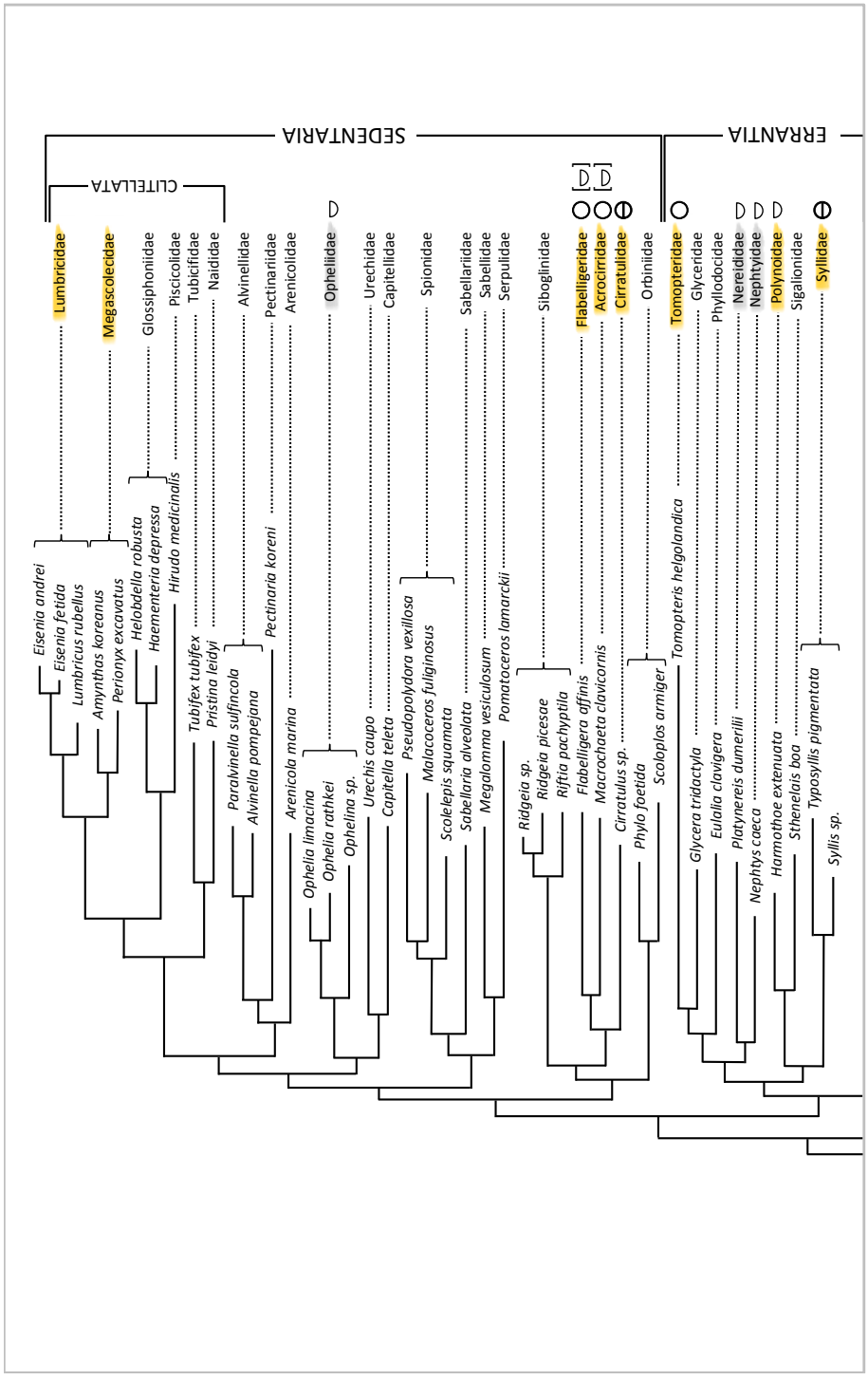
Although Bassot and Nicolas (1995) have evidenced the progressive recruitment of photosomes during light emission, the identity of these microsources as well as that of the photocytes have recently been called into question (PLYUSCHEVA AND MARTIN 2009, ANELI *ET AL.* 2014).

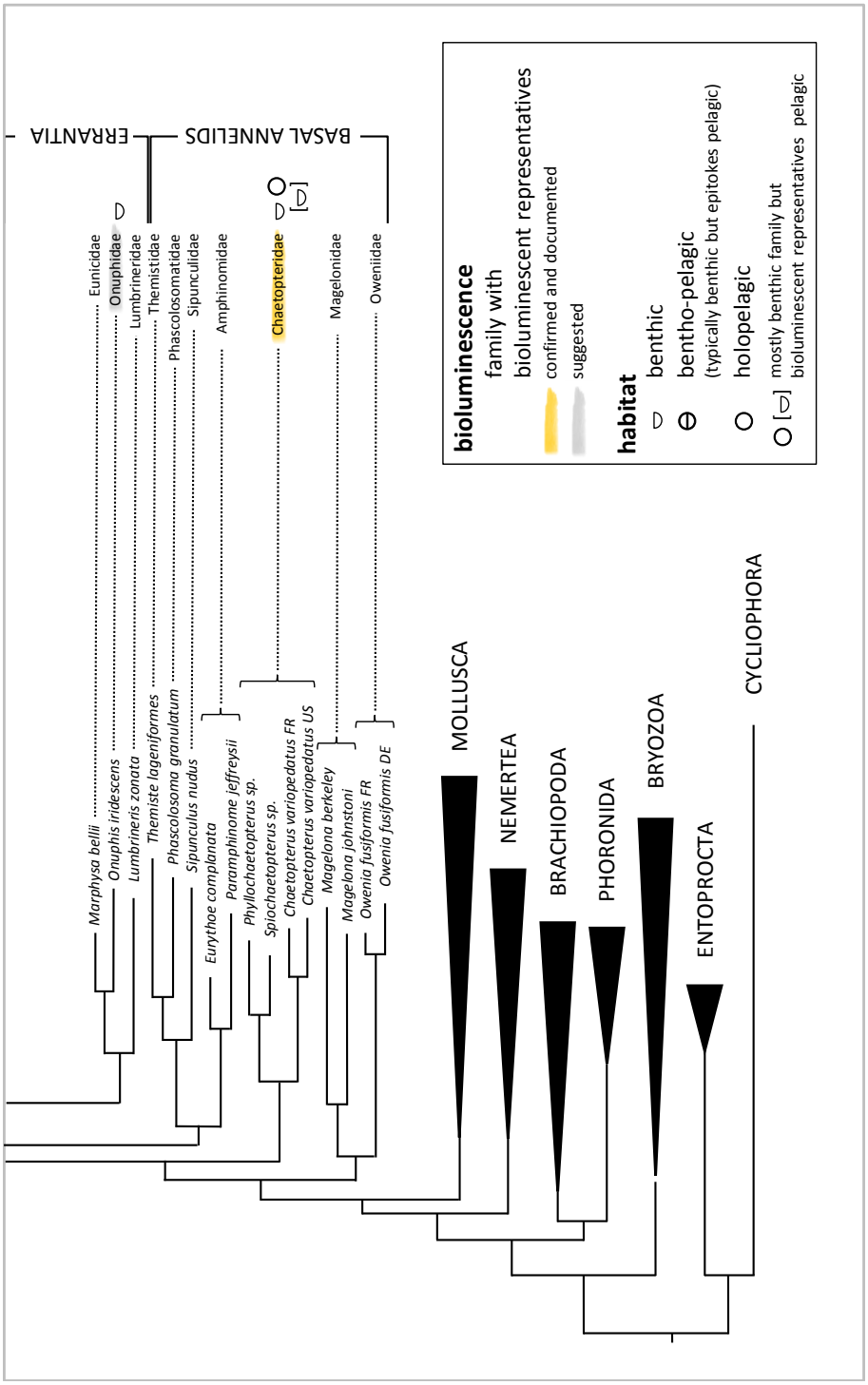
The ecological function of this bioluminescence remains unknown. However, it has been suggested that it could be either a warning or a distracting signal since the rhythmic flashing activity of autotomised elytrum was reported to last during minutes to hours (BONHOMME 1954, BASSOT AND NICOLAS 1995).

1.3.7.2. Terebellidae

Bioluminescence in terebellid polychaetes was first reported in *Polycirrus auranticus*, which emits a violet-blue light from its tentacles (DAHLGREN 1916). The λ_{max} of 445 nm, measured later from *P. perplexus*, is unusually short for a coastal organism (HUBER *ET AL.* 1989). Even though the histology of photogenic tissues was precisely analysed in *P. auranticus* and *P. caliendrum*, thus revealing photocytes of a glandular nature (DAHLGREN 1916, BONHOMME 1952a, 1954), the authors do not agree about the extracellular feature of bioluminescence, which is found incompatible with the emission of luminous flashes (HUBER *ET AL.* 1989). However, the emission kinetics seem to be very variable from one species to the other and also amongst a single species (BONHOMME 1954, JOHNSON AND JOHNSON 1959, HUBER *ET AL.* 1989). It could be that this variability is linked to the physiological state of the tested specimens, and especially to the exhaustion of their photogenic material, since its recovery seems to be particularly slow. They could also modulate their

Fig. 1.11. (next pages) Phylogenetic tree of bioluminescent annelids.
This phylogeny, based on the analysis of 77 taxa and 421 genes, is the last one available (WEIGERT *ET AL.* 2014, redrawn with permission). It has been annotated after the reviews of Harvey (1952) and Haddock *et al.* (2010). It clearly appears that bioluminescence evolved multiple times within Annelida. Interestingly, in Flabelligeridae and Acrocirridae which are fundamentally benthic famillies, bioluminescence is known only from holopelagic representatives. The two families Alciopidae (Phyllodocida: Errantia, pelagic) and Terebellidae (Terebellida: Sedentaria, benthic) which also contain bioluminescent species, do not appear in this analysis.


SEDENTARIA
ERRANTIA
CLITELLATA
Lumbricidae
Megascolecidae
Glossiphoniidae
Piscicolidae
Tubificidae
Naididae
Alvinellidae
Pectinariidae
Arenicolidae
Ophelidae
Urechidae
Capitellidae
Spionidae
Sabellariidae
Sabellidae
Serpulidae
Siboglinidae
Flabelligeridae
Acrocirridae
Cirratulidae
Orbiniidae
Tomopteridae
Glyceridae
Phyllodocidae
Nereididae
Nephtyidae
Polynoidae
Sigalionidae
Syllidae
Eisenia andrei
Eisenia fetida
Lumbricus rubellus
Amynthas koreanus
Perionyx excavatus
Helobdella robusta
Haementeria depressa
Hirudo medicinalis
Tubifex tubifex
Pristina leidyi
Paralvinella sulfincola
Alvinella pompejana
Pectinaria koreni
Arenicola marina
Ophelia limacina
Ophelia rathkei
Ophelina sp.
Urechis caupo
Capitella teleta
Pseudopolydora vexillosa
Malacoceros fuliginosus
Scolelepis squamata
Sabellaria alveolata
Megalomma vesiculosum
Pomatoceros lamarckii
Ridgeia sp.
Ridgeia piscesae
Riftia pachyptila
Flabelligera affinis
Macrochaeta clavicornis
Cirratulus sp.
Phylo foetida
Scoloplos armiger
Tomopteris helgolandica
Glycera tridactyla
Eulalia clavigera
Platynereis dumerilii
Nephtys caeca
Harmothoe extenuata
Sthenelais boa
Typosyllis pigmentata
Syllis sp.


ERRANTIA
BASAL ANNELIDS
Eunicidae
Onuphidae
Lumbrineridae
Themistidae
Phascolosomatidae
Sipunculidae
Amphinomidae
Chaetopteridae
Magelonidae
Oweniidae
Marphysa bellii
Onuphis iridescens
Lumbrineris zonata
Themiste lageniformes
Phascolosoma granulatum
Sipunculus nudus
Eurythoe complanata
Paramphinome jeffreysii
Phyllochaetopterus sp.
Spiochaetopterus sp.
Chaetopterus variopedatus FR
Chaetopterus variopedatus US
Magelona berkeley
Magelona johnstoni
Owenia fusiformis FR
Owenia fusiformis DE
MOLLUSCA
NEMERTEA
BRACHIOPODA
PHORONIDA
BRYOZOA
ENTOPROCTA
CYCLIOPHORA
bioluminescence
family with
bioluminescent representatives
confirmed and documented
suggested
habitat
benthic
bentho-pelagic
(typically benthic but epitokes pelagic)
holopelagic
mostly benthic family but
bioluminescent representatives pelagic

light output depending on the context that incites the use of bioluminescence. It has been suggested that the luminescence of *P. auranticus* is an aposematic signal since it reacts to the presence of a predator by presenting its tentacles which are the only bioluminescent and distasteful parts of its body (DAHLGREN 1916).

It may be that visual predators are capable of distinguishing their bioluminescence from the bioluminescence of *Chaetopterus variopedatus* (λ_{max} = 465 nm), with whom they can live in association (JOHNSON AND JOHNSON 1959, HUBER *ET AL.* 1989).

1.3.7.3. Chaetopteridae

Also known as "parchment worms", these annelids are filter-feeders living in vertical or U-shaped tubes of paper like fibrous aspect. They are found in shallow coastal habitats in both temperate and tropical locations. An extensive and multidisciplinary literature is devoted to these worms, especially to the bioluminescent species *Chaetopterus variopedatus* (HARVEY 1952). However, *C. variopedatus* is not a single cosmopolitan species, as argued by Hartman in 1957, but a species complex comprising 18 species (PETERSEN 1984, OSBORN *ET AL.* 2007). In general, the studied specimens were able to produce, like several other taxa (*e.g.* Terebellidae, Syllidae), both intracellular and secreted luminescence from various parts of their body (NICOL 1952, MARTIN AND ANCTIL 1984). However, Deheyn *et al.* (2013) claimed that it originates from the secreted mucus only. Discharge of luminous material would only be possible through a rupture in the photogenic glandular epithelium caused by the animal's reflex contraction during an outside attack on the tube (BROWN AND ROSEN 1978, MARTIN AND ANCTIL 1984). Bioluminescence seems to be a common feature in the genus *Chaetopterus* (HARVEY 1952) but it is also found in *Mesochaetopterus* and possibly in *Phyllochaetopterus* and *Spiochaetopterus* (HERRING 1987).

Finally, *C. pugaporcinus* has recently been described as a holopelagic paedomorphic species exhibiting a combination of both adult and larval features (IRVINE *ET AL.* 1999, OSBORN *ET AL.* 2007). Only one of the collected specimens produced a bright blue light after mechanical stimulation as well as a mucus cloud containing green luminescent particles.

1.3.7.4. Syllidae

One well-documented case of mating display is that of the syllid fireworms. The Bermudian worm *Odontosyllis enopla* inhabits shallow coastal areas and is normally benthic, except during spawning. Females reach the surface, swimming in narrow circles and secreting a green emerald luminous mucus (λ_{max} = 507 nm after

SHIMOMURA *ET AL.* 1963). Several males – whose visual sensitivity matches the luminous spectra (WILKENS AND WOLKEN 1981) – are commonly attracted to a single female, usually producing brief flashes, then all the worms rotate in a tight circle while discharging their gametes into the water (GALLOWAY AND WELCH 1911). This behaviour is known to be precisely rhythmed by lunar, seasonal and diurnal cycles (MARKERT *ET AL.* 1961). Similar behaviours were reported for other species of this genus: *O. octodentata* (ERDMAN 1965), *O. polycera* (DALY 1975), *O. luminosa* (GASTON AND HALL 2000) and *O. phosphorea* (POTTS 1913). However, their periodicity can be strongly influenced by local factors such as temperature and tidal regime (TSUJI AND HILL 1983).

In addition, light flashes elicited by mechanical disturbance have been observed outside spawning periods, suggesting multiple functions associated with light production (FISCHER AND FISCHER 1995, DEHEYN AND LATZ 2009). In *O. phosphorea*, only the largest worms appear to produce both internal and secreted bioluminescence, even though photogenic capabilities are acquired at early stages (DEHEYN AND LATZ 2009). Thus, syllid worms may use bioluminescence not only as a visual signal to attract mates, but also as a startle signal against predation, or even as an aposematic signal, since fishes were observed to regurgitate freshly ingested *O. luminosa* (GASTON AND HALL 2000).

Bioluminescent abilities were reported in several other species (*O. fulgurans*, *O. ctenostoma*) and genera including *Pionosyllis, Syllis*, *Eusyllis* and *Streptosyllis* (HARVEY 1952, BONHOMME 1954, HERRING 1987, POUPIN *ET AL.* 1999, ZÖRNER AND FISCHER 2006).

1.3.7.5. Accrocirridae

Swima bombiviridis (OSBORN *ET AL.* 2009), *S. fulgida* and *S. tawitawiensis* (OSBORN *ET AL.* 2011) are newly described species. They form a clade amongst Acrocirridae, from which they are distinguished by their pelagic mode of life. Collected below 2700 m, these transparent worms bear four pairs of elliptical, transformed segmental branchiae on anterior segments that produce green bioluminescence when autotomised. These “bombs”, which are not all released at once when the organisms are manually stimulated, seem able to regenerate. Osborn and associates (2009, 2011) suggested that the use of bioluminescent bombs is not related to reproduction, since bioluminescence is produced by both mature and immature specimens. The autotomy of these structures could act as part of a defensive behaviour, distracting a visual predator while the animal escapes. However, the size of the bombs (typically less than 1 mm) did not make *in situ* observation possible.

1.3.7.6. Cirratulidae

The cirratulid *Caulleriella bioculata* (*Heterocirrus bioculatus*) shows a seasonal variation in the yellow-green luminescence of large muco-photocytes progressively increasing in number with the approach of the spawning period (BONHOMME 1954). So it is possible that luminescence maintains the cohesion of the swarms in a similar fashion to that observed in Syllidae. It was also suggested that *C. fragilis* and *Chaetozone caputesocis* (*Caulleriella caputesocis*) produce a greenish light (GIBBS 1971, PETERSEN 1999). However, *C. parva* immature specimens have been observed to produce a bluish light when pinched or irritated (PETERSEN 1999). Thus, despite being little studied, this annelid family seems to exhibit a significant diversity of patterns and functions, which is expressed between species or possibly over ontogenesis.

Bioluminescence has also been reported in the related genera *Dodecaceria* and *Tharyx* (HARVEY 1952, HERRING 1978).

1.3.7.7. Flabelligeridae

Most of the bioluminescence in this family is unknown. It was found in deep-sea planktonic organisms, including *Poeobius meseres* and *Flota vitjasi* (HERRING 1978, HADDOCK *ET AL.* 2010). These species, morphologically very different, seem to have acquired their pelagic existence through two distinct lineages that have recently been proven to be phylogenetically related (BURNETTE *ET AL.* 2005, OSBORN AND ROUSE 2008).

1.3.7.8. Tomopteridae

Although the adult specimens range from 5 mm to 500 mm in length (ROUSE AND PLEIJEL 2001, FRANCIS W., *pers. comm.),* the tomopterids (gr. τόμος/tomos, section; πτερόν/pteron, wing) form a morphologically homogenous family of holopelagic polychaetes, living from the sea surface to at least 3000 m depth and distributed worldwide (OBIS Ocean Biogeographic Information System 2016). Their most distinctive characters are their apparent transparency, fin-like transformed parapodia and a pair of capilliform tentacular cirri directed posteriorly [**Fig. 1.12a**] (*e.g.* ROSA 1908, MALAQUIN AND CARIN 1922, DALES 1957, UŠAKOV 1972). As most annelids, they exhibit two coelomic cavities separated from each other by vertical mesenteries [**Fig. 1.12b**]. Each cavity forms a continuous space from the head to the tail that communicates with the parapodia. The sexes are separated and the coelomic fluid mainly contains the sexual products [**Fig. 1.12c,d**] floating freely after separation from the gonads, which are to be found attached inside the coelomic rami (MEYER 1929a). Endowed with an unarmed eversible proboscis, they

are known to be omnivores (phyto- and zooplankton) and predators from the very beginning of food uptake (*e.g.* tunicates, siphonophores, chaetognaths, copepods, fish larvae) (LEBOUR 1923, ÅKESSON 1962, RAKUSA-SUSZCZEWSKI 1968, FAUCHALD AND JUMARS 1979, KIRKEGAARD 1992, PHLEGER *ET AL.* 1998). For more biological and ecological elements concerning Tomopteridae, the reader may refer to section 3.1 of the methodological aspects and to Appendix A.

Tomopteridae are generally placed in the order Phyllodocida although little evidence is currently available (*e.g.* FAUCHALD 1977, FAUCHALD AND ROUSE 1997, ROUSE AND PLEIJEL 2003). According to recent molecular analyses, they form with Glyceriformia a sister clade to Phyllodociformia (STRUCK *ET AL.* 2007) but differ sharply from the latter in the structure of their anterior end (UŠAKOV 1972, FAUCHALD 1977). Although five extant genera have been originally described, only two are traditionally recognised: *Tomopteris* Eschscholtz 1825 (including *Briaraea*, *Escholtzia* and *Johnstonella* genera) and the monotypic genus *Enapteris* Rosa 1908 (FAUCHALD 1977). The distinction between two sub-genera, *Tomopteris* (*Tomopteris*) and *Tomopteris* (*Johnstonella*) is still maintained but not always recognised (DALES AND PETER 1972, FAUCHALD 1977). Given the large number of synonyms and considering that some *Tomopteris* species have never been observed since their original description (DALES AND PETER 1972), the number of 53 valid species recorded in the World Polychaeta Database (READ AND FAUCHALD 2015) is probably overestimated. Species identification should preferably be based on consideration of parapodium glands [**Fig. 1.13a**]. Criteria such as the size, the number of parapodia or the presence of a tail turned out to be less reliable as they may vary with age and maturity (ROSA 1908, MALAQUIN AND CARIN 1922). Only one fossil species*, Eotomopteris aldridgei* Briggs and Clarkson 1987 is known from specimens from the Lower Carboniferous of Scotland (BRIGGS AND CLARKSON 1987, CLARK 2014). However, this fossil rather belongs to Yndolaciidae (Phyllodocida *incertae sedis*), a poorly known family, bearing like *Eotomopteris* two pairs of long tentacular cirri with inner chaetae and parapodia supported by aciculae (BUZHINSKAJA 2004).

Definitely unclassifiable, their belonging to the group of gelatinous is also discussed. Compared with a given sample of gelatinous organisms, Johnsen and Widder (1998) discovered that tomopterids were amongst the least transparent (9.1% transparency at 480 nm). Actually, *T. carpenteri* was found to contain only 85% of water and to be intermediate in composition between typical gelatinous species (*e.g.* ctenophores, cnidarians) and non-gelatinous species (*e.g.* crustaceans) (CLARKE *ET AL.* 1992).

A total of twelve species were reported as light emitters: *Tomopteris anadyomene*, *T. apsteini*, *T. elegans*, *T. helgolandica*, *T. kefersteini*, *T. krampi*, *T. mariana*, *T. nationalis*, *T. nisseni*, *T. planktonis*, *T. rolasi* and *T. septentrionalis* (reviewed

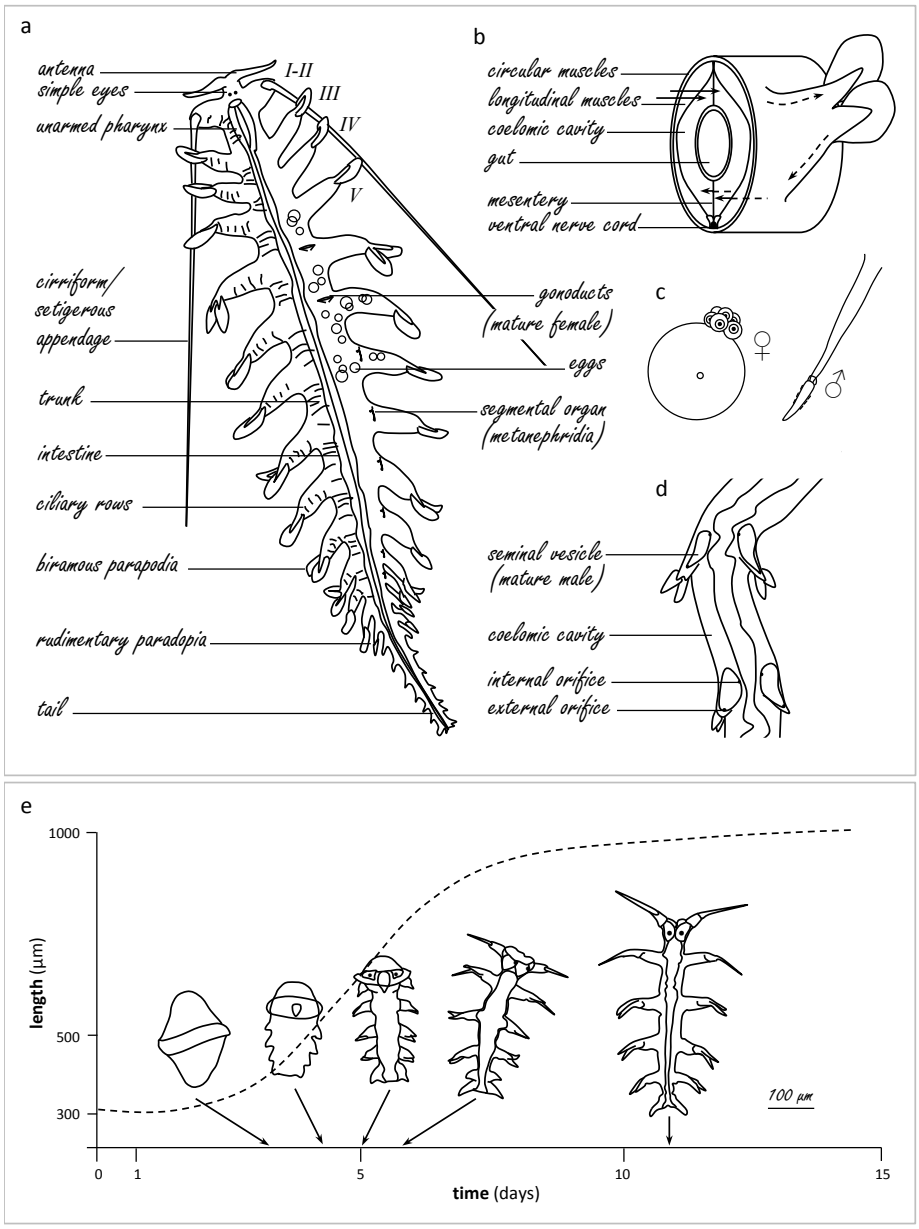


Fig. 1.12. Morphology and development of Tomopteridae
(a) General morpho-anatomy of *Tomopteris helgolandica* in ventral view; **(b)** Internal organisation and coelomic circulation (arrows) of *T. helgolandica* (redrawn from MEYER 1929a with permission); **(c)** An oosphere with seven attached micromeres (after FULLARTON 1895) and a biflagellate spermatozoon (after FRANZÉN 1982); **(d)** Caudal prolongation of a male *Tomopteris onisciformis* (redrawn from CARPENTER AND CLAPARÈDE 1860 with permission); **(e)** Growth curve of early life stages of *T. helgolandica* (after ÅKESSON 1962 with permission).

by POUPIN *ET AL.* 1999). Three of them are studied in this work (*T. helgolandica*, *T. planktonis* and *T. septentrionalis*) and we add to this account *T. pacifica* and the Antarctic species *T. carpenteri*, whose bioluminescence is described in the paper II. As reviewed by Harvey (1952), the first specimens of the genus *Tomopteris* were described in 1825 by Müller. Then in 1847, Busch described in *T. onisciformis* (*T. helgolandica*) parapodial pigmented rosette-shaped organs whose bioluminescent properties were suggested later in *T. rolasi* and *T. mariana* by Greeff (1882). According to him, the yellow cells may be photocytes that elaborate and accumulate lipoid products which would be the support of the photogenic substances (DAHLGREN 1916, BONHOMME 1954, TERIO 1960, 1964) [**Fig. 1.13b**]. The transparent cells may be either a lens structure or nutrition cells connected with the organ. In *T. mariana*, a nerve ganglion is to be found at the base of the transparent cells with fibres running between them to the putative photocytes [**Fig. 1.13b**] (GREEFF 1885, HARVEY 1952). Unfortunately, all of the descriptions by Greeff were made from free-swimming specimens or poor preparations, and none of the studies that have followed his work were able to deepen them sufficiently to confirm the nature and identity of the photogenic organs.

Five types of parapodial glands are known in Tomopteridae: the chromophile or chromatophile gland and the spur gland, the function of which is unknown, the tubular glands, presumably mucus glands, which are particularly abundant in the pinnae, and two types of rosette-like glands or hyalo-pigmented glands, which are supposed to be photogenic [**Fig. 1.13a,b**]. The study of their nature, their distribution and their position amongst the parapodium, which is helped by differential histological colourations, is of major importance as regards the identification of the species (MALAQUIN AND CARIN 1922). According to Rosa (1908), in the subgenus *Johnstonella*, rosette-like glands are generally rosettes *stricto sensu*, whereas species in the subgenus *Tomopteris* have hyaline glands. Despite the variation of their positions, these glands have the same essential composition and are considered to be homologous (MALAQUIN AND CARIN 1922). According to Meyer (1930), the rosettes II originate from the peritoneum and derive from the embryonary cœlomostomes which migrate during ontogenesis [**Fig. 1.13c**].

The bioluminescent tomopterids are commonly known as yellow light emitters (*e.g.* λ_{max} = 565 nm in *T. nisseni*, LATZ *ET AL.* 1988). However, most of the light emission patterns have never been described (POUPIN *ET AL.* 1999). Regarding the light emitting system, it appears to involve a photoprotein probably activated by superoxide anion (SHIMOMURA 2006). The presence of cœlenterazine (28 pmol in one specimen) and of a fluorescent emitter have also been evidenced from tissue homogenates (THOMSON *ET AL.* 1997, FRANCIS *ET AL.* 2014), suggesting that the colour

yellow is achieved by BRET (*supra*, p. 15). However, some of these works were conducted on unidentified specimens and the bioluminescent system is still unknown.

Although the luminescence was thought to be intracellular (HARVEY 1952), a secretory activity was also suggested in several species (Greeff 1885, Meyer 1929b, BONHOMME 1954, TERIO 1964). Terio (1964) and Salerno (1965), in particular, described a system of canaliculi presumably conducting the luminescent material formed in the light organs till the margin of the pinnae. Recently, tomopterid worms have been observed to release a luminous exudate while they tried to escape from a collecting device (video published by the MBARI on 1st July 2015 - https://www.youtube.com/watch?v=cdr1kWmSiiE). Thus, bioluminescence may be a defensive signal. However, the most commonly argued hypothesis suggests that the emission of a light of atypical wavelength implies some sort of intraspecific communication through a private communication channel (DALES 1971). Like in Syllidae and Cirratulidae, bioluminescence could be specifically used within the context of reproduction, since Salerno (1965) reported in *T. nationalis* an increase of the bioluminescent potential during the sexual activity period.

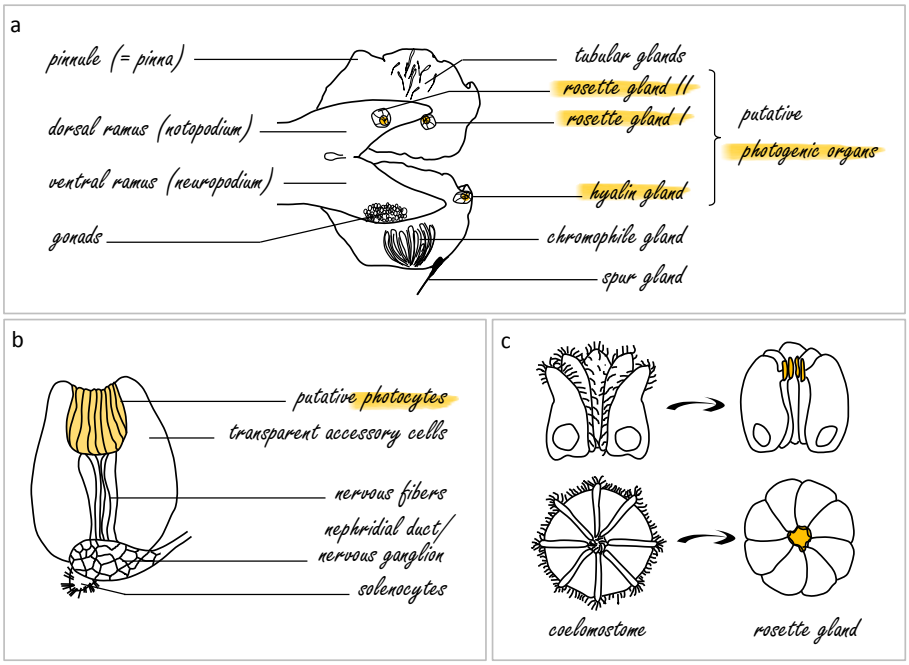


Fig. 1.13. Bioluminescence in tomopterids.
(a) Diagrammatic parapodium indicating the most common position of the five types of glandular organs including the putative light organs; **(b)** Rosette gland of *Tomopteris mariana* showing yellow-pigmented cells (redrawn from GREEFF 1885); **(c)** Morphological relationship between the cœlomostome and the rosette gland in tomopterid species (redrawn from MEYER 1929b with permission).

2. Aims of the study

The unusual yellow light emission displayed by some tomopterid annelids was first reported more than a century ago by Greeff (HARVEY 1952). Although little studied in comparison with bacteria, beetles, coelenterates or fishes, this bioluminescence is well known and often presented as an illustration of the intraspecific communication function (DALES 1971). Indeed, the lack of data concerning the biology and ecology of tomopterids and the underlying production- and control mechanisms of their light emission, impedes us to assess this hypothesis of major interest in pelagic ecology. Given that evolution has greatly favoured the emergence of blue light emission- and reception systems in this environment, how yellow light emission mechanisms evolved and were retained remains an open question. In addition, because their transparency and bioluminescence abilities may be interpreted as conflicting (invisibility *versus* visibility), tomopterids are convenient and original model organisms for studying how bioluminescence evolved and is used in the gelatinous plankton.

This study aimed to be multidisciplinary, combining pharmacological, morphological and behavioural observations. The main targets were (1) to characterise the bioluminescent display of *Tomopteris helgolandica* Greeff 1879 (the main model species presented in details in section 3.1.1.) and its control, (2) to identify and describe the photogenic structures involved, and (3) to provide a first ethological approach of colour perception by this species in order to find out, especially, whether it is able to perceive its own bioluminescence. In its present form, this work not only focuses on the species *T. helgolandica*, but also deals with the bioluminescence of four of its allies: *T. planktonis* Apstein 1900, *T. septentrionalis* Steenstrup 1849, *T. pacifica* Izuka 1914 and *T. carpenteri* Quatrefages 1866, the access to which was unexpected.

The light emission patterns of these species were thus compared and described for the first time in terms of colour, kinetics (time course, intensity) and dynamics (intra- or extracellular). (1) The analysis of emission spectra is a classical way to precisely reference the bioluminescence colour and to compare the known emission systems. Few tomopterids being known for their yellow light emission, this was a key step of this study. (2) Applying various pharmacological substances on isolated parapodia aimed to define the basis of the nervous control of the light emission as well as its effects on the emission kinetics. It is common to distinguish flash (brief signal) and glowing patterns (long-lasting signals), but they may be modulated in a finer and more complex. (3) At last, the description of the emission dynamics, when it was not clearly revealed by direct observation, might be helped by the histological analysis of the photogenic structures and their fluorescence.

Aside from this, the comparative histology of the putative light organs of all the available species, based on light microscopy of total mounts and semithin

sections of parapodia, scanning and transmission electron microscopy aimed to provide new insights into the light emission process and the evolution of light organs within the tomopterids. Then, to complete the pharmacological approach of bioluminescence control, the nervous circuitry of parapodia and the nervous supply of the light glands were investigated by immunohistochemistry.

Finally, the first ethogram of *T. helgolandica* was established and an automated video tracking and motion analysis system was implemented to quantify the tactic and kinetic movements induced by simulated yellow and blue bioluminescent signals.

The results are integrated at the end of this thesis in order to highlight the ecological value(s) of yellow-light emission within the Tomopteridae and hypotheses are discussed regarding its putative evolutionary pathway.

3. Methodological aspects

At the beginning of this work, tomopterid worms had never been studied in our laboratory and the latest research on their bioluminescence dated back to the 1960's. Consequently, many aspects of this research required preliminary optimisation. The objective of this chapter is to address some of these methodological issues and to suggest guidelines for future works.

3.1. Model species, collection and maintenance

This study is mainly devoted to the study of *Tomopteris helgolandica*. However, thanks to several encounters and collaborations, we were able to collect or exchange data for four other species from western Norway, but also from the Pacific- and Southern Oceans [**Fig. 3.1**]. Their identification was established after the dichotomous keys of Quatrefages (1866), Rosa (1908), Fauvel (1923), Monro (1930), Muus (1953), Dales (1955, 1957), Hartman (1964), Ušakov (1972), Pleijel and Dales (1991). A table summarising the diagnostic features used in this work in provided as supplementary material of the paper II (*infra*, p. 92).

3.1.1. *Tomopteris (Johnstonella) helgolandica* Greeff 1879

Tomopteris helgolandica is a North Atlantic species that can be abundant during the warm season off the Scandinavian coasts (ÅKESSON 1962, MATTHEWS AND BAKKE 1977, HANSSON 1987, KIRKEGAARD 1992) [**Fig. 3.1**]. Females are usually larger (50 to 100 mm) than males (up to 60 mm) and adults of both sexes have a long tail that carries rudimentary parapodia and reaches 3/4 of the body length [**Fig. 3.3a**]. Due to the lack of *in situ* observations, little is known about their reproductive biology and behaviour. Tomopterids are believed to have a continuous reproduction cycle (ÅKESSON 1962, SIMMONS 2009). However, spawning maximum was reported in this species in June (Gullmar Fjord, North sea, ÅKESSON 1962) and between May and August (Ireland, KIRKEGAARD 1992). The hypothesis of internal fertilisation, based on the observation of both male and female sexual products in the coelomic cavity of a female, has never been confirmed (CARPENTER AND CLAPARÈDE 1860, FULLARTON 1895, HACHFELD 1926, ÅKESSON 1962). The 7-days embryos measure less than 1 mm and can already look like miniatures of the adults [**Fig. 1.12e**]. According to Åkesson (1962), they are usually found near the surface, while the bigger and mature specimens live deeper.

The pinnal rosette glands (type II) are usually distinctive features located close to the tips and inner edges of both dorsal and ventral rami. Additional rosettes (type I) located on the trunks of the first two pairs of parapodia are often inconspicuous [**Fig. 3.3a**]. These organs have long been suggested to be photogenic despite the lack of experimental evidence (GREEFF 1885, MEYER 1929b, DALES 1971).

Specimens were regularly collected from October 2010 to November 2014 between 100 and 300 m depth from two connected fjords, Raunefjorden (N60°16'05'', E05°09'11'') and Korsfjorden (N60°11'24'', E05°13'20''), Western Norway, in collaboration with the Espegrend Marine Biological Station from the University of Bergen [**Fig. 3.2a-b**]. Two types of towed net samplers were used [**Fig. 3.2c**]: the Isaacs-Kidd midwater trawl (1.75 m wide × 1.30 m high mouth, 6.5 m long, 500 µm mesh aperture) (YASOOK *ET AL.* 2007) [**Fig. 3.2c1**] or a ring plankton net (1.5 m diameter mouth, 300 µm mesh aperture) [**Fig. 3.2c2**], depending on weather conditions. Additional specimens, used for histological examination were collected by Per R. Flood by vertical hauls of a 200 µm mesh aperture plankton net at 260-0 m depth in Herdlefjorden (N60°32'60", E5°01'30") and at 400-200 m depth in Sognefjorden (N61°04', E5°22'), Western Norway, during the period 1995-2006.

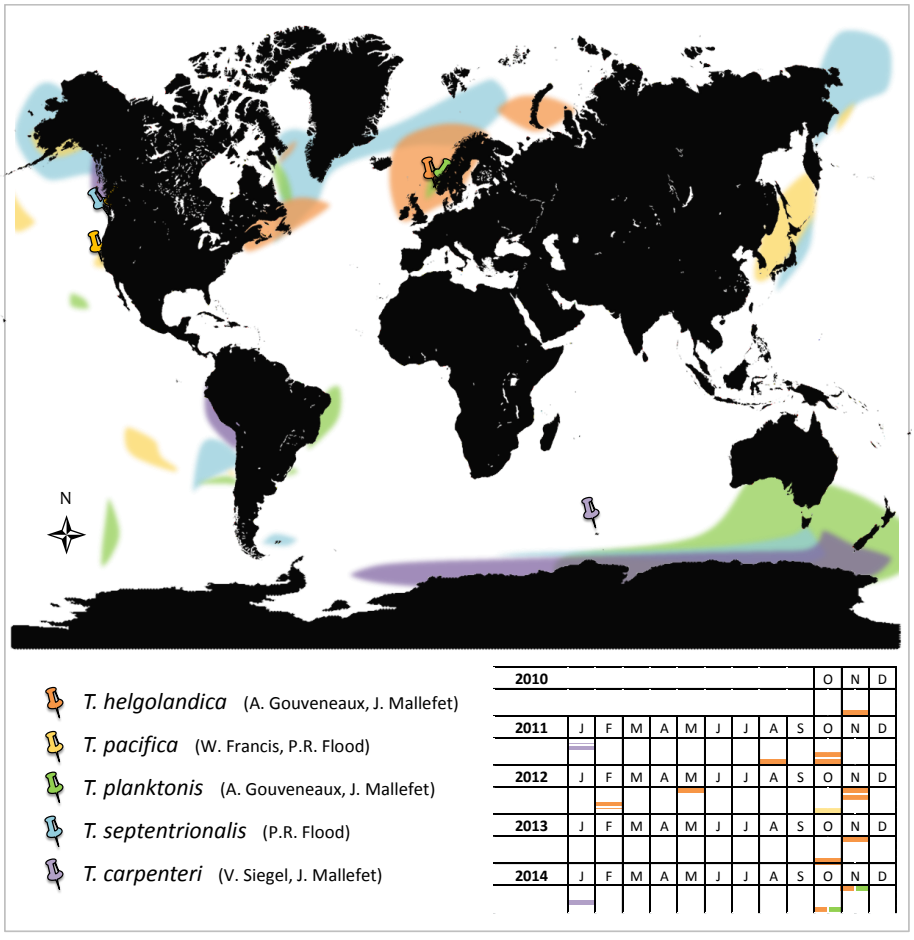
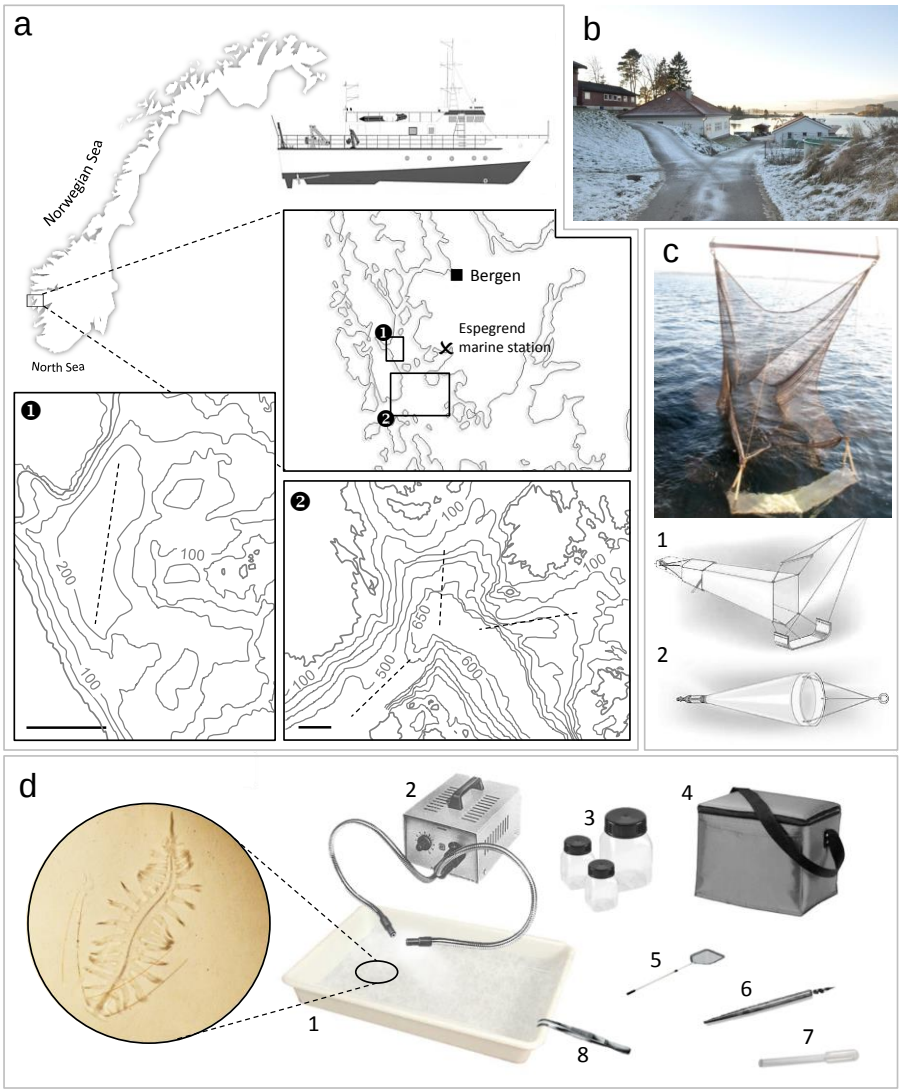


Fig. 3.1. (previous page) Global distribution and sampling sites of the studied tomopterid species (After OBIS 2016).

Fig. 3.2. Plankton sampling and sorting methods.
(a) Situation of sampling transects (dotted lines) in Raunefjorden (❶) and Korsfjorden (❷). Scale bars 1000 m. On the top right, image of the R/V Hans Brattström; **(b)** Espegrend Marine Biological Station (University of Bergen); **(c)** The Isaacs-Kidd midwater trawl (1) and a ring plankton net (2); **(d)** Sorting equipment aboard including a white tray (❶), an optic fiber lamp (❷), sampling containers to isolate the collected specimens (❸), an isotherm bag for transporting and keeping them away from heat and light exposure (❹) and useful tools for handling organisms such as a mini-dip net (❺), a woodcock feather (❻), a large opening pipette (❼) or curved forceps (❽).



The plankton was sorted aboard [**Fig. 3.2d**] and only undamaged individuals were later selected for analysis. Live specimens were housed in a permanent dark cold room (8°C) in aquariums of standing sea water, aerated or renewed daily. If the water was not agitated, the animals let themselves sink to the bottom of the aquariums, which should be clean of any sediment. The degeneration of the setigerous cirri sometimes observed after a prolonged captivity could be a consequence of this immobility. We tried to feed them with mysis shrimp larvae with apparently little success. No sign of cannibalism, as reported by Lebour (1923) and Åkesson (1962), was observed between the specimens housed together. Towed samplers sometimes being responsible for damaging of plankton and exhaustion of their bioluminescent capacities, all experimental protocols allowed recovery for minimum 24h prior to testing, a period reportedly sufficient to allow substantial recovery of luminescence potential in many planktonic organisms (LATZ *ET AL.* 1990, BOWLBY *ET AL.* 1991). It should also be noted that we observed spontaneous flashes until 30 days after capture.

In future research, the duration and the conditions of maintenance could be improved by using Kreisel tanks, which are classically used for keeping and growing gelatinous zooplankton (BAKER 1963, RASKOFF *ET AL.* 2003). We built a prototype of 200 mm in diameter based on diagrams published by Raskoff and colleagues (2003), but it needs to be optimised. If the cover is not perfectly sealed, microturbulences can disrupt the continuity of the flow and create zones of suction that can damage the worms. A pseudokreisel tank with a free water surface might be more appropriate.

3.1.2. *Tomopteris (Johnstonella) pacifica* Izuka 1914

One *Tomopteris pacifica* specimen preserved in formalin was provided by Warren Francis from the Monterey Bay Aquarium Research Institute (California, USA). It was collected in October 2012 by Steve D. H. Haddock using a ROV (remotely operated vehicle) equipped with suction samplers at 540 m depth in the Monterey Bay (N36°35'59", W122°09'02"). This species, apparently abyssal, is widely distributed in the colder waters of the North Pacific [**Fig. 3.1**] (IZUKA 1914, DALES 1957, UŠAKOV 1972).

This specimen was a female of 90 mm length including the tail, but its conspecifics can be larger. The differentiated caudal appendage, which is reddish brown, very long and devoid of parapodia, is characteristic of this species [**Fig. 3.3b**]. Rosette glands are present, like in *T. helgolandica*, on the stem of the two anterior parapodial segments, though often difficult to detect, and at the tips of the rami of the third and following segments.

The bioluminescence of this species was confirmed and documented by Per R. Flood who measured and photographed the light emitted by some specimens collected by night from the docks of Friday Harbour Laboratory in Puget Sound in January 1996 [**paper II**]. All of the tomopterids observed by W. Francis produced light in the form of an exudate. It is what Robison (1995) called "vomiting worms".

3.1.3. *Tomopteris (Tomopteris) planktonis* Apstein 1900

Although its presence in korsfjorden [**Fig. 3.2a2**] has been reported (MATTHEWS AND BAKKE 1977), *Tomopteris planktonis* was only collected exceptionally and with much less abundance [**Fig. 3.1**].

This species can easily be confused with *T. septentrionalis* because of their small size (less than 20 mm), their tail-less pygidium and their hyaline gland in the dorsal part of the ventral rami [**Fig. 3.3d**] (MALAQUIN AND CARIN 1922, UŠAKOV 1972, NEWELL AND NEWELL 1973).

Its bioluminescence ability was mentioned by its descriptor Apstein in 1900 without any additional comment. We described *T. planktonis* as the first known blue emitting *Tomopteris* in the second paper of this thesis.

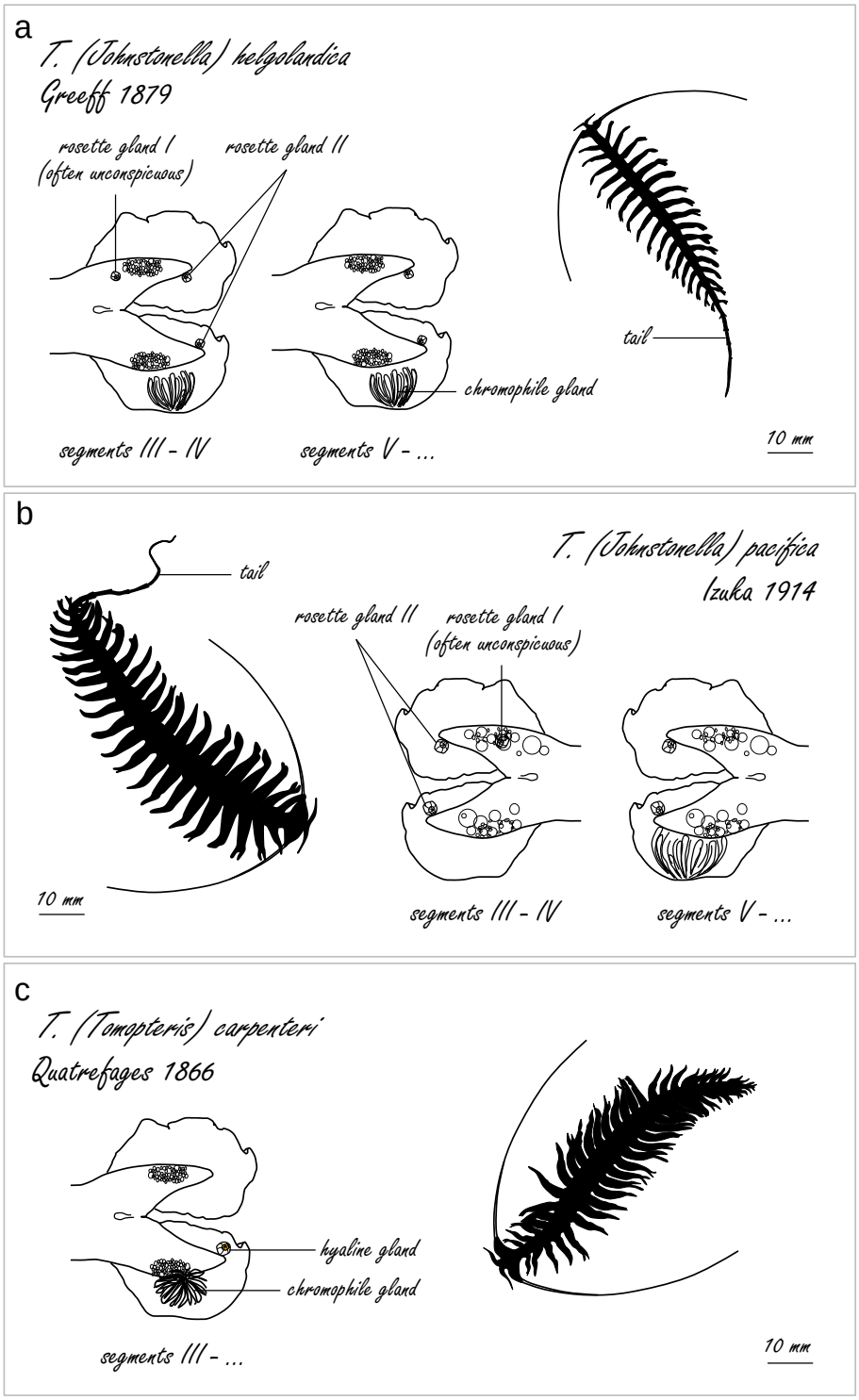
3.1.4. *Tomopteris (Tomopteris) septentrionalis* Steenstrup 1849

Tomopteris septentrionalis specimens came from the collections of Per R. Flood at Friday Harbour. He performed histological examinations and kindly accepted to share its results [**paper III**]. This species, more oceanic than *T. helgolandica*, is distributed worldwide, with the exception of the South Atlantic- and Indian Oceans, where its presence has never been reported [**Fig. 3.1**] (OBIS 2016). Their increased number in mid-summer and mid-winter in Monterey Bay (California) may be due to the local currents, but possibly also to a six monthly breeding cycle (DALES 1955).

Its bioluminescence was first reported by Terio (1960). According to this author, the lipofuscin-like material contained in the hyaline glands [**Fig. 3.3d**] may support the bioluminescence.

3.1.5. *Tomopteris (Tomopteris) carpenteri* Quatrefages 1866

Tomopteris carpenteri was collected in January 2014 aboard the N/O Marion Dufresne (France) at 125 m depth off the Kerguelen Islands, French Southern and Antarctic Lands (S49°53'72", E76°13'13"). Some preserved specimens collected in January 2011 and February 2013 aboard the R/V Polarstern (Germany) at 200 m depth off the Antarctic Peninsula (S61°45'00", W63°59'00") were donated by Dr.


a
T. (Johnstonella) helgolandica
Greeff 1879
rosette gland I
(often unconspicuous)
rosette gland II
tail
chromophile gland
segments III - IV
segments V - ...
10 mm
b
T. (Johnstonella) pacifica
Izuka 1914
tail
rosette gland II
rosette gland I
(often unconspicuous)
10 mm
segments III - IV
segments V - ...
c
T. (Tomopteris) carpenteri
Quatrefages 1866
hyaline gland
chromophile gland
segments III - ...
10 mm

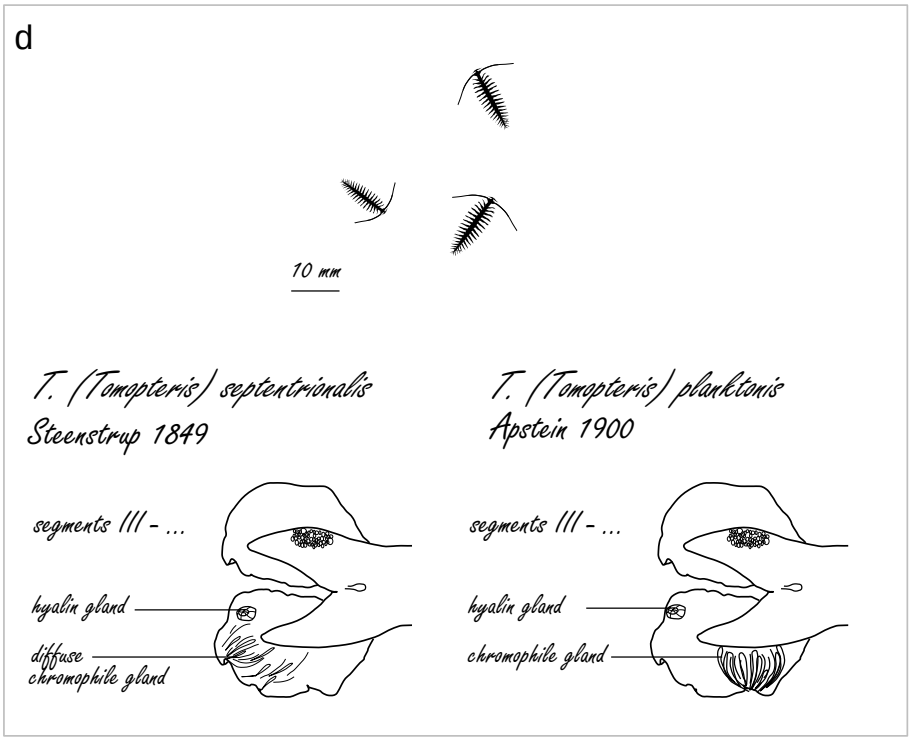


Fig. 3.3. General anatomy of the *Tomopteris* species studied in this work

Volker Siegel from the Thünen Institute (Germany). This species seems to be restricted to the Southern Ocean [**Fig. 3.1**].

Nearly all tomopterids are exceedingly transparent but a few of them are ornamented with highly-coloured chromatophores. *T. carpenteri* specimens, in particular, can show alternating orange and light coloured bands or red dark spots along the body axis. The rosette glands, present in all parapodia on the ventral ramus only, are also particularly large and dark.

We were the first to report and describe the bioluminescence of this species [**paper II**].

3.2. Experimental designs

3.2.1. Anaesthetisation

Anaesthetisation is necessary to immobilise invertebrates in order to facilitate and allow the performance of procedures that may be painful or stressful (COOPER 2011, ROSS AND ROSS 2008). Commonly applied prior to fixation, anaesthetic agents also

prevent the contraction and distortion of tissues (STEEDMAN 1976a, SMALDON AND LEE 1979, COSTA-PAIVA *ET AL.* 2007).

Menthol crystals were chosen and used according to the recommendations of Ross and Ross 2008 (comments in italics relate to our protocol):

General features of an anaesthetic agent:
☑ easy to administer: *immersion of the organism in a solution of diluted crystals*
☑ effective at low dose or exposure: *2.5 g.l^{-1} maximum*
☑ provides sedation or anaesthesia predictably: *see SMALDON AND LEE 1979, GALEOTTI ET AL. 2002*
☑ provides good analgesia: *unresponsive to mechanical stimulation*
☑ induces desired state quickly: *25 min maximum*
☑ easy to maintain the desired state: *in a low concentrated solution*
☑ easy to reverse the process: *recovery bath of sea water*
☑ uneventful rapid recovery: *complete recovery after 15 minutes maximum*
☑ wide safety margin: *recovery after double time exposure*
☑ low cost: *35€/100g*

When using drugs:
☑ the substance should be easily soluble in water or in a water-soluble solvent: *soluble in heated water*
☑ it should not produce hyperactivity during induction: *no*
☑ it should not induce a strong stress response: *no light emission, no muscle contractions*
☑ the drugs should be easy to obtain in bulk: *yes*
☑ drugs should be stable **×** *No data available*
☑ drugs should be safe to operators and non-carcinogenic: *irritating to eyes, skin and respiratory system (R37/38-41, after 67/548/CEE), toxic if ingested but classified by the U.S. Food and Drug Administration as safe (KAMATOU ET AL. 2013)*
☐ leaves negligible tissue residues after a short withdrawal time **×** *No data available*
☐ environmentally harmless or rapidly biodegradable **×** *No data available*

However, like for many other anaesthetics, little is known regarding the cellular and molecular mechanisms by which menthol is effective, especially on invertebrates. Menthol, which is a cyclic monoterpene alcohol, has long been known for its numerous biological properties (*e.g.* analgesic, antibacterial, antifungal) but most studies concerning its analgesic effect were done on mammal subjects (GALEOTTI *ET AL.* 2002, KAMATOU *ET AL.* 2013). It was shown that menthol inhibited the activity of voltage-gated Na^+ channels, which are critical for experiencing pain sensation (GAUDIOSO *ET AL.* 2012). The inhibition of Ca^{2+} currents in neuronal membranes and the selective activation of central κ-opioid receptors are also known to induce an increase of the pain threshold (GALEOTTI *ET AL.* 2002). Apart from the concentration and duration of application, it was proved that the stereochemistry of menthol molecules significantly influence its analgesic properties (GALEOTTI *ET AL.* 2002).

We statistically compared the luminous responses of tissue preparations from anaesthetised and non-anaesthetised specimens using a first-order

autoregressive covariance pattern model. It appeared that anaesthetisation alters neither the total quantity of light emitted (L_{tot}, P = 0.1084) nor the maximal light intensity (L_{max}, P = 0.0629) or the variance of data.

The specimens should not be transferred directly to the anaesthetising solution. A concentrated solution must be added drop by drop to the sea water containing the specimen until reaching a final concentration of 2.5 $g.l^{-1}$. The over-relaxation can induce a decrease of coelomic pressure, the formation of bubbles in the coelom or a collapse of tissues. Although the use of $MgCl_2$ (magnesium chloride) is preferred for many invertebrates including polychaetes (ROUSE AND PLEIJEL 2001), it proved to inhibit the bioluminescent capabilities of *T. helgolandica*. However, it was used with success on other luminous tomopterids (P.R. Flood, *pers. comm.*).

3.2.2. Pharmacology

The nervous control of bioluminescence was investigated by pharmacological tests in *T. helgolandica* [**paper I**] and to a lesser extent in *T. planktonis* [**paper II**].

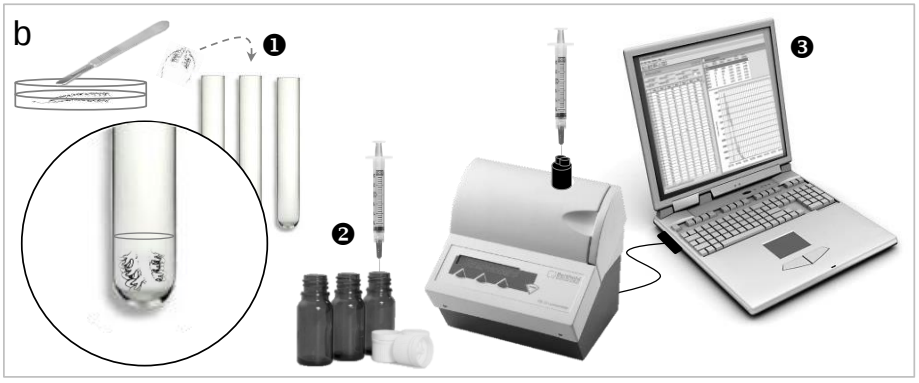
In practice, the effect of various drugs [**Fig. 3.4a**] on light emission was tested on living tissues with a single tube luminometer (FB12, Berthold Detection Systems, Germany) [**Fig. 3.4b❸**]. Anaesthetised organisms were dissected from the head to the tail into serial preparations including three parapodial segments. Each preparation was placed into 50 µl of artificial seawater (ASW: 400.4 mM NaCl, 9.6 mM KCl, 52.3 mM $MgCl_2$, 9.9 mM $CaCl_2$, 27.7 mM Na_2SO_4, 20 mM Tris, final pH 8.3) [**Fig. 3.4b❶**]. The bioluminescence was then triggered by adding 50 µl of a given solution [**Fig. 3.4b❷**]. For each tested specimen, one preparation was treated with the control stimulus (*i.e.* 200 mM potassium chloride (KCl) or 1 mM cholinergic agonist) whereas the other preparations were treated with the various pharmacological substances to be tested [**Fig. 3.4a**]. The parameters of the light responses [**Fig. 3.4c**] were standardised and expressed as a percentage of the control light response. It should be noted that all of the experiments were designed following the latin square principle [**Fig. 3.4d**]: from one specimen to another, the preparation from an identical position is never treated twice by the same solution to eliminate possible interindividual or interpreparation variability.

KCl is a depolarising agent known to maximise the luminescent reaction. It was suggested to act through a voltage-dependent physiological pathway (BILBAUT AND BASSOT 1977, DEHEYN *ET AL.* 2000). It was demonstrated in several luminous species that, used in doses such as 200 or 500 mM, KCl induced little or no morphological changes compared to pharmacological stimulation (DEHEYN *ET AL.* 2000, GERMAIN AND ANCTIL 1988).

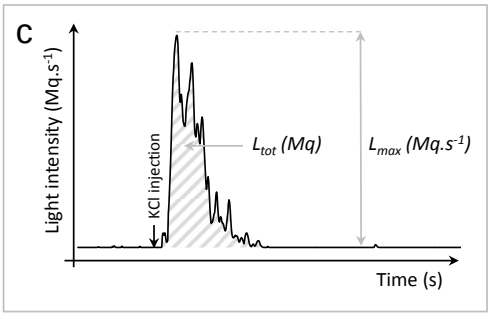
a

drug			concentration
usual name	**commercial name**	**pharmacology**	*mmol.l^{-1}*
nervous control			
KCl	potassium chloride	depolarising agent	200
extrinsic control (screening)			
serotonin	5-hydroxytryptamine	serotoninergic neurotransmitter	1
epinephrine	(±)-epinephrine	adrenergic neurotransmitter	1
GABA	gamma-aminobutyric acid	GABAergic neurotransmitter and neuromodulator	1
nitric oxide	sodium nitroprusside	guanylyl cyclase activator	1
cholinergic system			
carbachol	carbamoylcholine chloride	cholinergic agonist	1
DMPP	1,1-dimethyl-4-phenyl-piperazinium iodide	nicotinic agonist	1
acetylcholine	acetylcholine chloride	cholinergic neurotransmitter	1
eserine	eserine	cholinesterase inhibitor	1
tubocurarine	d-tubocurarine chloride	cholinergic nicotinic receptor antagonist	1
atropine	atropine sulfate	cholinergic muscarinic receptor antagonist	1
intrinsic control (screening)			
8br-cGMP	8-bromoguanosine 3',5'-cyclic monophosphate	cGMP analogue	1
db-cAMP	2'-O-dibutyryladenosine 3',5'-cyclic monophosphate	cAMP analogue	1
forskolin	7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydro-xylabd-14-en-11-one	adenylyl cyclase activator (↑ cAMP)	1
U73122	1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione	phospholipase C inhibitor (↓IP3 and DAG)	1
U73343	1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-2,5-pyrrolidinedion	inactive analogue of U73122	1
calcio-dependance			1
nifedipine	nifedipine	calcium channel blocker	1
diltiazem	diltiazem chloride	calcium channel blocker	1
trifluoperazine	trifluoperazine dihydrochloride	calmodulin inhibitor	1
verapamil	(±)-verapamil	calcium channel blocker	1

b



c



d

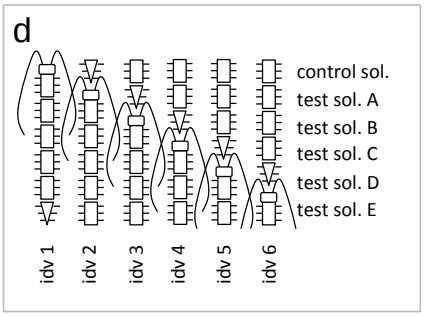


Fig. 3.4. (previous page) Light intensity measurements upon chemical stimulation
(a) List of chemical and pharmacological substances tested; **(b)** Experimental setup: each anaesthetised worm was dissected in preparations of three pairs of parapods placed in 50 µl of ASW (❶), light emission was triggered by adding 50 µl of a given solution (❷) and the luminescent response was recorded with a single tube luminometer (FB12, 2005, Berthold Detection Systems, Bad Wildbad, Germany) connected to a laptop, using the FB12 sirius protocol manager software (❸); **(c)** Typical curve shape of maximal KCl-induced light emission (L_{max} = maximal light intensity, L_{tot} total quantity of light emitted, 1 Mq = 1 megaquanta = 10^6 photons); **(d)** Latin square principle.

3.2.3. Spectrometry

Because the light signal of tomopterids is brief and emitted from distant and punctual sources, the spectral distribution was usually difficult to measure from live emissions without a collimator-equipped device. We therefore measured them from long-exposure digital pictures [**Fig. 3.5a**]. In order to validate this unusual acquisition method, we have first tested, for several bioluminescent organisms, the linear correlation between the emission maxima obtained by live measurements and those obtained by reflectance measurements taken on various digital pictures [see **paper II**]. However, the reliability of the shape of reflectance spectra, especially the presence of secondary peaks, remains to be confirmed.

Another method designed by Per R. Flood to capture the bioluminescent display of the worms was to make what he called “contact lumigrams”. Specimens were relaxed and laid on diapositive colour film protected by Saran wrap in total darkness [**Fig. 3.5b**]. The bioluminescence was chemically induced to expose the film, which was carefully rinsed and dried in total darkness before development [**paper II**].

3.2.4. Morphology

3.2.4.1. Fixation and preservation

Preserved tissues from each species were used for different purposes, especially the morphological analysis of the photogenic structures and their fluorescence. However, the microscopic examination of parapodial glands is also a key step in species identification (ROSA 1908) [**Fig. 3.3**].

According to the observation and histochemical techniques implemented thereafter [**see the next section**], a number of fixatives were employed: 4% buffered formaldehyde was used for light and epifluorescence microscopy and 4% paraformaldehyde in phosphate buffer for immunohistochemical stainings.

The use of aldehyde-based fixatives is common both on annelids (Rouse and Pleijel 2001) and on the gelatinous plankton (JOHNSON AND ALLEN 2012, STEEDMAN 1976b, 1976c, HARRIS *ET AL.* 2000), and was successfully tested on *Tomopteris* (TERIO

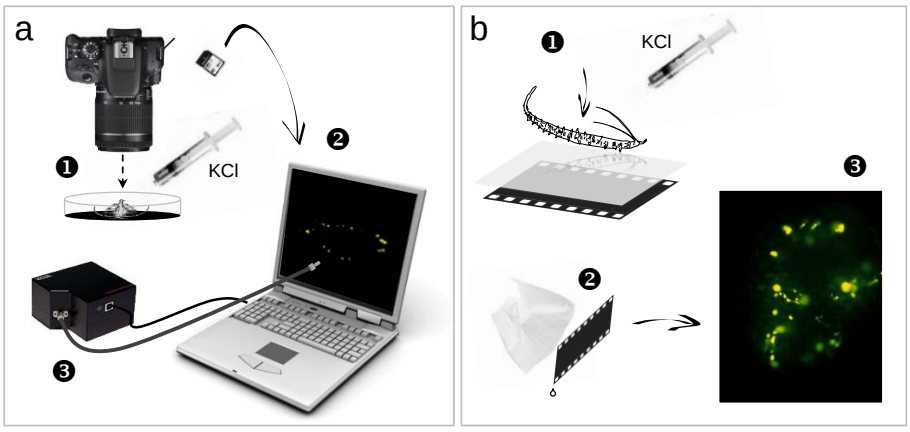


Fig. 3.5. Pictures and spectral measurements
(a) The KCl-induced bioluminescence is photographed in the dark with exposure time adjusted to the light intensity of the specimen (1-30 s depending on the sensitivity of the camera 3200-25000 ISO) (❶), then the spectra is measured directly on the digital picture (❷) using a mini-spectrometer (C10083CA, Hamamatsu photonics, Japan) equipped with an optical fiber (model A976201) (❸); **(b)** Alternatively, "contact lumigrams" were realised: the worm was laid on a protected diapositive colour film (Fuji RFP 50 ASA) and a maximal light emission was triggered in total darkness (❶), then the film was rinsed, dried (❷) and developped (❸).
Photo is courtesy of P.R. Flood (Bathybiologica).

1960, ÅKESSON 1962, FRANZÉN 1982, BARTOLOMAEUS 1997). However, although satisfactory to conduct general observations on whole mounted preparations, they were usually poor for epifluorescent microscopy and for the detailed histological description of the photogenic organs.

The autofluorescence exhibited by most bioluminescent systems (HASTINGS 1996) is a natural marker currently used to identify the light emitting structures (BASSOT AND BILBAUT 1977b, SWEENEY 1980, ROBISON *ET AL.* 2003, RENWART *ET AL.* 2014). Pinnal tissues being thin and transparent enough to transmit the light, fluorescence was researched in total mounts of both living and fixed parapodia. Its spatial distribution was observed with an epifluorescence microscope (Leitz Diaplan; excitation band-pass filter 420-490 nm, dichroic mirror 510 nm, emission high-pass filter 515 nm) while its dynamics and its spectral distribution were described using an inverted laser scanning or confocal microscope (LSM-710, Carl Zeiss Microscopy, Jena, Germany; scanning module including 34 spectral detection channels from 400 to 700 nm) [**paper III**]. However, aldehyde fixatives can enhance autofluorescence from cell components such as flavins, which may interfere with the fluorescent signal of interest (BILLINTON AND KNIGHT 2001). In addition, these fixatives can lead to lipid dissolution (JONES 1976, STEEDMAN 1976c). In this regard, it should be recalled

that the putative light organs contain a lipoid substance (*supra*, p. 45) possibly playing a key role in the light emission. As pointed out by Jones (1976), if the lipoid substances are lost or modified, their localisation and reactive properties remain of doubtful veracity. Consequently, since we experienced difficulties to preserve the yellow vesicles of rosettes glands, especially in sections, it is likely that the diffuse fluorescent signal we observed from preserved material [**paper III**] reflects the overspread of lipoid substances accumulated in these vesicles (as a result of aldehyde-fixation, but also possibly because of KCl maximal stimulation).

To facilitate further investigations, it should be taken into account that (1) neither aldehydes nor alcohol (75% and higher) are good preservatives for lipids over a long period of time, (2) a preserving fluid with a basic pH (8.4 or higher) leads to faster lipid breakdown compared to neutral solutions and (3) tissue sections must preferably be cut in refrigerated conditions because lipids of low melting point, such as those found in plankton, cannot be made solid by fixation (STEEDMAN 1976c). In the same way, freezing or freeze-drying methods could advantageously replace a classic fixation. For lack of fast-freezing device, some fixatives such as Carnoy's, Helly's and Flemming's fluids as well as osmic acid in chloroform were reported as giving good results (BONHOMME 1954, TERIO 1960, ÅKESSON 1962, FLOOD P.R., *pers. comm.*).

3.2.4.2. Histochemical and observation techniques

Apart from topographic (trichromic) stainings of tissue sections, specific colourations proved to be useful for the detection and distinction of various pinnal glands of both sectioned and whole mounted parapodia. The chromophile gland, as its name indicates, stained well with haematoxylin and derivatives solutions. However, the tubular cells of which it is formed are known to stain more or less intensely depending on their functional state (ROSA 1908, MALAQUIN AND CARIN 1922, BONHOMME 1952b). Stainings by Janus green B or by Alcian blue at pH 1 and 2.5, which indicated the presence of acidic mucopolysaccharides, also produced positive results [**paper III**]. The hyaline glands do not stain at all with haematoxylin nor do they darken with osmic acid, as do the lipoid vesicles of the rosettes glands.

To complete the pharmacological investigation presented in the next section, marking the nervous system of *T. helgolandica* aimed to clarify the innervation circuitry of parapodia and particularly of the photogenic organs. Various histochemical methods exist, such as the silver impregnation method of Bielschowsky (GABE 1968) or the thiocholine-ferricyanide method of Karnovsky and Roots (1964), but we focused on immunohistochemical labelling (α-acetylated tubulin antibodies, choline acetyltransferase antibodies). The experiments developed in collaboration with the Department of Biological and Environmental

Sciences from the University of Gothenburg (Sweden) did not achieve the expected specificity and resolution (some micrographs are nevertheless presented in section 4.2.2.3). However, the α-acetylated tubulin antibodies labelled the tubular gland cells, which had not been considered since the work of Greeff (1885). These observations are presented in the paper III.

3.2.5. Ethology

Arena experiments were optimised and conducted on *T. helgolandica* in order to determine its behavioural sensitivity to luminous signals of various types and colours.

All experiments were performed in a dark cold room. Each tested specimen was placed in a circular acrylic tank [**Fig. 3.6a❹**] and filmed by a mixed light imaging system comprising an infra-red sensitive video camera and a low-light-level video camera [**Fig. 3.6a❶**]. Since the transparency of *T. helgolandica* naturally reduces its contrast relative to the background (JOHNSEN AND WIDDER 1998, JOHNSEN 2014), infra-red illumination (IREDs, λ_{max} = 945 nm, FWHM (full width at half maximum intensity) = 60 nm, P (power) = 300 mW) [**Fig. 3.6a❸**] was used to break this camouflage with minimal disturbance to the animal (WIDDER *ET AL.* 2005). The infra-red video camera recorded the movement of the subject while the low-light-level video camera was set to detect any bioluminescent displays (REYNOLDS 1978, BUSKEY AND SWIFT 1985, WIDDER 1992).

Manually controlled light signals were then applied through a 40 mm long fake worm in silicone elastomer (Sylgard® 184, Dow Corning Corporation, Midland, MI, USA) with optic fibres producing luminous spots that mimic the distribution pattern of *T. helgolandica* photophores [**Fig. 3.6a❺**]. This model was immersed in the aquarium at different places (left/right) and two light colours (blue, λ_{max} = 464 nm, FWHM = 22 nm and yellow, λ_{max} = 587 nm, FWHM = 36 nm) as well as two types of signals (flashes 0.2 s^{-1} or continuous) were tested. The chosen light intensity (15 $Mq.s^{-1}$) approximated that produced by 10^{-4} M carbachol-stimulated tissues. Each recording lasted 2 minutes.

The acquired digital signals were treated using EthoVision XT v.10.0.828 (© 2013 Noldus Information Technology b.v., Wageningen, The Netherlands) which is an automated video tracking and motion analysis system. The software detected the subject and extracted the position of its central body point [**Fig. 3.6b❹**]. This datum was then transformed into a series of dependent variables quantifying the behaviour of the subject.

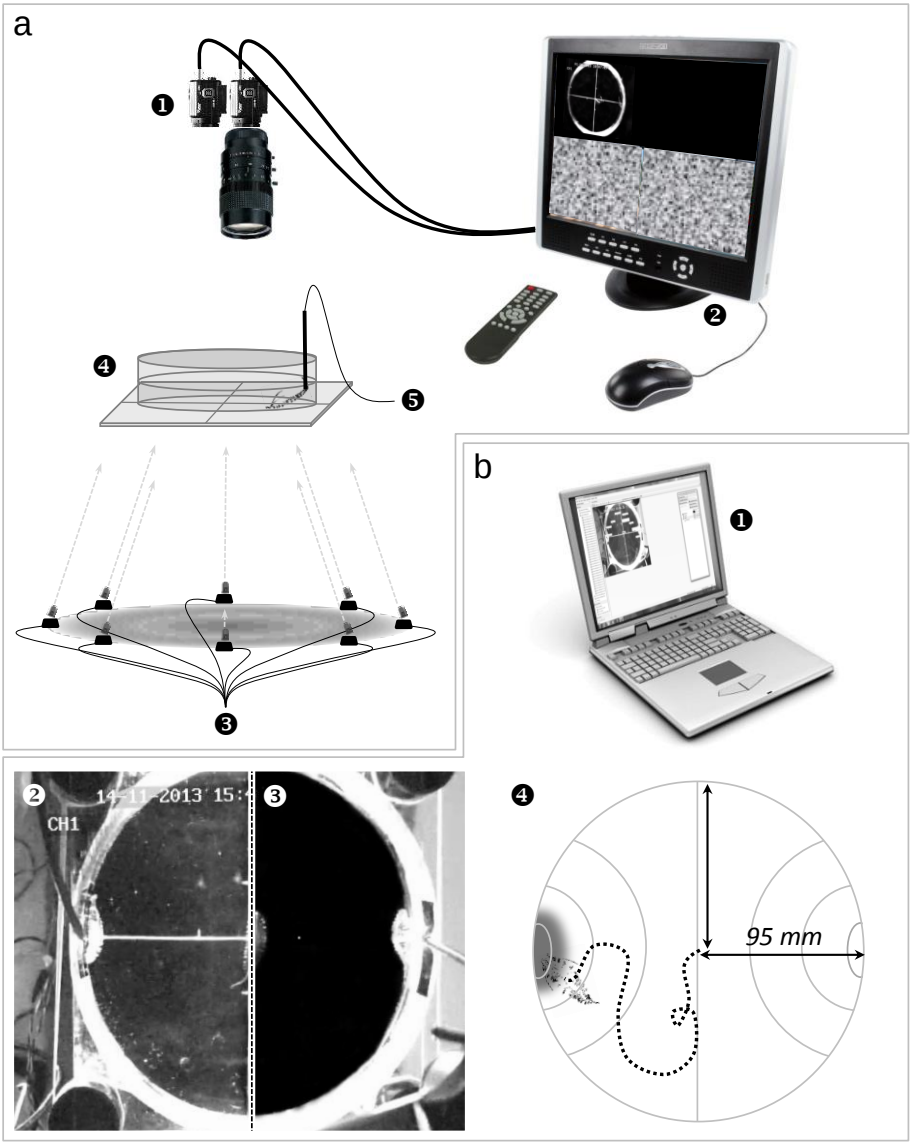


Fig. 3.6. Ethology: video recording and analysis.
(a) Experimental setup: mixed video recording system (infra red camera: Watec 902H ultimate, lens 5-50 mm, F/1.4; low-light-level camera: Watec 902H ultimate, auto-iris lens HZ848DC, F/1.0) (❶), IREDs (❷), DVR device (SEC-DVRMON30, König electronic, Netherlands) (❸), plexiglass round aquarium (❹), manualy controlled luminous model (❺); **(b)** Video analysis with Ethovision: ❶ Ethovision softwaree, ❷ View of the arena as recorded with the current setup, ❸ View on the arena as expected for future researchs, ❹ Diagram of the arena showing the tracking path of a worm.

Due to the poor quality of some video recordings [**Fig. 3.6b②**], considerable time was spent to manually correct the tracking errors. For future works, some simple modifications of the experimental design could significantly improve the quality of tracking. For example, every potential source of light reflection (*e.g.* particles in the water, scratches of the acrylic tank) must be avoided as much as possible; the timer has to be visible on the screen but must appear out of the arena, the orthogonal frame used for the calibration must be restricted to several reference points [**Fig. 3.6b③**]. Most importantly, the contrast of the worm could be increased by post-processing the videos or by applying a vital stain of low toxicity such as the Bismarck Brown 1:50 000 used by Åkesson 1962. However, this practice should be regarded with caution since the impact of this substance on the physiology and the behaviour of *Tomopteris* is unknown. Finally, some more sophisticated visualisation techniques, such as the shadowgraph and Schlieren techniques (see SETTLES 2001 for a detailed introduction), can be used. Briefly, these techniques highlight the optical inhomogeneities (*i.e.* refractive index gradients) in transparent media. Although the refractive index of gelatinous organisms is known to match that of seawater (JOHNSEN AND WIDDER 1999, JOHNSEN 2001), some equipment can detect angles of refraction of fewer than 0.1 seconds (SETTLES 2001). These highly sensitive optical systems, which can be implemented in infra-red light and in 3D, were used especially to characterise behaviours and interactions in free-swimming organisms like copepods (BAGØIEN AND KIØRBOE 2005a, 2005b, KIØRBOE *ET AL.* 2005). A video sequence of *Tomopteris* specimens recorded by these techniques was recently published on fishlarvae.org/galleries/ (Norwegian Institute of Marine Research) or available at this address: https://www.youtube.com/watch?v=XBPmf6cJV3w.

3.3. Statistics

All statistics presented in this work were conducted by or with the support of the internal consultancy service in statistics (SMCS) of the *Université catholique de Louvain*. All the tests were performed using JMP® Pro v.11.2.0, SAS (Statistical Analysis System Institute Inc., Cary, North Carolina, USA) or R (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria) and based on a minimum of 6 replicates. For future work, the number of replicates should be increased as interindividual variability can be significant.

4. Results and discussion

4.1. Patterns and control of light emission in *T. helgolandica* and allies

As previously mentioned, although numerous authors have observed and reported the ability of some *Tomopteris* species to emit light (*e.g.* MALAQUIN AND CARIN 1922, BONHOMME 1952b, HARVEY 1952, Dales 1971), basic experimental data such as direct measurements of this bioluminescence are missing. Therefore, this chapter firstly aims to characterise and compare the bioluminescent patterns of *T. helgolandica* [**paper I**] with those of some of its relatives, namely *T. planktonis*, *T. septentrionalis*, *T. pacifica* and *T. carpenteri* [**paper II**]. These two articles also address and compare the neural control pathways involved in the light emission of the sympatric species *T. helgolandica* and *T. planktonis*.

4.1.1. Physiological control of bioluminescence in a deep-sea planktonic worm, *Tomopteris helgolandica* (Paper I)

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Abstract

Tomopteris helgolandica Greeff 1879 (Tomopteridae) is a transparent holoplanktonic polychaete that can emit a bright light. In this study, we investigated the emission pattern and control of this deep-sea worm's luminescence. Potassium chloride depolarisation applied on anaesthetised specimens triggered a maximal yellow light emission from specific parapodial sites, suggesting that a nervous control pathway was involved. Pharmacological screening revealed a sensitivity to carbachol, which was confirmed by a dose–light response associated with a change in the light emission pattern, where physiological carbachol concentrations induced flashes and higher concentrations induced glows. The light response induced by its hydrolysable agonist, acetylcholine, was significantly weaker but was facilitated by eserine pretreatment. In addition, a specific inhibitory effect of tubocurarine was observed on carbachol-induced emission. Lastly, KCl- and carbachol-induced light responses were significantly reduced when preparations were pre-incubated in Ca^{2+}-free artificial seawater or in different calcium channel blockers (verapamil, diltiazem) and calmodulin inhibitor (trifluoperazine) solutions. All of these results strongly suggest that *T. helgolandica* produces its light flashes *via* activation of nicotinic cholinergic receptors and a calcium-dependent intracellular mechanism involving L-type calcium channels.

Introduction

Planktonic organisms can achieve almost perfect invisibility *via* transparency, making this one of the most valuable and fascinating adaptations to pelagic environments (JOHNSEN AND WIDDER 1998). Paradoxically, at first sight, some of these cryptic organisms are also brightly luminous (HADDOCK AND CASE 1999, POUPIN *ET AL.* 1999).

The tomopterid holoplanktonic polychaetes belong to a particularly diverse family that is extremely transparent (JOHNSEN AND WIDDER 1998) and includes at least 12 bioluminescent species (POUPIN *ET AL.* 1999). Although the spectral distribution has been measured in only one species (λ_{max} = 565 nm for *Tomopteris nisseni*) (LATZ *ET AL*. 1988), these pelagic worms are often described as yellow emitters, in contrast to most bioluminescent marine organisms, which emit a blue–green light. Additionally, given the species-specific luminous organ distribution observed in this family, this unusual luminescence has been suggested to be an intraspecific communication signal (HARVEY 1952, DALES 1971, LATZ *ET AL.* 1988). Since the review of Dales (1971), this elegant hypothesis is often highlighted despite the lack of evidence, given basic experimental data are missing such as emission pattern and associated physiological control mechanisms. Here, we focused on these fundamental aspects and present a pharmacological approach to investigating the luminescence control of *T. helgolandica* Greeff 1879, a widespread, previously established bioluminescent East Atlantic species (HARVEY 1952).

Materials and method

Organism collection

Tomopteris helgolandica specimens (1.5-6.0 cm in length), were collected from October 2010 to November 2012 at a 200–300 m depth from two connected fjords, Raunefjorden and Korsfjorden (Western Norway), using two types of towed net samplers: the Isaacs-Kidd midwater trawl (1.75 m wide × 1.30 m high mouth, 6.5 m long, 500 µm mesh aperture) or ring plankton nets (1.5 m diameter mouth, 300 µm mesh aperture) depending on the weather and the vessel's equipment. Live specimens were housed in classical aquariums or in Kreisel tanks commonly used for maintaining gelatinous zooplankton (BAKER 1963, RASKOFF *ET AL.* 2003) in a permanent dark cold room (6-8°C) at the Espegrend Marine Biological Station of the University of Bergen, Norway.

Anaesthesia

Several anaesthetics that have previously been used on polychaetes (SMALDON AND LEE 1979, COSTA-PAIVA *ET AL.* 2007, ROSS AND ROSS 2008, Cooper 2011), including magnesium chloride, propylene phenoxetol and tricaine mesylate, were unsuccessful with *T. helgolandica*. However, preliminary experiments demonstrated that menthol efficiently anaesthetised the animals within 30 min and reduced the interindividual variability of the emitted light compared with non-treated specimens without affecting light emission parameters. Thus, before each experiment, the organisms were relaxed for 30 min in a menthol solution applied at increasing concentrations (0.25-2.5 $g.l^{-1}$).

Pharmacology

The organisms were dissected from the head to the tail into serial preparations that comprised three parapod pairs. Each preparation was placed into 50 µl of artificial seawater (ASW: 400.4 $mmol.l^{-1}$ NaCl, 9.6 $mmol.l^{-1}$ KCl, 52.3 $mmol.l^{-1}$ $MgCl_2$, 9.9 $mmol.l^{-1}$ $CaCl_2$, 27.7 $mmol.l^{-1}$ Na_2SO_4, 20 $mmol.l^{-1}$ Tris, final pH 8.3). Next, light production was triggered by adding 50 µl of a given test solution. For each studied specimen, one preparation was treated with the control stimulus (200 $mmol.l^{-1}$ KCl or, depending on the tested effect and based on preliminary results, 1 $mmol.l^{-1}$ cholinergic agonist) whereas the other preparations were treated with different pharmacological substances [**Table 1**]. The light responses were standardised and expressed as a percentage of the control light response. All of the experiments were designed following the Latin square principle: from one specimen to another, the preparation from an identical position is never treated twice by the same solution to eliminate possible interindividual or interpreparation variability. The pharmacological solutions were prepared in ASW buffered at pH 8.3 just before the experiments were performed. The light intensity was measured for 10-20 min with a single tube luminometer (FB12, 2005, Berthold Technologies, Bad Wildbad, Germany). To avoid light stress or artefactual measurements, all handling and experiments were performed in partial darkness or under red lighting.

Statistics

All of the statistical analyses were performed with JMP software (v10.0.0, 2012, SAS Institute Inc., Cary, NC, USA) on logtransformed data [$\Sigma\log(x(1\rightarrow n))/n$] for relative values >1. Variance normality and equality were first tested by the Shapiro–Wilk test and Levene's test, respectively. When these parametric assumptions were not met, a one-way ANOVA was replaced by a non-parametric Kruskal–Wallis ANOVA to assess the significant difference between more than two groups. All of the pairwise comparisons were tested using a *post hoc* Student's *t*-

drug			concentration
usual name	**commercial name**	**pharmacology**	**(mmol l^{-1})**
KCl	potassium chloride	depolarizing agent	200
serotonin	5-hydroxytryptamine	serotoninergic neurotransmitter	1
epinephrine	(±)-epinephrine	adrenergic neurotransmitter	1
GABA	gamma-aminobutyric acid	GABAergic neurotransmitter and neuromodulator	1
nitric oxide	sodium nitroprusside	guanylyl cyclase activator	1
carbachol	carbamoylcholine chloride	cholinergic agonist	1
DMPP	1,1-dimethyl-4-phenyl-piperazinium iodide	nicotinic agonist	1
acetylcholine	acetylcholine chloride	cholinergic neurotransmitter	1
eserine	eserine	cholinesterase inhibitor	1
tubocurarine	d-tubocurarine chloride	cholinergic nicotinic receptor antagonist	1
atropine	atropine sulfate	cholinergic muscarinic receptor antagonist	1
nifedipine	nifedipine	calcium channel blocker	1
diltiazem	diltiazem chloride	calcium channel blocker	1
trifluoperazine	trifluoperazine dihydrochloride	calmodulin inhibitor	1
verapamil	(±)-verapamil	calcium channel blocker	1

Table 1. Detailed list of chemical and pharmacological substances (Sigma-Aldrich Co.) tested to investigate the nervous control of luminescence in *Tomopteris helgolandica*.

test (each pair), Dunnett's test (with control) or a *post hoc* Dunn's test, as appropriate. Each difference was considered to be significant at 0.05. For clarity, the graphically illustrated values are expressed as the geometric means [$\sqrt{}(x_1 \times x_2 \times \ldots \times x_n)$] with the corresponding s.e.m..

Results

Light emission pattern

When applied on whole specimens, potassium chloride (200 mmol.l^{-1} KCl) triggered a maximal yellow light emission at 1 megaquanta (Mq) = 10^6 photons) [**Fig. 1A,C**]. Because of the linear relationship between the total emitted light (L_{tot}) and the maximal light intensity (L_{max}) of the control light responses [**Fig. 1B**], only L_{tot} is presented.

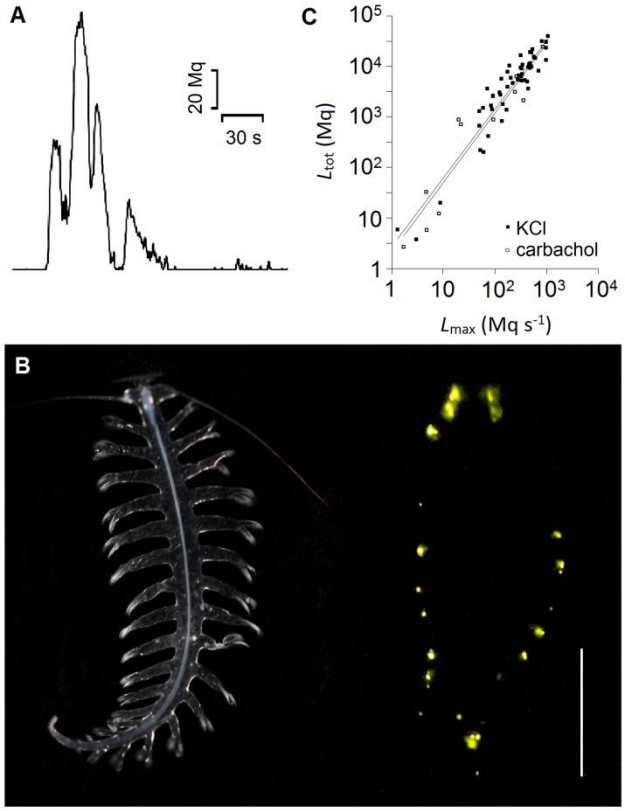

Fig. 1. Light emission pattern of *T. helgolandica*.
(A) Typical curve shape of maximal KCl-induced light emission; **(B)** Linear relationship between the total quantity of light emitted (L_{tot}) and the maximal light intensity (L_{max}) for 0.2 mol.l^{-1} KCl- and 10^{-3} mol.l^{-1} carbachol-induced luminescence. R^2-value are 0.8944 and 0.8822 respectively, slopes (P = 0.6921) and intercepts (P = 0.6609) are significantly equal; **(C)** *T. helgolandica* photographed in natural light and its KCl-induced bioluminescence photographed in the dark. Scale bar 1 cm.

Screening of neurotransmitters

The primary neurotransmitter families known to mediate bioluminescence throughout invertebrates were pharmacologically screened on *T. helgolandica* parapods (NICOL 1960, CASE AND STRAUSE 1978, ANCTIL 1979, GARDNER AND WALKER 1982, ANCTIL 1987, WALKER *ET AL.* 1996) [**Table 2**]. Only carbachol elicited a luminescence higher than the ASW-induced emission (mechanical stimulus).

Extrinsic cholinergic control

The amount of emitted light increased with increasing carbachol concentration [**Fig. 2A**], and different emission patterns were observed at various concentrations.

At low carbachol concentrations, the pattern consisted of a series of weak intensity flashes [**Fig. 2A**, top left panel], which was in contrast to the monophasic shape of 1 mmol.l^{-1} carbachol light emission [**Fig. 2A**, top right panel], which was similar to the KCl-induced light response [**Fig. 1A**]. However, the tissue preparations generally responded poorly to acetylcholine compared with its non-hydrolysable agonist carbachol. Thus, an eserine pretreatment was tested. This cholinesterase inhibitor induced a weak light response (0.04 ± 0.03% of KCl) and significantly facilitated acetylcholine-induced emission but did not affect carbachol-induced light emission [**Fig. 2B**]. Lastly, the specificity of the cholinergic receptors was evaluated using nicotinic and muscarinic blocking agents, tubocurarine and atropine, respectively [**Table 1**]. Only tubocurarine significantly inhibited the carbachol-induced emission [**Fig. 2C**]. This observation was confirmed with the nicotinic agonist dimethyl-phenylpiperazinium (DMPP), which triggered an intense light emission (9519.55±8490.38% of KCl; not shown).

Intrinsic control: calcium requirement

Given that the nicotinic control pathway suggests that Ca^{2+} is a second messenger, we aimed to investigate the calcium dependence of the reaction. Pre-incubating the tissue preparations in Ca^{2+}-free ASW significantly inhibited the KCl- and carbachol-induced luminescence responses by 95 and 100%, respectively [**Fig. 3A**]. The luminescence was also significantly reduced by different calcium channel blockers, verapamil (phenylalkylamines) and diltiazem (benzothiazepine), and by trifluoperazine, a calmodulin inhibitor [**Fig. 3B**].

drug	**L_{tot} (% KCl)**	***P*-value**	**n**
ASW (control)	4.67 ± 4.22		5
serotonin	16.56 ± 15.13	1.000	5
epinephrine	53.71 ± 52.76	1.000	5
GABA	15.76 ± 10.60	1.000	5
nitric oxide	89.40 ± 79.55	1.000	4
carbachol	1129.88 ± 482.90	0,2839	5

Table 2. Screening of neurotransmitters' effect on isolated tissue preparations of *T. helgolandica*.
Light intensities are expressed as a function of KCl-induced luminescence (7627.07 ± 2172.73 Mq).

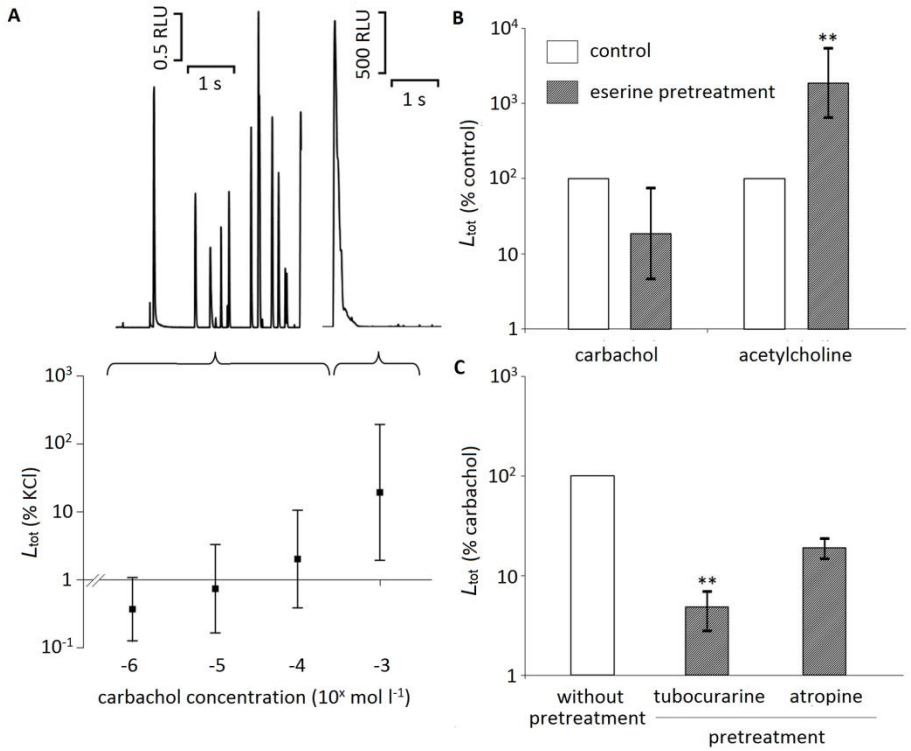

Fig. 2. Bioluminescence physiological control in *T. helgolandica*.
(A) Carbachol dose-light response – the total quantity of light emitted during the experiment (L_{tot}) is expressed as a percentage of the value obtained with KCl application (theoretical physiological maximum) – and parapod luminescent response patterns in response to low (top left) and high (top right) carbachol application; **(B)** Effect of an eserine pretreatment on the luminescence induced by carbachol and acetylcholine application. Values represent the L_{tot} increase expressed as a percentage of each activator injected alone (without eserine pretreatment). Eserine significantly increased the luminescent response to acetylcholine but had no effect on carbachol-induced luminescence; **(C)** Effect of atropine and tubocurarine pretreatments on carbachol-induced luminescence. Tubocurarine significantly decreased the luminescent response to carbachol but atropine had no significant effect on this luminescence. (n = 6; ** $P < 0{,}01$).

Discussion

Although some bioluminescent organisms produce a continuous glow, most light emission signals are transient events mediated by specific control mechanisms (NICOL 1960). Two control levels are commonly distinguished: an extrinsic control represented by peripheral control pathways and an intrinsic control that includes the photogenic reaction and the related intracellular signalling pathways (CASE AND STRAUSE 1978). In self-luminescent metazoans characterised by differentiated

photogenic structures, emission is controlled either by hormones (CLAES AND MALLEFET 2009) or *via* coupling mechanisms between photocytes and excitable cells, including neural, muscular or epithelial cells (HERRING AND MORIN 1978, ANCTIL 1987, HASTINGS AND MORIN 1991, KRÖNSTRÖM *ET AL.* 2009). Although luminescence can originate in a nerve-free bioluminescent epithelium, such as in the conducting epithelia of some Hydrozoa (BASSOT *ET AL.* 1978, DUNLAP *ET AL.* 1987) and Anthozoa (GERMAIN AND ANCTIL 1996), it is most frequently controlled by neural pathways (NICOL 1960, CASE AND Strause 1978). In addition to turning the light emission on and off, nervous control abilities can modulate and adjust the intensity, duration, frequency or angular distribution of a light signal and thus generate diversity and specificity. However, a large proportion of the functional diversity of the existing emission patterns and control systems is unknown, especially in annelids, where the most detailed bioluminescence control studies have been limited to polynoid and chaetopterid benthic species (GARDNER AND WALKER 1982, ANCTIL 1987). Therefore, the luminescence control of pelagic worm species has been poorly documented (HARVEY 1952, HADDOCK *ET AL.* 2010).

According to our results, *T. helgolandica* luminescence is under nervous control, as revealed by yellow luminescence induced by KCl depolarisation in nervous fibres, which directly or indirectly causes a photogenic structure response (DE BREMAEKER *ET AL.* 1996). Furthermore, the pharmacological screen revealed a dose-dependent carbachol sensitivity. In fact, carbachol and acetylcholine both induced light emission, but the tissue preparations demonstrated a low

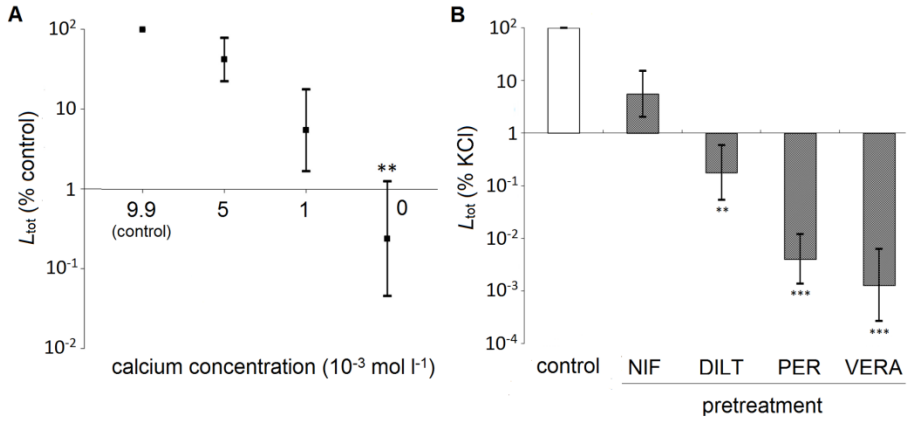

Fig. 3. Bioluminescence intrinsic control in *T. helgolandica*.
(A) Dose-dependant inhibitory effect of calcium depletion. Values represent the L_{tot} decrease expressed as a percentage of those obtained in complete artificial sea water (10^{-2} mol.l^{-1}); **(B)** Effect of calcium organic inhibitors (NIF = nifedipine, DILT = diltiazem, PER = trifluoperazine, VERA = verapamil) on KCl-induced luminescence. (n = 6; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

responsiveness to acetylcholine. Pharmacological carbachol concentrations (over 0.1 mmol.l^{-1}) elicited a monophasic signal, similar to KCl-induced light emission, but 1 mmol.l^{-1} acetylcholine failed to elicit such a pattern. However, eserine pretreatment significantly facilitated acetylcholine-induced light emission, which attained similar intensities to those emitted in response to low carbachol concentrations (< 0.1 mmol.l^{-1}, nearest physiological concentration) and suggested an involvement of cholinesterase activity. Lastly, the flash trains (L_{max} = 400 Mq.s^{-1}) observed at the lowest concentrations were probably more representative of the naturally expressed signal. The specific inhibitory effect of tubocurarine on carbachol-induced emission not only indicates an involvement of the cholinergic pathway but also demonstrates the nicotinic receptor prevalence. These observations were supported by the sensitivity of the samples to the nicotinic agonist DMPP and by their calcium-dependent light response, which suggested that L-type calcium channels were also involved. *Tomopteris helgolandica* produces yellow flashes from each parapod through neural control that activates nicotinic cholinergic receptors and a calcium-dependent intrinsic mechanism.

Numerous and widespread cholinergic control mechanisms exist in annelids (GARDNER AND WALKER 1982, WALKER *ET AL.* 1996). The control of bioluminescence is relatively well known in Polynoidae and Chaetopteridae (NICOLAS *ET AL.* 1978, GARDNER AND WALKER 1982, ANCTIL 1987) and is reinforced by the present study of one Tomopteridae. However, because of its ubiquity, the specific mode, level and site of action of acetylcholine in the bioluminescent process remain unclear. Muscarinic cholinergic and serotoninergic mechanisms have been described in benthic scale-worms (Polynoïdae) as part of the excitatory pathway of elytral luminescence (NICOL 1954, NICOLAS *ET AL.* 1978, MIRON ET AL. 1987, ANCTIL *ET AL.* 1989). The tube-worm *Chaetopterus variopedatus* (Chaetopteridae) produces a glowing blue mucus in response to the contractile action of the adjacent epithelio-muscular cells, which are controlled by muscarinic cholinergic and GABAergic pathways (NICOL 1952, ANCTIL 1981, MARTIN AND ANCTIL 1984, ANCTIL 1987). Given that some photocytes in other organisms are not directly innervated but are controlled by adjacent supportive cells that trigger light emission by epithelial conduction (ANCTIL 1987, DUNLAP *ET AL.* 1987), the calcium entry *via* L-type channels we observed could act at both the neuro-photocyte level and an intermediate level. The presence of parapod nerve fibres, revealed by the histological studies of Greeff (1882, 1885) and Bonhomme (1952) on *T. mariana* and *T. kefersteini*, respectively, suggested that the bioluminescence of these worms was under nervous control. In particular, Greeff exhibited a scheme of photogenic organs with direct nerve connections. However, the characterisation of photogenic structures remains ambiguous (MALAQUIN AND CARIN 1922, BONHOMME 1952).

Despite the differences observed in the light emission pattern, its control and the bioluminescence characteristics between polynoid and chaetopterid worms, the luminescence in all cases has been associated with defensive functions. The same hypothesis has been suggested for the blue luminescence of the benthic polychaete *Polycirrus perplexus* (Terebellidae) (HUBER *ET AL.* 1989) and for the 'green bombs' expelled by the recently described deep-sea pelagic specimens belonging to Acrocirridae (OSBORN *ET AL.* 2011). Only syllid worms, whose behaviour has been well studied, use their green light emission for both deterrence and intraspecific communication during mating swarms (WILKENS AND WOLKEN 1981, TSUJI AND HILL 1983, FISCHER AND FISCHER 1995, GASTON AND HALL 2000, DEHEYN AND LATZ 2009). Given that the open ocean does not facilitate contact between planktonic organisms, an atypical emission wavelength would be highly advantageous for Tomopteridae. Although the emission of yellow light has been interpreted by numerous authors as a specific signal that involves a private communication channel (HARVEY 1952, DALES 1971, LATZ *ET AL.* 1988), the spectral sensitivity of *T. septentrionalis* is rather centred on blue (BUSKEY AND SWIFT 1985).

Nevertheless, it is likely that *T. helgolandica*'s yellow light may play different roles, as suggested by the observation of different emission patterns – flash *versus* glow – according to the stimulus applied. Flash is often associated with a deterrent function, whereas glows are considered attractive, suggesting that the worms modulate the light output depending on the context that incites the use of bioluminescence. However, in the absence of further experimental data, this hypothesis remains speculative.

Conclusions

Our results strongly support the hypothesis that *T. helgolandica*'s bioluminescence is under nervous control, revealing new insight into the pathways involved. The yellow light flashes are produced *via* activation of nicotinic cholinergic receptors and a calcium-dependent intracellular mechanism involving L-type calcium channels. However, the understanding of tomopterid bioluminescence at an ecological level is beyond our current knowledge. In addition to studying the intrinsic mechanisms of light emission, an assessment of the mechanisms that govern their visual capabilities as well as their reproductive biology and behaviour will be performed in our future research.

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Author contributions

A.G. and J.M. designed the experiments, and collected the samples and data. A.G. performed the experiments and analysis, and wrote the manuscript. J.M. revised the manuscript.

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4.1.2. Unexpected diversity of bioluminescence in planktonic worms (Paper II)

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Abstract

The vast majority of pelagic bioluminescent organisms emit a blue light with emission maxima (λ_{max}) ranging from 450 to 490 nm. Among the known outliers, the tomopterids (Annelida: Polychaeta) are usually described as yellow emitters (λ_{max} = 565-570 nm) for which bioluminescence functions as a specific recognition signal. However, here we report the first data of colours emitted by four different tomopterid species, *Tomopteris pacifica*, *T. carpenteri*, *T. septentrionalis* and *T. planktonis*. Surprisingly, among them, *T. planktonis* is a blue emitter (λ_{max} = 450 nm). Our pharmacological results on *T. planktonis* support a cholinergic control, as recently demonstrated in the yellow emitter, *T. helgolandica*. Moreover, as revealed by epifluorescence microscopy, the light seems to be produced in both species from the same yellow-pigmented parapodial glands. Despite these similarities, tomopterids express an unexpected diversity of bioluminescent colour patterns. This leads us to reassess the ecological value of bioluminescence within this group.

Introduction

Bioluminescence is a widespread phenomenon in the Ocean, particularly in the mesopelagic realm (200-1000 m). The emission maxima (λ_{max}) of most bioluminescent organisms living in this habitat fall within the blue range (λ_{max} = 450-490 nm) (HERRING 1983). However, despite the evolutionary forces that led to the near homochromatic nature of pelagic bioluminescence, some species have developed other colours. Among them, the tomopterids (Annelida: Polychaeta), represented by transparent, strictly planktonic species, are commonly described as yellow light emitters (*e.g.* DALES 1971, GOUVENEAUX *ET AL*. 2013, FRANCIS *ET AL*. 2014). These worms, deeply nested within the Errantia, are isolated from the few families where bioluminescence has been documented (*e.g.* the most studied: Chaetopteridae, Syllidae, Polynoidae) (WEIGERT *ET AL.* 2014). The remarkable presence of yellow pigmented cells, yellow fluorescence and yellow bioluminescence in the parapodia of various species has often led authors to generalise this colour and the ability to emit light to all tomopterids (DALES 1971, LATZ *ET AL.* 1988). Until recently, some studies tended to neglect the interspecific differences that may exist at biochemical, physiological or ecological levels (*e.g.* FRANCIS *ET AL.* 2014). However, 53 species, including at least 12 luminous ones (POUPIN *ET AL.* 1999), have been described and the emission spectrum is currently known in only one species: *Tomopteris nisseni* Rosa, 1908 (LATZ *ET AL.* 1988). The only specimen examined (λ_{max} = 565, FWHM = 55 nm) appeared to be responsive to both mechanical and electrical stimuli.

Here we report the first documented observation of a blue emitting species, *T. planktonis* Apstein, 1900 (λ_{max} = 450 nm). The latter was collected in South West Norway, where we usually collect the yellow emitter *T. helgolandica*. As revealed by fluorescence microscopy, light emission seems to originate from the yellow-pigmented parapodial glands as previously hypothesised in other species including *T. rolasi* Greeff, 1885, *T. kefersteini* Greeff, 1879, *T. nationalis* Aptein, 1900 (GREEFF 1885, BONHOMME 1954, TERIO 1964). These observations are compared with newly acquired data about three related yellow emitting species: *T. septentrionalis* Steenstrup, 1849 and *T. pacifica* Izuka, 1914 collected in the North Pacific Ocean, and *T. carpenteri* Quatrefages, 1866 from the Southern Ocean. Regarding the pharmacological aspect, our results suggest a cholinergic control of light emission.

Experimental

Specimen collection

T. planktonis, *T. helgolandica*, *T. septentrionalis*, *T. pacifica* and *T. carpenteri* specimens were collected by trawling (ring plankton nets 300-500 µm, or Isaacs-Kidd Midwater Trawl) from various locations at distinct times of the year during the period 1995-2016 [**Tab. 1**]. A list of diagnostic features used for species identification is provided as supporting information [**Tab. SI**]. All specimens were maintained in a dark cold room (6-8°C) in aquariums of standing, aerated sea water. They kept swimming actively and retained their bioluminescent abilities for

species	sampling			use	n
	location	depth	period		
T. planktonis	Raunefjorden, Western Norway N60°15'51'', E5°08'28''	180-0 m	Nov. 2014	- picture and reflectance spectra - pharmacology and fluorescence microscopy	3 6
T. helgolandica	Korsfjorden and Raunefjorden, Western Norway N60°16'15'', E5°09'59'', N60°16'02'', E5°08'13''	250-0 m	2011-2016	- picture and reflectance spectra - live spectra	3 1
T. septentrionalis	San Juan Island, Washington state, USA N48°32'42'', W123°0'44''	surface	Jan. 1996	- contact lumigram and reflectance spectra	3
T. pacifica	San Juan Island, Washington state, USA N48°32'42'', W123°0'44''	surface	Jan. 1996	- contact lumigram and reflectance spectra	3
T. carpenteri	Kerguelen islands, French Southern and Antarctic Lands S49°53'72", E76°13'13"	125-0 m	Jan. 2014	- picture and reflectance spectra - live spectra	6 3

Table 1. Sampling locations and experimental methods implemented for each model species.

weeks after collection. They were anaesthetised in menthol (*T. planktonis*, *T. helgolandica*, *T. carpenteri*) or magnesium chloride ($MgCl_2$) (*T. septentrionalis*, *T. pacifica*) solutions before experimentation. Menthol anaesthetisation was applied for 20 minutes at increasing concentrations (0.25-2.5 $g.l^{-1}$) obtained by the gradual addition of a stock solution (25 $g.l^{-1}$) in the sea water. Then the specimens were rinsed in sea water during 10 minutes. There was no light response during the anaesthetisation process and the quantity of light induced in relaxed specimens was comparable to that produced without anaesthetisation. The other worms were immobilised by adding 1/3 to 1/2 volume of 0.33 M $MgCl_2$ (isotonic to sea water) to the sea water and waited seconds to minutes for the critters to stop responding to mechanical stimulation by a touch of a dissection needle. Experiments were then performed immediately after.

Bioluminescence induction

The bioluminescence was induced by a variety of stimuli. In most cases, the light production was triggered either by 200-500 mM potassium chloride solutions (KCl; pH 8.3) or by 1% Triton X-100 solutions. KCl, a depolarising agent which has been suggested to act through a voltage-dependent physiological pathway, maximises the luminescent reaction and is commonly used as a standard stimulus (GERMAIN AND ANCTIL 1988, DEHEYN *ET AL*. 2000). The non-ionic surfactant Triton X-100 is known to solubilise cell membranes, hence releasing luminous compounds to react in the medium.

In *T. carpenteri*, KCl and electrical stimulations (9V, continuous, tungsten electrode in sea water and on the specimen) triggered similar bioluminescence displays. Electrical stimulations have not been performed on the other species.

Spectra characterisation

The direct measurement of the emission spectrum from a living organism is undoubtedly the most reliable technique. However, when this live measurement could not be performed, we measured the emission maxima either from digital pictures or from what we call “contact lumigrams”.

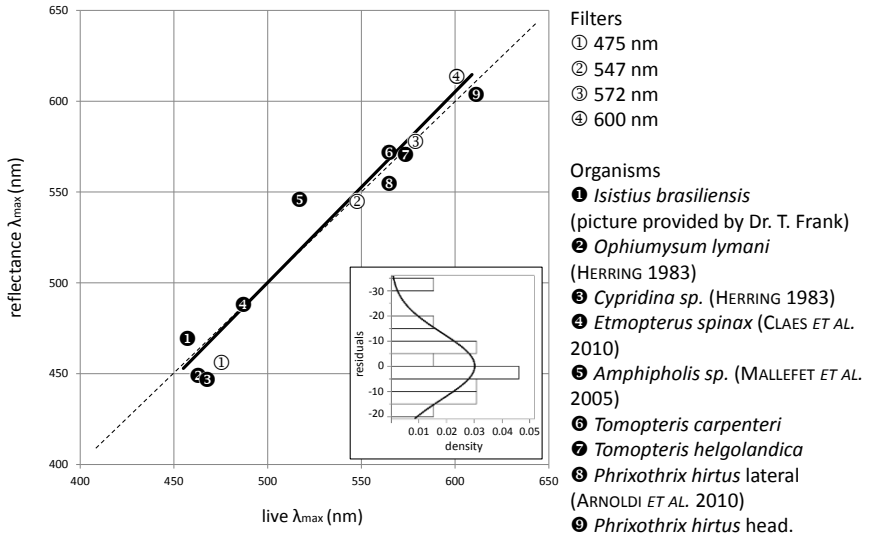


Fig. 1. Linear relationship between the emission maxima (λ_{max}) acquired by live measurements (Live λ_{max}) retrieved from different sources or done in the laboratory and from pictures (Reflectance λ_{max}).
The linear regression (solid line) used the standard least squares method (y = 1.0499x – 24.457 ; R^2 = 0.9521, *P* < 0.0001). At the bottom right, a density histogram of the residuals illustrates their normal distribution (*P* = 0.1941, Shapiro-Wilk W test). The reference regression line (dashed line) considers a perfect match between live and reflectance measurements (y = 1x). The slopes (*P* = 0.1562) and intercepts (*P* = 0.1718) of both regressions are not significantly different (ANCOVA).

Digital pictures (*T. planktonis*, *T. carpenteri*), were taken in total darkness, with a fullframe Digital Standard-Lens Reflex (DSLR, EOS Canon 1Dx and 50 mm f1.4 lens on macro extension tubes). Exposure time was adjusted according to the light intensity of the specimens. Then untreated raw images were transferred to a computer. To make "contact lumigrams", specimens of *T. septentrionalis* and *T. pacifica* were immobilised in 0.3 M $MgCl_2$ and laid on diapositive colour film protected by Saran wrap in total darkness (Fuji RFP 50 ASA). The bioluminescence was chemically induced to expose the film. Afterwards, careful rinsing and drying in total darkness preceded the film development.

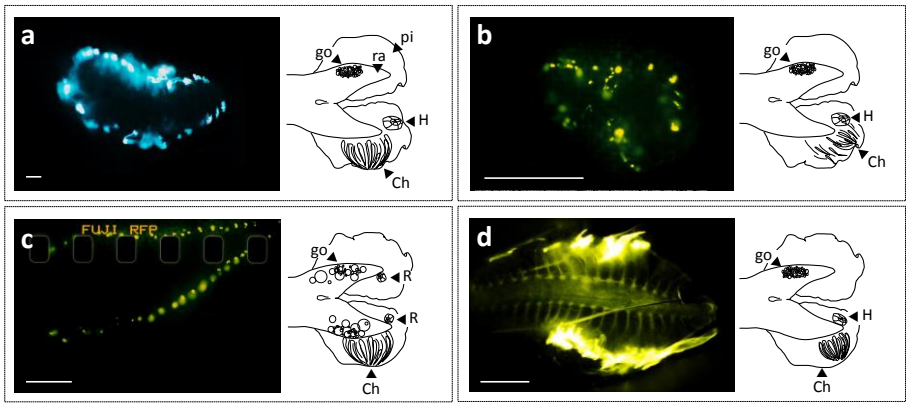
Spectra were acquired using a Hamamatsu mini-spectrometer (C10083CA, Hamamatsu photonics, Hamamatsu city, Japan) equipped with an optical fibre (model A976201). Spectra of the room in darkness, or of a dark area on the picture surrounding the luminous specimens, were recorded prior to the spectral acquisition of luminescent signal. This dark background signal was subtracted from each acquired spectrum. Then, Gaussian fittings (adjusted R^2 > 0.90) were applied using the free MagicPlot software (2013, MagicPlot Systems, LLC, St Petersburg, Russia).

Fluorescence microscopy

Microscopic examination of endogenous fluorescence was performed on KCl-stimulated parapodia from *T. planktonis* mounted *in toto*. We used the following filter set: excitation band-pass filter 420-490 nm, dichroic mirror 510 nm, emission long-pass filter 515 nm (Leitz Diaplan, Leitz, Wetzlar, Germany).

Pharmacology

Finally, six *T. planktonis* specimens were used to investigate the cholinergic control of their bioluminescence emission. The light response was triggered by direct immersion of sections of two pairs of parapodia in different pharmacological solutions and measured with a single tube luminometer (FB12, Berthold detection



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	method	λ_{max} (nm)	FWHM (nm)	n
T. planktonis (a)	digital picture	450 ± 0.5	23 ± 1.0	3
T. septentrionalis (b)	lumigram	557 ± 3.0	95 ± 4.5	3
T. pacifica (c)	lumigram	549 ± 9.0	85 ± 13.5	3
T. carpenteri (d)	digital picture	574 ± 1.5	139 ± 2.0	6
	live	564 ± 3.5	35 ± 18.0	3
T. helgolandica	digital picture	573 ± 2.0	130 ± 1.0	3
(Gouveneaux and Mallefet 2013)	live	573	51	1
T. nisseni (Latz *et al.* 1988)	live	565	55	1

Fig. 2. Diversity of bioluminescent patterns within tomopterids (Annelida: Polychaeta). Bioluminescent patterns of **(a)** *T. planktonis* under 200 mM potassium chloride (KCl) stimulation; **(b)** *T. septentrionalis* under 1% triton X-100 stimulation; **(c)** *T. pacifica* under 500 mM KCl stimulation; **(d)** *Tomopteris carpenteri* under electrical stimulation (9V, continuous). Scale bars 0.5 mm (a-c) and 5 mm (d). Species identification is based on the distribution of pinnal glands [**Tab. SI**] as illustrated by the diagrams of the 4th parapodia. pi: pinnule, fin-like parapodial appendage; ra: ramus; Ch: chromophile gland; H: hyaline gland; Ro: rosette gland; go: gonads. Picture credits: J. Mallefet (a, d) and P. R. Flood (b, c); **(e)** Spectral distribution parameters measured either from pictures or from living specimens (λ_{max} = average maximum wavelength, FWHM = average full width at half maximum intensity, mean ± s.e.m., n = 3-6).

systems, Pforzheim, Germany). The results are here expressed as a percentage of carbachol- or acetylcholine-induced control responses. The statistical analyses were performed with JMP Pro software (v11.2.0, 2013, SAS Institute Inc., Cary, NC, USA) on log-transformed data. In order to be consistent with our previous pharmacological investigation (GOUVENEAUX AND MALLEFET 2013), the graphically illustrated values are expressed as the geometric means [$\sqrt[n]{(x1 \times x2 \times \ldots \times xn)}$] with the corresponding standard error of the mean (s.e.m.).

Results and discussion

Diversity of light emission colours

In order to validate the spectral acquisition method, we have first tested, for several organisms, the linear correlation between the emission maxima obtained by live measurements found in the literature and those obtained by reflectance measurements taken on various digital pictures [**Fig. 1**]. Moreover, live and reflectance spectra measurements using 4 transmittance filters, with λ_{max} at 475, 547, 572 and 600 nm respectively, were used to calibrate our photographic equipment. According to the regression analysis, λ_{max} is minimally affected by the methodological differences. The linear fitting is good ($R^2 = 0.9521$, $P < 0.0001$) and not significantly different from the theoretical line y = 1x (Analysis of Covariance, or ANCOVA). In addition, this method appears to be robust with respect to the variation of shooting methods (digital camera, exposed diapositive films). The density histogram showing the residuals of linear regression [**Fig. 1**, bottom right] gives an order of magnitude on the measurement uncertainties, which include, among other things, the spectrometer resolution (6 nm), the possible Schwarzschild effect or the reciprocity law failure that can occur on diapositive film under long exposure at very low light level (MARTIN *ET AL.* 2003), and other technical limitations possibly due to RGB (Red, Green, Blue) technologies from DSLR sensors and LEDs screens.

In order to avoid any luminous capability loss, worms were stimulated as soon as possible after collection. The brightest light emissions obtained with various stimulators are illustrated in figure 2. Conspicuous spectral differences exist among *Tomopteris*: *T. planktonis* appears to produce a bright blue light emission, whereas *T. helgolandica*, *T. carpenteri*, *T. septentrionalis* and *T. pacifica* emit a yellow-green light [**Fig. 2a-d**]. *T. planktonis* emission maxima ($\lambda_{max} = 450 \pm 0.5$ nm) is thus about 100 nm below those of the known yellow-green tomopterid emitters (including *T. nisseni*), whose emission peak in a narrow range of about 20 nm (557-574 nm) [**Fig. 2e**].

From biochemical and evolutionary perspectives, our results suggest that the yellow light emitting systems might have derived from a common blue emitting system, as suggested in the red emitting stomiatoid fishes (WIDDER *ET AL.* 1984, HERRING AND COPE 2005). Similarly, this important chromatic shift may result from the radiationless transfer of the luminescent energy produced in the blue range to an accessory yellow fluorescent protein. This is a phenomenon described as

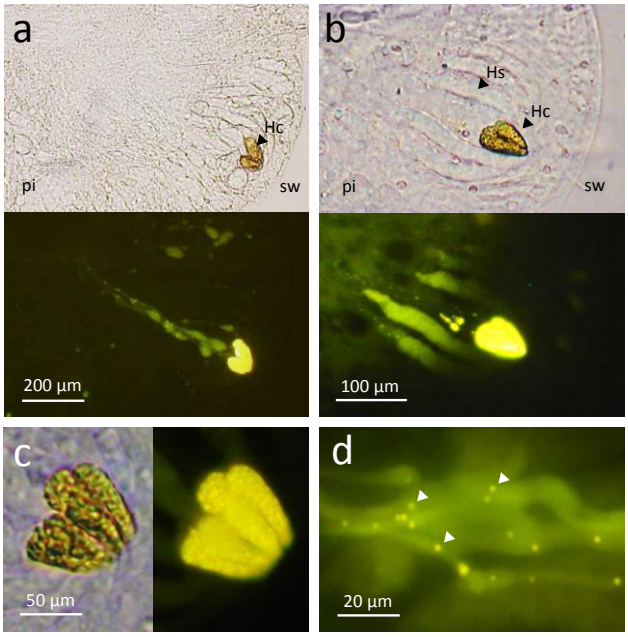

Fig. 3. Autofluorescence of stimulated hyaline glands from *Tomopteris planktonis*.
(a-b) Hyaline glands of 200 mM KCl-stimulated parapods: *in toto* mounts in plain bright-field illumination (upper halves) and autofluorescence in blue excitation light (420-490 nm, emission long-pass filter 515 nm) of the same fields of view (lower halves). Hc = core structure of the hyaline gland, Hs = transparent sacculi of the hyaline gland, pi = pinnule; sw = sea water; **(c)** Grains inside the yellow-pigmented core structure of the hyaline glands: in plain bright-field illumination (left half) and in blue excitation light (right half); **(d)** Isolated fluorescent grains (white arrows) as observed in the fluid released by the core structure of the gland in the surrounding pinnal tissues.

Bioluminescence Resonance Energy Transfer (BRET) which has been evidenced especially in anthozoans and hydrozoans using a green fluorescent protein or GFP (SHIMOMURA 2006). This hypothesis was recently supported by the observations of Francis and colleagues (2014), according to whom a fluorescent compound extracted from *Tomopteris spp.* presented an absorption peak at 430 nm and a fluorescence emission at 580 nm. Moreover, epifluorescence microscopy of KCl-stimulated tissues of *T. planktonis* [**see the next section**] revealed an intense yellow fluorescent signal in both pigmented and transparent structures that constitute the putative light organs [**Fig. 3**]. Terio (1960) has also observed and measured similar *in situ* fluorescence (λ_{max} = 560-580 nm, 450 nm shoulder) in preserved material from *T. septentrionalis* Steenstrup, 1849. Although the rosette-like organs of *T. planktonis* clearly demonstrate yellow fluorescent properties [**Fig. 3**], for unknown reasons, the energetic transfer might not be sufficient to trigger BRET effect, resulting in the emission of blue light [**Fig. 2a**]. Whatever the case may

be, further spectral and biochemical analyses are needed in order to confirm this hypothesis. According to previous studies (SHIMOMURA 2006, THOMSON *ET AL.* 1995), the light emitting substance of *Tomopteris* would be a cœlenterazine-based photoprotein probably activated by superoxide anion. The first discovered BRET system in *Aequorea victoria* also involves a cœlenterazine-based photoprotein.

Putative light glands

The bioluminescent reaction of tomopterids is thought to occur in rosette-like parapodial glands located in the fin-like appendages that border the dorsal and ventral rami (GREEFF 1882, 1885, DAHLGREN 1916, HACHFELD 1926, MEYER 1926, 1930, BONHOMME 1954, TERIO 1964). These glands, which may be of two types, rosette *stricto sensu* or hyaline glands, are typically formed by a yellow-pigmented core structure surrounded by a layer of transparent large cells. They are considered structurally homologous despite the fact that they divide the *Tomopteris* into two subgenera (*Johnstonella* and *Tomopteris* respectively) (ROSA 1908, DALES 1955). Although interspecific differences regarding the repartition of these glands [**Fig. 2a-d**] and the intra- or extracellular nature of the emission have been noted (DALES 1971), the functional role of each structural element composing this rosette-shaped organ in the light production has never been investigated.

T. planktonis is a *Tomopteris stricto sensu* carrying one hyaline gland at the end of the ventral pinnule [**Fig. 2a**, right half]. Tissues have been examined by epifluorescence microscopy (excitation band-pass filter 420-490 nm, emission long-pass filter 515 nm) [**Fig. 3**]. The light excitation in the blue range revealed a strong yellow autofluorescence in stimulated tissues only (here KCl stimulation). The other parts of the parapodia did not show any fluorescence. The most intense fluorescent signal is located in some sort of grains densely packed inside a yellow-pigmented core structure [**Fig. 3c**]. In a lesser density, these grains are also to be found scattered in a fluid spread from the centre of the rosette-like glands in the direction of the tip of the ramus [**Fig. 3a,d**]. These observations may illustrate several stages in the emission process. Thus, in *T. septentrionalis* and *T. pacifica*, the light spots found on the lumigrams [**Fig. 2b-c**] varied much in size, but many of them were smaller than 20 µm in diameter, which is an order of magnitude smaller than the overall size of the rosette-like glands. We interpret this size difference as an indication of light being emitted from grains in, or secreted from the yellow-pigmented bodies, rather than from the transparent sacculi which constitute the peripheral part of the hyaline glands. However, it should be noted that, since the fluorescence appears in response to bioluminescent emission, it is due to a breakdown product rather than to the luminescent material itself. The presence of similar granular structures has been noted in other bioluminescent organisms (*e.g.* the anthozoa *Renilla*, the tunicate *Pyrosoma*, the snail *Dakia*) (SWEENEY 1980). Some small fluorescent vesicles have also been observed in epidermal cells of the bioluminescent polychaete *Odontosyllis phosphorea* (Syllidae) (HARVEY 1952). The organelles were found to coalesce then burst to the surface of the cells, releasing fluorescent mucus (DEHEYN AND LATZ 2009). In *T. planktonis*, as the grains are found outside the yellow-pigmented core structure, they could corroborate the

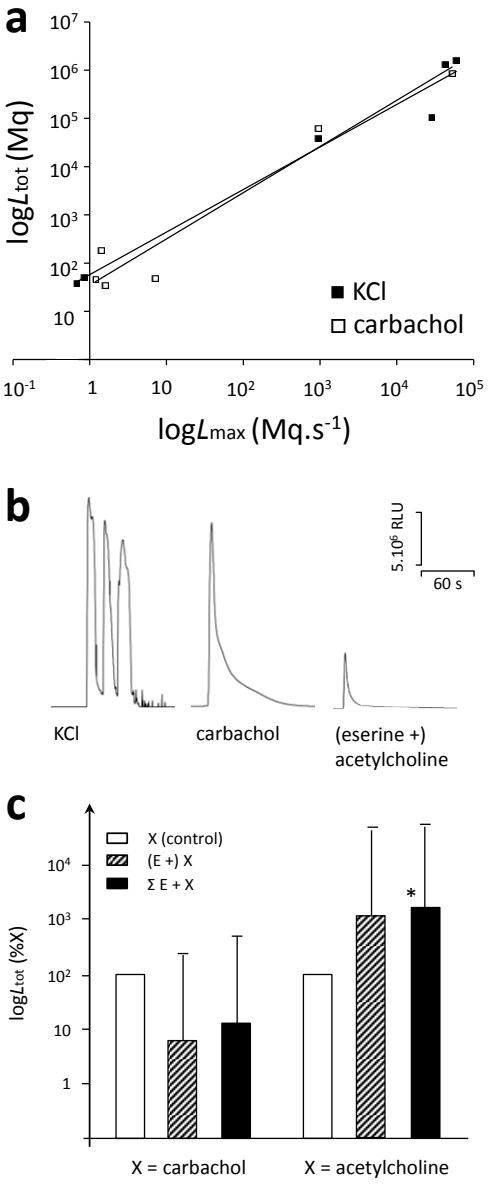

Fig. 4. Physiological control of bioluminescence by *Tomopteris planktonis*.
(a) Linear relationship between the total quantity of light emitted (L_{tot}) and the maximal light intensity (L_{max}) for 200 mM KCl- and 1 mM carbachol-induced luminescence. 1 megaquanta (Mq) = 10^6 photons. R^2-values are 0.971 and 0.944, respectively, and the slopes (P = 0.4696) and intercepts (P = 0.5083) are significantly equal (ANCOVA); **(b)** Light emission profiles induced by bath application of 200 mM KCl, 1 mM carbachol and 1 mM acetylcholine preceded by a 1 mM eserine treatment. (RLU: relative light unit); **(c)** Activatory effect of the 1 mM eserine pretreatment (E+) on 1 mM carbachol- and acetylcholine-induced light emissions (L_{tot} = total emitted light, mean ± s.e.m., n = 6, Wilcoxon test, * $P < 0.05$).

hypothesis of an extraglandular light emission. However, the absence of fluorescent residue in the coelomic cavity does not support the hypothesis of intracoelomic emission as suggested by Meyer (1929) in *T. helgolandica*. Preferably, the apical position of the hyaline glands in *T. planktonis* suggests the clearance of bioluminescent material directly out of the body. Such bioluminescent display has been observed in the other hyaline gland bearing species *T. carpenteri* (by J.M., *pers. obs*.) and in some unidentified species (FRANCIS *ET AL.* 2014). Yet, the photograph of the bioluminescence pattern of *T. planktonis* remains inconclusive as its quality may have been altered by the movements of the animal.

Since the photogenic structures observed in annelids are believed to derive from pre-existing epithelio-muscular and mucus cells (ANCTIL 1987), structural and functional parallels are probably to be made among the photogenic secretory structures of tomopterids and those of other bioluminescent annelids (DEHEYN AND LATZ 2009, DEHEYN *ET AL.* 2013, BRANCHINI *ET AL.* 2016). However, the diversity of secretory tissues and products, both in tomopterids' parapodia and in annelids in general (HAUSEN 2015), makes comparisons between taxa as distant as the Syllidae or Chaetopteridae rather hazardous ones. In order to clarify the specific issue of the emission dynamics in the Tomopteridae, a comparative descriptive and functional morphological study must be implemented. This work in currently in progress.

Cholinergic nervous control

The involvement of cholinergic mechanisms in the control of the bioluminescence of annelids is relatively well established. It is known especially in some polynoids and chaetopterids (GARDNER AND WALKER 1982, ANCTIL 1987) and in *T. helgolandica* (GOUVENEAUX AND MALLEFET 2013). Therefore, following the experimental design used by Gouveneaux and Mallefet (2013), a possible cholinergic activity has been investigated in *T. planktonis*. Because of the linear relationship between the total emitted light (L_{tot}) and the maximal light intensity (L_{max}) of the control light responses [**Fig. 4a**], only L_{tot} values are presented. As shown in the figure 4b, [(I)] the carbachol (non-hydrolysable cholinergic agonist) induces a luminous response comparable to the one emitted under KCl stimulation (maximal light intensity: L_{max} = 300-20,000 $Mq.s^{-1}$ per pair of parapodia; 1 Mq = 1 Megaquanta = 10^6 photons), whereas [(II)] the acetylcholine, even applied after eserine pretreatment (cholinesterase inhibitor), induces only 35% of the total quantity of light emitted by KCl on average [**Fig. 4b**]. However, eserine induces a weak luminous response and increases significantly the effect of acetylcholine [**Fig. 4c**]. The emission induced by carbachol is not modified by eserine. Similarly to what was described in *T. helgolandica* (GOUVENEAUX AND MALLEFET 2013), a cholinergic pathway seems to be involved in the control of bioluminescence for *T. planktonis* as well.

Conclusion

The exceptional access to several species of the genus *Tomopteris* enabled us to establish a first comparative analysis of bioluminescence within this group.

According to these new results, similarities do exist between bioluminescent tomopterids at morphological, physiological and, possibly, biochemical levels. However, the yellow luminescence was inappropriately attributed to all tomopterids, which actually exhibit a significant diversity of emission patterns. The observation of an unidentified small, tailless, blue emitting tomopterid from the Gulf of California was also recently mentioned by Haddock and co-workers (*pers. comm.* to A.G.). The fact that sympatric *T. helgolandica* and *T. planktonis* produce different bioluminescent colour signals in the same optical environment supports the hypothesis of specific recognition signals. Alternatively, these signals could fulfil different functions. For instance, the blue bioluminescence of *T. planktonis* could act as a deterrent signal, as suggested in numerous pelagic gelatinous invertebrates (*e.g.* HADDOCK AND CASE 1999). However, without behavioural observations and an extensive study of their visual spectral sensitivity, that remains purely hypothetical.

From an evolutionary perspective, the biochemical system enabling most of the known bioluminescent *Tomopteris* to produce a yellow light may have derived from a common blue light emitting system. After Shagin and colleagues (2004), the evolution history of cnidarian GFP-like would have been punctuated by 15 colour diversification events. According to this hypothesis, spectral divergence would have appeared among the Tomopteridae while they shared similar photogenic structures and control mechanisms. Finally, the observation of yellow light emission from unidentified small tailless tomopterids from other fjords of western Norway (FLOOD P.R., *pers. obs.*) suggests the presence of another undescribed species in these waters. Undoubtedly, these results emphasise the need for further biochemical investigations and the taxonomical revision of the Tomopteridae.

Symbols and Abbreviations

λ_{max}	maximal emission wavelength
$MgCl_2$	magnesium chloride
KCl	potassium chloride
DSLR	Digital Standard-Lens Reflex
R^2	coefficient of determination
s.e.m.	standard error of the mean
ANCOVA	ANalysis of COVAriance
RGB	Red, Green, Blue
LED	Light emitting Diode
FWHM	Full Width at Half Maximum intensity
BRET	Bioluminescence Resonance Energy Transfer
GFP	Green Fluorescent Protein
L_{tot}	total emitted light
L_{max}	maximal light intensity
Mq	Megaquanta
RLU	Relative Light Unit

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Author contributions

A.G., P.R.F. and J.M. designed the experiments, collected the samples and data. A.G. performed the analysis and wrote the manuscript. J.M. and P.R.F. revised the manuscript.

Tab. SI. (next pages) Diagnostic features used for identification of the studied tomopterid worms.

Depending on the sampling location, various keys were used and compared. Our specimens showed good agreement with every point reported in this table. Their distribution is usually not restricted to the areas mentioned which only confirm the presence of each species where they were collected.

subgenera	*Johnstonella*	
species	*T. helgolandica*	*T. pacifica*
length	40-50 mm	30-40 mm
number of parapodia	18-21 pairs	21-25 pairs
1st cetigerous appendage	absent	present (absent or broken off in large specimens)
2nd cetigerous appendage	up to 3/4 of the body length	almost as long as the body
rosette glands	at the tips of the rami of the 3rd and following pairs of parapodia + on the trunks of the first two pairs of parapodia (indictinct)	at the tips of the rami of the 3rd and following pairs of parapodia + on the trunks of the first two pairs of parapodia (indictinct)
hyaline glands	absent	absent
chromophile glands	on the ventral pinnea, inferior to the ramus	on the ventral pinnae, in the 3rd and following pairs of parapodia
gonads	in both notopodia and neuropodia	in both notopodia and neuropodia
tail	long with rudimentary parapodia	long and slender, devoid of parapodia, orange in colour
distribution	North Atlantic	North Pacific
references	Fauvel 1923, Muus 1953, Dales 1957, Ušakov 1972, Pleijel and Dales 1991	Dales 1955, 1957, Ušakov 1972

Tomopteris		
T. planktonis	*T. septentrionalis*	*T. carpenteri*
3-11 mm	10-30 mm	45-70 mm
13-18 pairs	22-24 pairs	29-37 pairs
absent	absent	absent
up to 3/4 of the body length	1/2 to 4/5 of the body length	up to 3/4 of the body length
absent	absent	absent
on the neuropodial pinnae, at the apex of the ventral ramus, in the 3rd and following pairs of parapodia	on the neuropodial pinnae, at the apex of the ventral ramus, in the 3rd and following pairs of parapodia	on the neuropodial pinnae, somewhat above and beyond the apex of the ventral ramus, in the 3rd and following pairs of parapodia, brown in colour
on the neuropodial pinnae, inferior and at the inner edge of the ramus, in the 4th and following pairs of parapodia	on the ventral pinnea, at the apex of the ramus, in the form of parallel glandular tubes (indistinct), in the 4th and following pairs of parapodia	on the ventral pinnae, below the apex of the ventral ramus, in the 4th and following pairs of parapodia
in the notopodia only	in the notopodia only	in both notopodia and neuropodia
absent	absent	absent
North Atlantic	North Pacific	Southern Ocean
Rosa 1906, Fauvel 1923, Muus 1953, Dales 1957, Ušakov 1972, Pleijel and Dales 1991	Rosa 1906, Fauvel 1923, Muus 1953, Dales 1957, Ušakov 1972, Pleijel and Dales 1991	Quatrefages 1866, Monro 1930, Hartman 1964

4.1.3. Additional results and discussion

4.1.3.1. Effects of mechanical, electrical and chemical stimulations

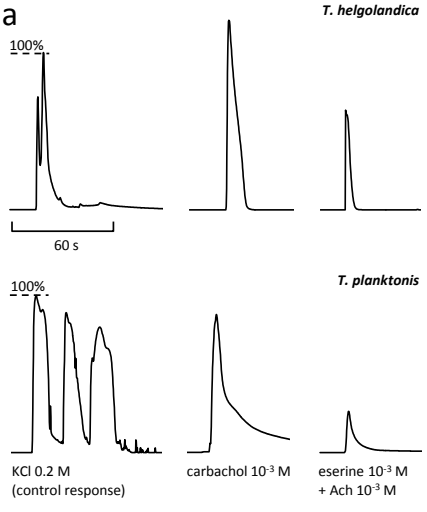
The best way to describe the natural bioluminescent display of an organism is probably to refer to spontaneous light emissions or, failing that, emissions induced by minimally invasive stimuli (*e.g.* mechanical, photic stimuli). Intact *T. helgolandica* specimens were found to emit microflashes of very short duration (< 2 s) when disturbed by squirting sea water (FLOOD. P.R.*, pers. comm.*). In response to rough handling (emersion, forceps), some individuals have also been observed to produce a light that persists up to seven seconds in all the parapodia simultaneously [**paper IV**]. Complete emersion of the animal was used by Francis W. (*pers. comm.*) in order to induce in some specimens the expulsion of a luminescent exudate and collect it. He also obtained a luminous response by gently agitating the animals in a tube (FRANCIS *ET AL.* 2014). However, this type of procedure, hardly repeatable and reproducible from distinct individuals or species, is of limited value when it comes to making comparisons. Indeed, quantifying and standardising the excitation strength for techniques used in the laboratory for mechanical stimulating luminescence (including pulling, pinching or shocking the specimen, agitating, stirring or bubbling the water) is quite difficult (LATZ *ET AL.* 1994). Moreover, we know that the stimulus intensity – regardless its nature – can significantly influence the kinetics and the type (intracellular/secreted) of light response (NICOL 1960, MARTIN AND ANCTIL 1984, DEHEYN AND LATZ 2009). Lastly, in addition to its variations between closely related species (*e.g.* in dinoflagellates: LATZ *ET AL.* 1994), the excitation threshold may be affected by the condition of the animals (NICOL 1960). Alternatively, electrical and chemical stimuli, although invasive to varying degrees (ZÖRNER AND FISCHER 2006), enable us to investigate a given luminescent pattern under controlled experimental conditions as well as the underlying nervous regulation mechanisms.

Electrical stimulations have been widely used on bioluminescent organisms including polychaetes (*e.g.* in Polynoidae: HERRERA 1979, BILBAUT 1980; Chaetopteridae: NICOL 1952, MARTIN AND ANCTIL 1984; Syllidae: ZÖRNER AND FISCHER 2006). The annelid body plan being typically characterised by a metamerisation and a more or less pronounced regionation, the spatial pattern of bioluminescence can be strongly constrained by these anatomical subdivisions. This is particularly true for *Chaetopterus variopedatus* in which the responsiveness of the various regions depends on the intensity and frequency of electrical pulses (MARTIN AND ANCTIL 1984). In the syllid *Eusyllis blomstrandi*, the light response spreads along the worm from the stimulated zone by the recruitment of segments (ZÖRNER AND FISCHER 2007). Electrophysiological investigation would also be interesting to develop in a recently discovered terricolous oligochaete which was observed to produce light

from 1/2, 1/4 or 1/8 of segments (KOKEN M., *pers. comm.*). Concerning *T. helgolandica* and *T. carpenteri*, they both appeared to be responsive to 9V continuous current delivered through extracellular electrodes laid on the surface of their bodies [**paper II: Fig. 1A**]. However, in our experience and to our knowledge, the production of luminescent mucus by *T. carpenteri* (MALLEFET J., *pers. comm.*) has never been observed in *T. helgolandica*. We did not test the facilitation, summation, and fatigue effects to which most neurally controlled bioluminescent systems are subject (NICOL 1960, ANCTIL 1987).

Lastly, a variety of chemicals can be used to trigger light responses: hypotonic solution (diluted sea water, fresh water), detergent agents (triton X-100), salt solutions (chlorides of Na, K) or drugs (neurotransmitters, blockers/inhibitors, second messengers). However, their mode of action is more or less specific and sometimes unknown. KCl depolarisation, which is frequently used in various taxa (*e.g.* GERMAIN AND ANCTIL 1988, DEHEYN *ET AL.* 2000, 2013), induced in the *Tomopteris* we studied a monophasic response of maximal intensity which merely reflects the luminous potential of the stimulated animal [**Fig. 4.1a, paper I: Fig. 1A,C, paper II: Fig.2a-d**]. Despite the precautions taken in standardising the method (such as the recovery period of luminous capacities, or anaesthesia), we generally observed an important interindividual variability, which is not correlated with the size of the individuals. The drugs can be applied in pharmacological dose in order to induce a maximal effect or in physiological dose, which induces a less pronounced but more realistic response [**paper I: Fig. 3A**].

a



b

treatment	*T. helgolandica*	*T. planktonis*
potassium chloride	++	++
serotonin	0	Ø
epinephrine	0	Ø
GABA	0	Ø
nitric oxide	0	Ø
carbachol	++	++
DMPP	++	Ø
ACh	+	+
eserine	±	±
tubocurarine	-	Ø
atropine	0	Ø
nifedipine	0	Ø
diltiazem	-	Ø
trifluoperazine	-	Ø
verapamil	-	Ø
calcium depletion	-	Ø
8br-cGMP	(+)	Ø
db-cAMP	(+)	Ø
forskolin	(+)	Ø
U73122	(+)	Ø
U73343	(+)	Ø

Ø: not tested, 0: no effect, - : inhibitory effect, + : activatory effect, ++ : light production, (·) results to be confirmed

Fig. 4.1. (previous page) Cholinergic control of bioluminescence in *T. helgolandica* and *T. planktonis*
(a) Typical time course of light emissions of parapodia from *T. helgolandica* and *T. planktonis* in response to KCl, carbachol and acetylcholine (after eserine pretreatment) stimulations; **(b)** Synthesis of the effects of various substances used to characterise the neural control of their light emissions. The propreties of each drug can be found in Fig. 3.4a.

4.1.3.2. Involvement of cholinergic control mechanisms

The figure 4.1 summarises and completes the pharmacological results presented in papers I and II. Two main effects were revealed in both *T. helgolandica* and *T. planktonis*: (1) 10^{-3} M carbachol induced a light response similar in intensity to KCl standard emission [**Fig. 4.1a**] and (2) the cholinesterase inhibitor (eserine) was found to significantly facilitate acetylcholine-induced light emission [**papers I and II: Figs. 2B**]. In *T. helgolandica*, the sensitivity to carbachol was confirmed by a dose-dependent response associated with a change in the time course of the emission pattern [**paper I: Fig. 2A**]. These results strongly support the hypothesis of a cholinergic activity involved in the control of bioluminescence for both species. This feature is shared by several bioluminescent annelids, especially the polynoids and chaetopterids (CASE AND STRAUSE 1978, GARDNER AND WALKER 1982, ANCTIL 1987). However, for them as for *Tomopteris*, the details of neuro-photocyte transmission are still unresolved. Moreover, our results do not exclude the implication of other regulation mechanisms, including antagonistic or synergic effects.

According to further experiments conducted on *T. helgolandica*, the receptors for the response to ACh appeared to be nicotinic (nAChR). Compared to muscarinic receptors, the nicotinic ion channels are known to be rapid-acting receptors which short-circuit the transduction cascade and possibly enhance the efficiency of a reflexive light response. The calcium-dependence of KCl-induced luminescence was also demonstrated, with specific involvement of L-type voltage-gated calcium channels [**paper I**]. The calcium requirement for light emission has already been evidenced in several taxa (*e.g.* in Cnidaria: SHIMOMURA 1995, Echimodermata: MALLEFET *ET AL.* 1998, DEWAEL AND MALLEFET 2002b, Annelida: ANCTIL 1987, RODIONOVA *ET AL.* 2002) and can be effective at various levels. In the photoprotein systems of some cnidarians (*Aequorea*, *Renilla*, *Obelia*) and ctenophores (*Mnemiopsis*, *Beroë*), Ca^{2+} ions act as reactional cofactors (HASTINGS AND MORIN 1969, SHIMOMURA 1995, 2006) [**Fig. 1.4**]. Sensitive to Ca^{2+} concentration, these proteins constitute one of the most valuable probes for detection of intracellular calcium (BLINK 1990). Other pathways of calcium mobilisation during light emission may include high-affinity transduction processes mediated by transient variations of intracellular Ca^{2+} concentrations (in this case, it is referred to as second messenger) or the stimulation of neurotransmitters' exocytose from the

presynaptic neurons (ELDEFRAWI AND ELDEFRAWI 1988). The voltage-gated calcium channels form a family of channels known as key transducers of cell surface depolarisation into intracellular events (CATTERALL *ET AL.* 2005). L-type Ca^{2+} currents typically require a strong membrane depolarisation for activation but are usually long-lasting. While only nAChRs would have relayed the signal produced by low concentrations of carbachol, thus producing trains of flashes [**paper I, Fig. 2A**], the L-type Ca^{2+}-channels probably reached their threshold of excitability in response to higher concentrations which amplified and altered the emission pattern (glowing response, > 5 s). Amongst the cellular functions of these channels may be found excitation-contraction coupling and neurotransmitter release from sensory cells (including photoreceptors). In the studied system, they might be part of a signalling pathway initiated from mechanoreceptors (the bioluminescence being mechanically stimulable, see *supra*, p. 95 and paper IV) or photoreceptors (*infra*, p. 164).

Finally, according to a screening of several other second messengers [**Fig 3.1b**], 8br-cGMP, db-cAMP, and forskolin had no effect on KCl-light emission while they significantly increased the total quantity of light emitted ($P < 0.01$). Bioluminescence activity would therefore be triggered by protein phosphorylation under the mediation of cGMP- and cAMP-dependent protein kinases. Calcium-calmodulin-dependent protein kinase could also be involved in this pathway. The phospholipase C inhibitor (U73122) also enhanced KCl-induced luminescence (L_{tot} only, $P < 0.01$), but this effect seems to be unspecific since U73343, the inactive analogue of U73122, equally activated the quantity of light emitted ($P < 0.05$). For rigorous assessment of these transduction pathways, additional tests should include supplementary drugs producing opposite effects (*e.g.* guanylyl cyclase activator, adenylyl cyclase inhibitor, phosphodiesterase inhibitor) and various concentrations (in order to evidence possible dose-dependent responses). Due to the low number of specimens available, most of the drugs tested on *T. helgolandica* have not been tested on *T. planktonis*.

In addition, the pharmacological results provided useful tools to develop a morphological approach of the nervous control of bioluminescence, which is complementary to the first one. Immunohistochemical stainings have been performed in collaboration with the Department of Biological and Environmental Sciences from the University of Gothenburg (Sweden). Some micrographs are presented in the paper III [**Fig. 5**] and in the section 4.2.2.3 as supplementary results.

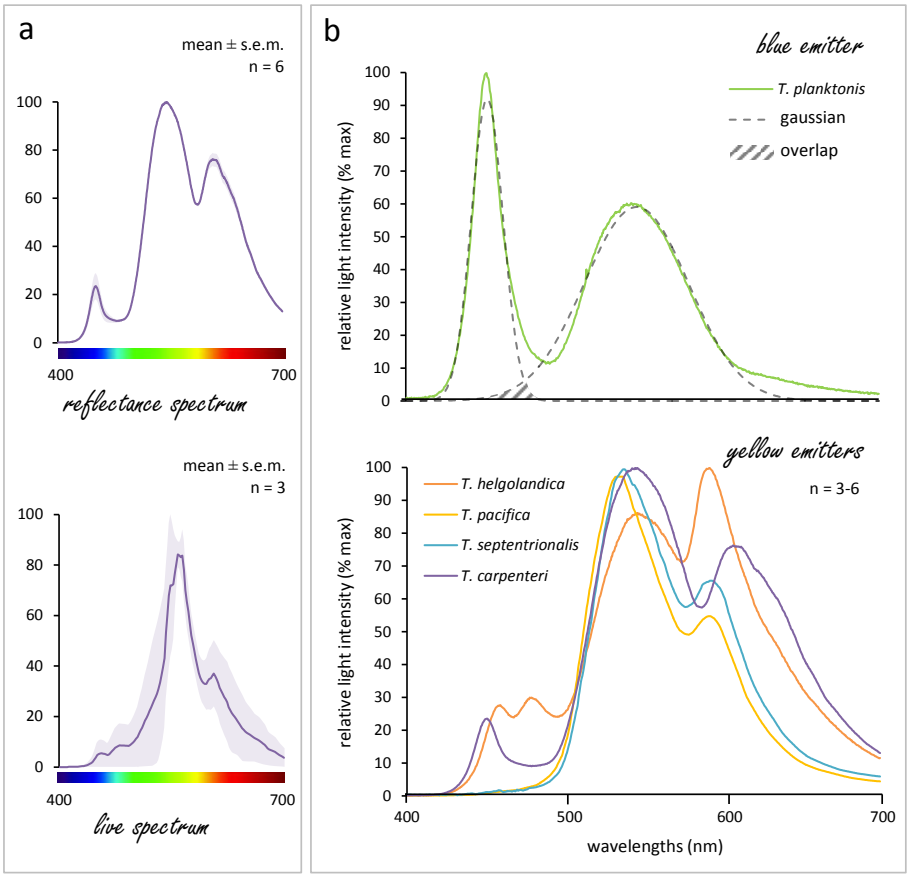


Fig. 4.2. Emission spectra of five Tomopteris species.
(a) Comparison between reflectance and live spectra in *T. carpenteri* [**see paper II for methodological details**]. Both are similar including the secondary peaks; **(b)** Reflectance spectra of *T. planktonis* (double gaussian fitting), *T. helgolandica*, *T. pacifica*, *T. septentrionalis* and *T. carpenteri*. Each curve is the average of 3-6 replicates.

4.1.3.3. Unexpected diversity of light emission patterns within the Tomopteridae

Although *T. planktonis* specimens were about five times smaller in size than *T. helgolandica* [**Fig. 3.3a,d**], their light glands were of comparable size [**paper III**] and *T. planktonis* produced higher light intensities. This was probably not due to the physiological condition of the worms, which have always been captured, treated and tested under the same conditions. However, this is neither the only nor the most remarkable difference between their bioluminescent displays. *T. helgolandica* is a yellow emitter (λ_{max} = 573 nm, FMHW = 130 nm), like most bioluminescent

Tomopteris spp. observed until now (λ_{max} = 565 nm in *T. nisseni* (LATZ *ET AL.* 1988), λ_{max} = 565 nm in *Tomopteris spp.* (FRANCIS *ET AL.* 2014)), but *T. planktonis* was found to be a blue emitter (λ_{max} = 450 nm, FMHW = 23 nm) [**paper II**]. This is common as a marine and pelagic organism but also unusual in Tomopteridae (HERRING 1983, WIDDER *ET AL.* 1983, HASTINGS 1996). Consequently, we suggested that the yellow light emitting biochemistry and structures might have derived from an original blue emitting system. This hypothesis is mentioned but not developed in the paper II since the multimodal reflectance spectra suggesting the involvement of a secondary fluorescent emitter (BRET) should be confirmed by live measurements. Indeed, although reflectance spectra were compared to live spectra for several species (*T. helgolandica*, *T. carpenteri*) [**paper II: Fig. 2; Fig. 4.2a**], the reliability of secondary peaks [**Fig. 4.2b**] and their matching between species should be investigated further. In the dragonfish *Malacosteus*, spectral and morphological evidence suggests that its red emitting suborbital photophore derived from the blue emitting postorbital photophore (HERRING AND COPE 2005). The same bioluminescence chemistry is believed to produce the blue and the red light emissions by filtering and energy transfer to a red fluorescent protein (WIDDER *ET AL.* 1984). Although the rosette glands of *T. planktonis* demonstrated yellow fluorescent properties [**paper II: Fig. 4**], the energetic transfer might be partial as show by the secondary peak around 544 nm [**Fig. 4.2b**] but not sufficient to trigger BRET effect. It may be that one of the prerequisites is not fulfilled: theoretically, BRET is achieved only if the emission spectrum of the donor molecule and the absorption spectrum of the acceptor molecule overlap sufficiently and if the distance between these molecules ranges between 10 and 100 Å (MORIN AND HASTINGS 1971, BOUTE *ET AL.* 2002). If only a partial energy transfer occurs in *T. planktonis*, this may also results from a defect related to stability, concentration or distribution of the fluorescent protein.

This newly evidenced spectral diversity invites us to revise the generally accepted hypothesis of intraspecific communication. Given that *T. helgolandica* and *T. planktonis* live in the same environment, species-specific bioluminescent colours may facilitate recognition and communication between individuals of the same species. Alternatively, these emission patterns may provide distinct ecological advantages: an intense blue signal may be associated with a defensive behaviour against a wide range of visual predators while a less intense yellow signal is more likely to be adapted to attract conspecifics (assuming that they possess yellow sensitive visual pigments). Nevertheless, one cannot exclude a dual function for light production, even for a single species. That specific question will be addressed in the third results section [**4.3**] in light of behavioural observations of *T. helgolandica*. Further, to investigate the spectral sensitivity of tomopterids and their predators remains essential.

Finally, although most of the *Tomopteris* observed by Francis and colleagues (2014) have been found to release glowing material from their parapodia into the water, only one of our five species, *T. carpenteri*, was observed to do so. This specific issue will be developed in the next section [**4.2**].

In conclusion, the differences observed in terms of intensity, frequency, spectral distribution and expression dynamics (internal *versus* secreted light production) probably reflect an underestimated diversity of patterns in tomopterids and underline the importance of taking interspecific differences into consideration.

4.2. Morphology and fluorescence of light glands in *T. helgolandica* and allies

One of the key steps in the study of bioluminescence is to explore the morphology and organisation of the photogenic structures. The identification of these structures, their description and the study of changes in both morphological and fluorescent characteristics operated during the light emission process are three lines of research that could be particularly informative about the emission dynamics (DEHEYN *ET AL.* 2000, RENWART *ET AL.* 2014). Indeed, apart from eye-like photophores showing a typical pigment cup-photocyte-lens sequential structure [**Fig. 1.7a**], the identity of less simple photogenic structures can only be confirmed by a thorough investigation.

As already mentioned and reiterated in the paper III, the bioluminescent properties of parapodial rosette glands are, since the works of Greeff (1882, 1885), widely acknowledged, despite the fact that he did not mention any conclusive microscopic observation of fluorescence or bioluminescence. Thus, in five *Tomopteris* species, we aimed to describe and compare the histology of four types of parapodial glands (rosette, hyaline, chromophile and tubular) with emphasis on the putative light organs. In whole mounted parapodia isolated from *T. helgolandica*, we also analysed the distribution and temporal dynamics of their endogenous fluorescence (common proxy of bioluminescence) in response to KCl- and carbachol-induced light emissions.

4.2.1. Morphology and fluorescence of the parapodial light glands in *Tomopteris helgolandica* and allies (Phyllodocida: Tomopteridae) (Paper III)

(Submitted)

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Abstract

The histology of putative light organs in the parapodia of five species of *Tomopteris* (pelagic annelids) is examined and compared using light, epifluorescence and scanning electron microscopy. The structural homology of rosette glands in the parapodial pinnae of the tail-bearing species *T. helgolandica* and *T. pacifica*, and hyaline glands of the tail-less species *T. carpenteri*, *T. planktonis* and *T. septentrionalis* is highlighted. However, the rosette glands point towards the ramus of the coelomic cavity inside the parapodia, whereas the hyaline glands point towards the surrounding water and penetrate the pinnal surface on the posterior side of the parapodia. Further, in order to assess the photogenic properties of rosette glands from *T. helgolandica*, we analysed the distribution and the temporal dynamics of their endogenous fluorescence in isolated parapodia in response to light emission induced by KCl and carbachol. The gradual extinction of bioluminescence was combined to the centrifugal spread of fluorescence from the core of the gland. We suggest this fluorescence to be produced by a breakdown product of the chemiluminescent reaction. Finally, both gland types probably evolved from a common light-emitting structure and differentiated along a functional and migrational axis extending from endocrine secretion close to the coelomic ramus to exocrine secretion close to the lateral margin of the pinna.

Introduction

The yellow bioluminescence displayed by some tomopterid annelids is well known since it is believed to act as an intraspecific communication signal (DALES 1971, GOUVENEAUX AND MALLEFET 2013, FRANCIS *ET AL.* 2014). However, many aspects of this unusual light emission remain poorly documented. Being strictly holoplanktonic, these annelids have a worldwide distribution in ocean waters from the surface down to 5,000 m deep (OBIS 2016). To date, fourteen species among 53 described ones, have been reported as bioluminescent (DALES AND PETER 1972, POUPIN *ET AL.* 1999, WORMS 2016, GOUVENEAUX *ET AL.*, in press). As reviewed by Harvey (1952), the yellow-pigmented rosette-shaped organs of these annelids have been described in 1847 by Busch in *Tomopteris onisciformis*, subsequently identified as *T. helgolandica* (GREEFF 1879). Although situated on the parapodia, these organs were first proposed as visual organs by Vejdowsky (1878). It was in the extensive work of Greeff on *T. rolasi* (GREEFF 1882) and *T. mariana* (GREEFF 1885) that their glandular and bioluminescent properties were suggested. However, neither Greeff nor any of the authors who succeeded him (*e.g.* DAHLGREN 1916, HACHFELD 1926, MEYER 1926, 1930, BONHOMME 1954, TERIO 1960, 1964), have demonstrated these light-emitting properties. Indeed, already Malaquin and Carin in 1922 rightly pointed out the lack of microscopic observations explicitly showing the bioluminescence of these organs. In spite of this, bioluminescent properties are currently attributed to three types of parapodial gland: the rosette glands I and II, and the hyaline glands (HARVEY 1952, DALES 1971). The distinction between the rosette- and hyaline glands is sometimes difficult (ROSA 1908, BONHOMME 1954), which prompted some authors to consider these organs as structurally homologous (MALAQUIN ET CARIN 1922). However, no detailed morphological comparison has been conducted to date, nor have the bioluminescent properties of these glands been demonstrated which is the reason why the present comparative morpho-functional study was undertaken.

In this work, the histology of putative light organs in the parapodia of five bioluminescent tomopterids including the two tail-bearing species *T. helgolandica* and *T. pacifica* (IZUKA 1914) and the three tail-less species *T. planktonis* (APSTEIN 1900), *T. septentrionalis* (STEENSTRUP 1850) and *T. carpenteri* (QUATREFAGES 1866) is compared for the first time. In order to assess the photogenic properties of rosette glands, we also analysed the distribution and temporal dynamics of their endogenous fluorescence (common proxy of bioluminescence) in isolated parapodia from *T. helgolandica* in response to light emission induced by potassium chloride (KCl) and carbachol.

The overall aim of this dual approach, both structural and functional, is to provide new insight on the light emission process and the evolution of light organs within the tomopterids.

species	location	depth	period
T. helgolandica	Korsfjorden (N60°16'15", E5°09'59") Raunefjorden (N60°16'02", E5°08'13") Herdlefjorden (N60°32'60", E5°01'30") Sognefjorden (N61°04', E5°22'), Western Norway	400-0 m	1995-2014
T. pacifica	San Juan Island, Washington state, USA from the docks of Friday Harbor Laboratories	surface	Jan. 1996
	Monterey Bay (N36°35'59", W122°09'02"), California, USA	540 m	Oct. 2012
T. septentrionalis	San Juan Island, Washington state, USA from the docks of Friday Harbor Laboratories	surface	Jan. 1996
T. planktonis	Raunefjorden (N60°15'51", E5°08'28") Herdlefjorden (N60°32'60", E5°01'30"), Western Norway	300-0m	Nov. 2014
T. carpenteri	Kerguelen islands (S49°53'72", E76°13'13"), French Southern and Antarctic Lands	125m	Jan. 2014

Table 1. Sampling locations and depths for each studied species.

Material and methods

Specimen collection and maintenance

This work results from the sampling of biological material and results of several campaigns [**Table 1**]. All specimens were kept in a permanent dark cold room (8°C) in aquariums of standing, aerated sea water and were not fed during their captivity. The experimental protocols allowed recovery for minimum 24h prior to fixation or testing, a period reportedly sufficient to allow substantial recovery of luminescence potential in many planktonic organisms (LATZ *ET AL.* 1990, BOWLBY *ET AL.* 1991).

The various observation- and histochemical methods were implemented according to the available biological material for each studied species [**Table 2**]. Each observation was made on a minimum of three specimens, unless specified otherwise.

General histology

Whole mounts

Given the transparency and thinness of pinnal tissues, the distribution and morphology of the various parapodial glands could be described from whole mounted material. Individual parapodia were isolated from unfixed, newly caught specimens, mounted on microscope slides and photographed in dark field illumination under a dissecting or compound microscope. Similar wholemounts from specimens fixed for some hours to several years in 4% formaldehyde and 1% glutaraldehyde (Merck, Darmstadt, Germany) in 0,5% sodium borate and 60% filtered seawater were examined in dark- or bright field illumination, unstained or after staining in a 0,1% solution of Janus green B (Sigma-Aldrich Company, St Louis, MO, USA) in distilled water.

Histological sections

Examination of histological sections was done for *T. helgolandica* and *T. septentrionalis* only. Most specimens were fixed for 2 to 4h in ice-cold 2.5% glutaraldehyde in 40 mM sodium cacodylate buffer and 60% filtered seawater. Later they were rinsed in several changes of isotonic buffer and post-fixed in 1% osmium tetroxide (OsO_4) in isotonic buffer for 2 hours before they were rinsed in distilled water and dehydrated in 70, 90 and 96% ethanol. After equilibration in 100% propylene oxide embedding took place in epoxy resin (Agar Scientific Ltd, Stansted, Essex, UK). Other newly caught specimens were immersed for two hours in ice-cold 4% OsO_4 dissolved in chloroform, followed by several rinses in 1 M sucrose before dehydration and embedding as mentioned above.

To ease orientation of the tissue, isolated pinnae were embedded flat on microscope slides under acetate cover slips. After polymerisation, the acetate film was stripped off and blank epoxy resin blocks glued on top of selected pinnae in the desired orientation. Later, these were detached from the glass slide by gentle heating and trimmed for sectioning at 0,5 µm thickness on a Reichert ultramicrotome. The sections were stained with 1% toluidine blue in borate buffer and examined in a Nikon Labophot microscope.

Scanning electron microscopy

For SEM *T. helgolandica* and *T. planktonis* were aldehyde fixed, postosmicated and dehydrated as mentioned above. Later they were critical point dried from liquid CO_2 (COHEN 1974). Individual parapodia and pinnae were mounted on specimen supports. Some parapodia were also mounted between two layers of adhesive tape, which were later pulled apart to cause dry fracturing (FLOOD 1975) to expose the ramus of the coelomic cavity inside the pinna. The specimens were subsequently sputter-coated by gold-palladium 60-40% alloy, mounted on specimen supports and examined in a Zeiss Supra 55VP field emission SEM operated at 5 kV with an "in-lens" secondary electron detector.

Immunohistochemistry

Antibodies against acetylated α-tubulin are typically used to label microtubule-rich neuronal structures (MÜLLER AND WESTHEIDE 2000, ORRHAGE AND MÜLLER 2005, MÜLLER 2006) but a variety of eukaryotic cells such as secretory- or ciliated cells show tubulinergic immunoreactivity (HELM AND CAPA 2015). Whole *T. helgolandica* specimens were fixed at 4°C for 18h in 4% paraformaldehyde in a 10 mM PBS (0.8% NaCl, 0.02% KCl, 0.14% Na_2HPO_4, 0.02% KH_2PO_4, pH 7.4), then stored at 4°C in PBS with 0.05% sodium azide (NaN_3) until use. *T. carpenteri* and *T. pacifica* specimens were fixed and stored for a few months in 4% formalin before analysed. To reduce non-specific staining, dissected parapodia were preincubated with normal donkey serum (10% in PBS with 2.0% NaCl, 0.2% Na_2HPO_4, 1.1% NaH_2PO_4, 0.2% NaN_3, 0.2% Triton X-100, 0.1% BSA, pH 7.2) for 30 min. Primary antibodies (acetylated α-tubulin T-6793, mouse anti-sea urchin, 1:1000, Sigma-Aldrich Company, St Louis,

tissues	observed feature	observation method	model species				
			Tomopteris helgolandica	*Tomopteris pacifica*	*Tomopteris planktonis*	*Tomopteris septentrionalis*	*Tomopteris carpenteri*
fresh material	structure (whole mounts)	light microscope	✓	✓	✓	✓	✓
	autofluorescence (whole mounts)	epifluorescence microscope	✓	×	✓*	×	×
		laser scanning microscope (cLSM)	✓	×	×	×	×
preserved material	structure (sections)	light microscope	✓	×	×	✓	×
	structure (dry fractures)	scanning electron microscope (SEM)	✓	×	×	×	×
	autofluorescence (whole mounts)	epifluorescence microscope	✓	×	✓	×	×
	immunofluorescence (whole mounts)		✓	✓ (n = 1)	×	×	✓

Table 2. Observation methods of structural features and luminescence displays in *Tomopteris helgolandica, T. planktonis, T. septentrionalis, T. carpenteri and T. pacifica*. *GOUVENEAUX *ET AL.*, in press

MO, USA) were applied and the preparations incubated overnight. They were rinsed (3 x 5 min in PBS with 2.0% NaCl) and incubated for 60 min with the secondary antibody conjugated with the fluorophore fluorescein isothiocyanate (FITC, donkey anti-mouse 715-095-150, 1:100, Jackson ImmunoResearch Laboratories, West Groce, PA, USA). The preparations were rinsed (3 x 10 min in PBS with 0.9% NaCl) and mounted in Vectashield antifade mounting medium (Vector Laboratories, Burlingame, CA, USA). All incubations were made in a humid chamber at room temperature.

The preparations were examined in an epifluorescent microscope (Leica Digital Modul R, excitation band-pass filter 450-490 nm, dichroic mirror 510 nm, emission high-pass filter 515 nm). Specificity of secondary antibody binding was controlled by treating the preparations as described above but omitting the primary antibody. It did reveal no non-specific staining of any of the secondary antibodies.

Patterns and dynamics of endogenous fluorescence

Specimens of *T. helgolandica* were relaxed (menthol 0.25-2.5 $g.l^{-1}$, 30 min) and parapodia were isolated and whole mounted in artificial sea water (ASW: 400.4 mM NaCl, 9.6 mM KCl, 52.3 mM $MgCl_2$, 9.9 mM $CaCl_2$, 27.7 mM Na_2SO_4, 20 mM Tris, final pH 8.3). Then the light emission was triggered by application of a buffered solution (either 200 mM KCl or 1 mM carbachol (Sigma-Aldrich Company, St Louis, MO, USA), pH 8.3) close to the sample using a non-metallic syringe needle (Microfil, World Precision Instruments, Sarasota, FL, USA) set between the two microscope slides. KCl is a depolarising agent maximising the luminescent reaction, and carbachol is a non-hydrolysable cholinergic agonist, both of which proved to trigger light emission in *T. helgolandica* (GOUVENEAUX AND MALLEFET 2013). The endogenous fluorescence of the preparation was simultaneously recorded (0.4 s of

light excitation and 1.93 s of acquisition per frame) using a confocal laser scanning microscope (Zeiss cLSM 710, excitation 488 and 514 nm, detection channels from 400 to 700 nm, maximal detection channel 536 nm) up to 10 min from the bioluminescence induction.

The light emission kinetics were measured from distinct parapodia with a luminometer (FB12, Berthold detection systems, Pforzheim, Germany) and are only indicative. Additional observations of fixed stimulated tissues were carried out using both epifluorescence- (Leitz diaplan, excitation band-pass filter 420-490 nm, dichroic mirror 510 nm, emission high-pass filter 515 nm) and cLSM.

Results

Morphology of the parapodial pinnal glands

As the name *Tomopteris* (Neo-Latin from Greek meaning "split wing") implies, each parapodium ends in a dorsal and ventral pinna or noto- and neuropodial pinna. Each of these contains a branch (ramus) of the coelomic cavity and a variety of pinnal glands embedded in their epithelium [**Fig. 1**]. We focused our attention on the putative bioluminescent pinnal glands, *i.e.* the rosettes (or rosettes II) which are present in both the noto- and neuropodial pinna of every parapodium of the

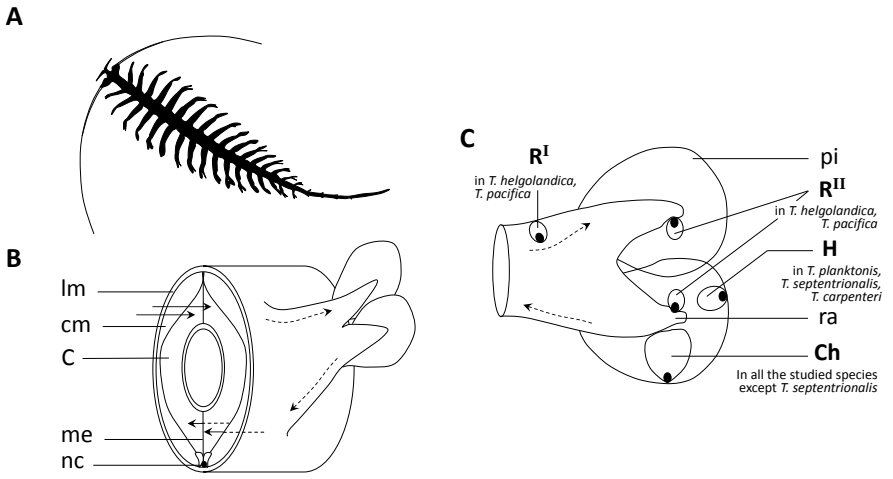


Fig. 1. General features of the tomopterid annelids.
(A) Shape of a *Tomopteris helgolandica* specimen. Their total length ranged from 1 cm to 6 cm; **(B)** Diagram of a trunk section (redrawn from Meyer, 1929a with permission). Arrows indicate the circulation of the coelomic fluid through the coelomic cavity; **(C)** Diagram of a biramous parapodium showing the distribution of the various pinnal glands according to the species. The apical pole of the gland is indicated by a black point. (C, coelomic cavity; Ch, chromophile gland; cm, circular muscles; H, hyaline gland; lm, longitudinal muscles; me, mesentery; nc, ventral nerve cord; nr: neuropodium; nt: notopodium; pi, pinna; ra, ramus; R^{I}, rosette gland I; R^{II}, rosette gland II).

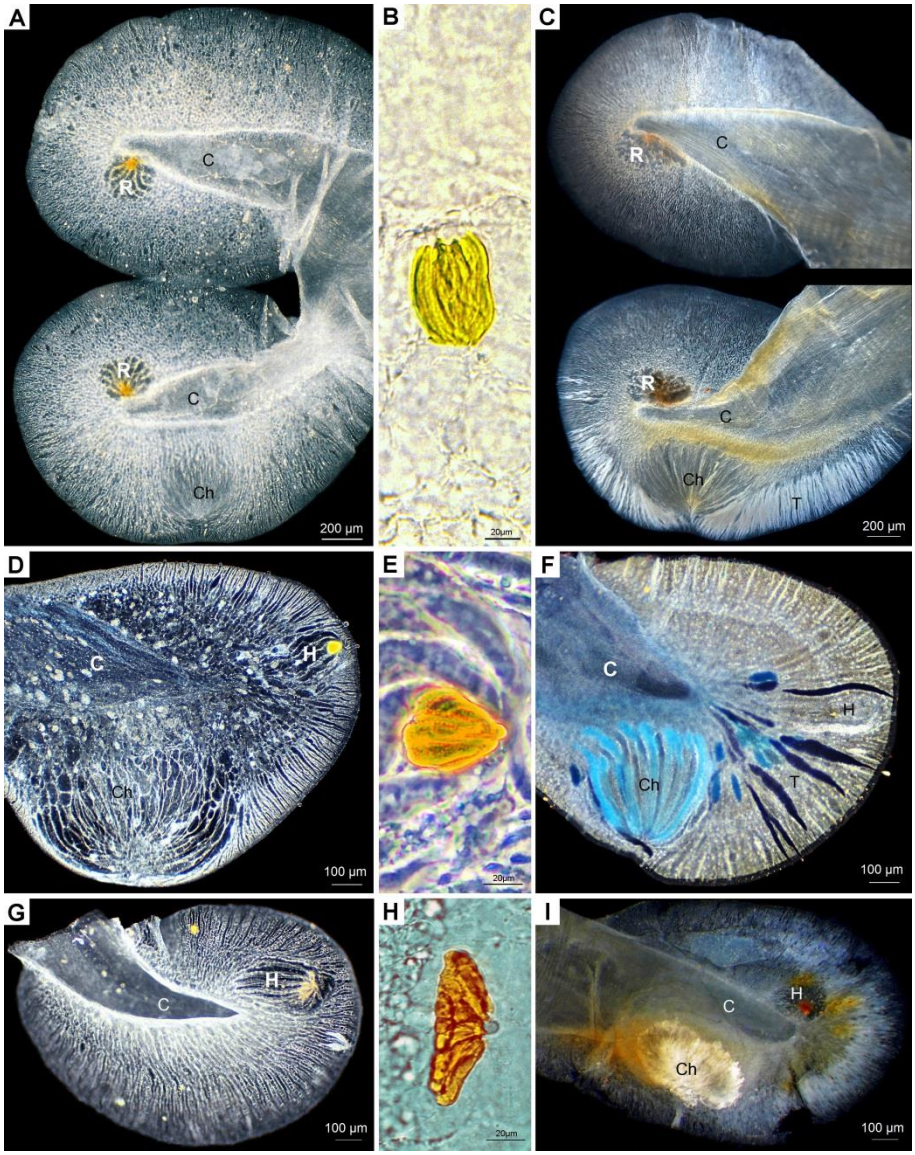


Fig. 2. Whole mounts of parapodial pinnae of the examined species.
(A) Fresh (unfixed) noto- and neuropodial pinnae of *Tomopteris helgolandica*; **(B)** Detail of the yellow-pigmented core structure of a notopodial rosette gland; **(C)** Glutaraldehyde-fixed noto- and neuropodial pinnae of *T. pacifica*; **(D)** Fresh neuropodial pinna of *T. planktonis*; **(E)** Detail of the pigmented core of the hyaline gland of the same specimen; **(F)** Formaldehyde-fixed and Janus green stained neuropodial pinna of *T. planktonis*; **(G)** Fresh neuropodial pinna of *T. septentrionalis*; **(H)** Detail of the pigmented core of the hyaline gland of a similar specimen; **(I)** Formaldehyde-fixed neuropodial pinna of *T. carpenteri*. (C, coelomic cavity; Ch, chromophile gland; H, hyaline gland; R, rosette gland; T, tubular glands).

tail-bearing species *T. helgolandica* and *T. pacifica*, and the hyaline glands which are present only in the neuropodial pinna of every parapodium of the tail-less species *T. planktonis*, *T. septentrionalis* and *T. carpenteri* [**Fig. 1C**]. In all species the posterior parapodia were usually less developed and carried smaller rosette- or hyaline glands. Nevertheless, light emission was induced even from these most posterior segments [**Fig. SI**].

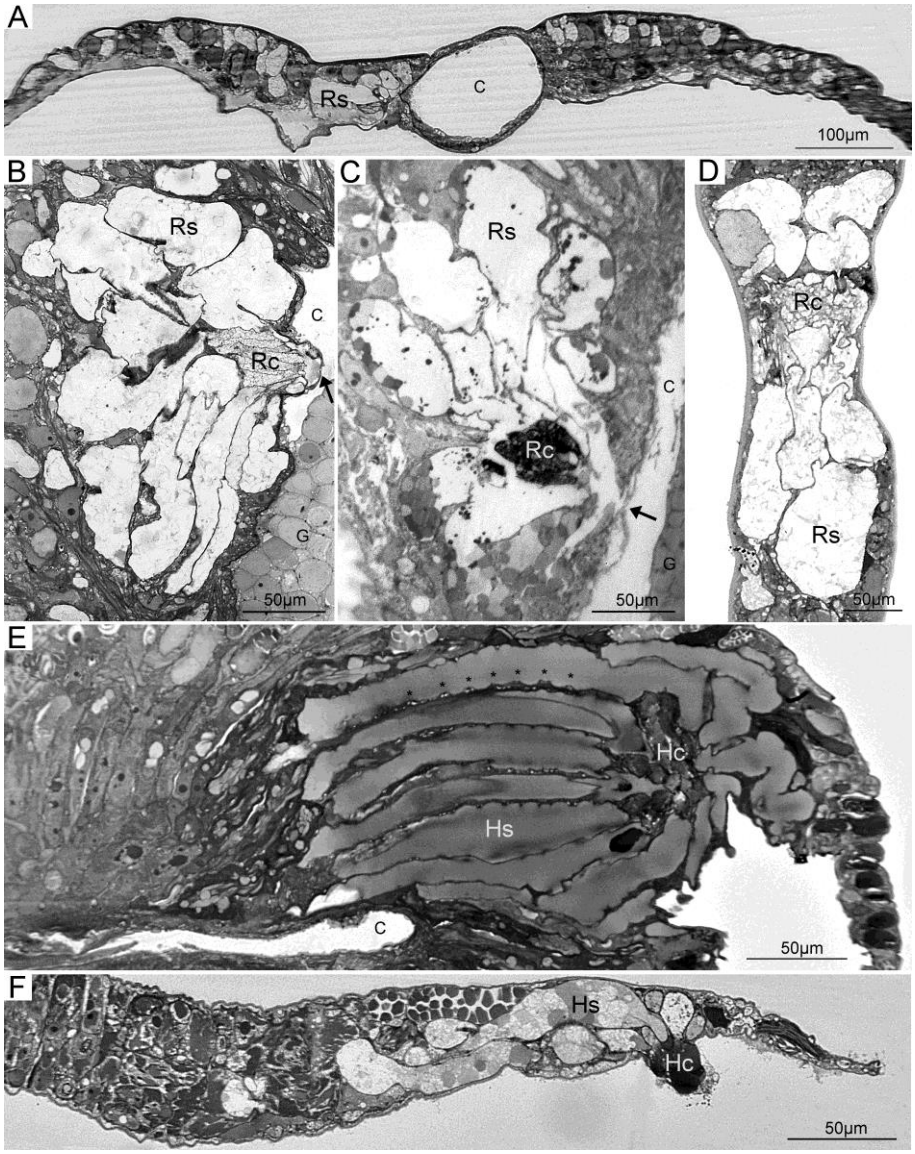
Morphology of the rosette glands in tail-bearing *Tomopteris*

In both *T. helgolandica* and *T. pacifica* each parapodium had two rosette glands. In the notopodial pinna the gland was located inferiorly (ventral) and slightly proximal to the apex of the coelomic ramus and in the neuropodial pinna in a comparable position on the top side (dorsal) of the ramus.

In the plane of the parapodial pinna, these glands were shaped like semicircular fans rather than radially symmetrical (circular) rosettes and occupied less than half the diameter of the pinna [**Fig. 2A, C, 3B, C**]. Their overall diameter ranged between 200 and 350 µm and were slightly larger in *T. pacifica* than in *T. helgolandica* [**Fig. 2A, C**].

In the antero-posterior diameter the rosette glands of *T. helgolandica* occupied the entire thickness of the pinna, except for a thin cuticular surface layer on both sides [**Fig. 3A, D**]. The center of each fan consisted of a yellow-pigmented core which in live material consisted of some 20 closely apposed, elongated, refractive bodies less than 10 µm wide and 50-80 µm long [**Fig. 2B**]. In glutaraldehyde fixed, post-osmicated, ethanol dehydrated and epoxy embedded and sectioned material, the yellow pigment was gone and the core structure consisted of some 20 irregularly rounded cytoplasmic profiles with a faint vesicular content [Rc in **Fig. 3B, D**]. In material fixed in 4% osmium tetroxide in chloroform, but otherwise treated in a similar way, the cytoplasmic profiles of the core structure contained granules of 1 to 3 µm diameter, heavily blackened by osmium [Rc in **Fig. 3C**].

Fig. 3. (next page) Semithin (0,5 µm) sections of epoxy resin embedded material stained by 1% toluidine blue in 0.2% sodium borate solution and examined in a compound light microscope operated in bright field mode. Fig. 3C from material fixed directly in 4% OsO4 in chloroform, the rest from material fixed in formaldehyde and glutaraldehyde in isotonic buffer, before osmication and embedding.
(A) Vertical transverse section of notopodial pinna of *Tomopteris helgolandica* at the level of the rosette gland; **(B)** Section through a rosette gland of *T. helgolandica*, parallel to the pinnal surface; **(C)** Similar section of material fixed in 4% osmium tetroxide in chloroform. Note the blackened core structure of the rosette gland (Rc); **(D)** Horizontal section through rosette gland of *T. helgolandica*; **(E)** Section through a hyaline gland of *T. septentrionalis* parallel to the pinnal surface. Note the presence of tiny profiles interposed between the glandular cells at fairly regular intervals (asterisks); **(F)** Horizontal section of the neuropodial pinna of *T. septentrionalis* at the level of the secretory pore of the hyaline gland. (C, coelomic cavity; G, gonads; Hc, core structure of the hyaline gland; Hs, sacculi of the hyaline gland; Rc, core structure of rosette gland; Rs, sacculi of the rosette gland) Arrows point to the thin coelomic lining facing the extracellular space next to the core structure of the rosette gland.



The cytoplasmic profiles of the glandular core were gathered in a cone-like structure which faced the lining of the coelomic ramus. However, even if this lining was exceedingly thin and the cone sometimes bulged into the coelomic cavity, the resolution achieved by our light microscopy did not allow us to positively identify any pore in the lining at this point [arrows in **Fig 3B, C**]. Rather, an extracellular space of variable size usually separated the apical glandular tissue from the coelomic lining and cavity [**Fig. 3B, C**].

The peripheral layer of the rosette gland consisted of transparent sacculi radiating from the yellow-pigmented core structure. Although transparent, faint

contours of large vesicles, which contained a finely granular substance, were present in these sacks in most embedded samples [Rs in **Fig. 3A-D**]. The number of saccular profiles observed in the peripheral region of most sectioned rosette glands ranged between 12 and 18 and was therefore comparable to the number of

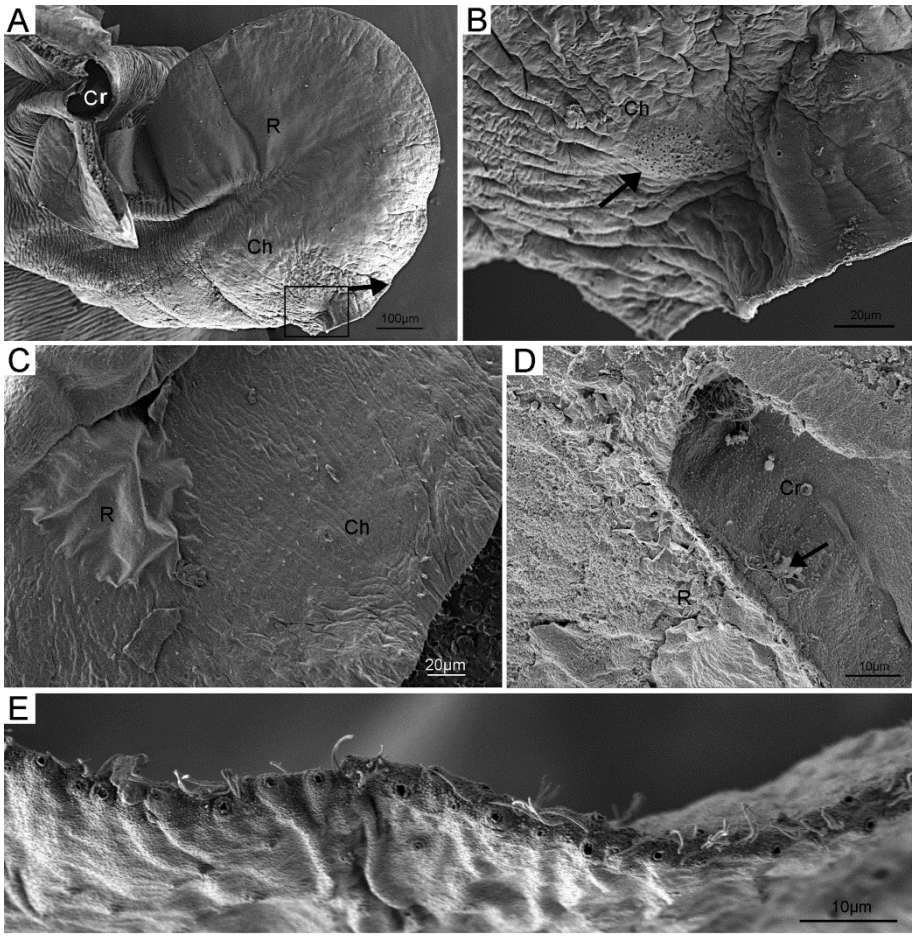


Fig. 4. Scanning electron micrographs of *Tomopteris helgolandica*.
(A) The 5th left parapodium showing the anterior surface of the neuropodial pinna with the position of the rosette gland and chromophile gland indicated by R, respectively Ch. Further, the notopodial pinna is fractured to reveal its coelomic ramus (Cr). The framed area is shown at higher magnification in fig 4B; **(B)** The chromophile gland ends in a cluster of secretory pores (arrow). Scattered pores of tubular mucus glands are also present on the remaining surface; **(C)** The posterior side of the neuropodial pinna reveals a smooth and pore-less cuticle covering the rosette gland; **(D)** A neuropodium dry-fractured to reveal its coelomic ramus (Cr). A ciliated pocket (arrow) is present where the underlying (hidden) rosette gland (R) is likely to face the lining; **(E)** The pinnal rim revealed groups of cilia and numerous secretory pores for the underlying mucus glands. (Cr, coelomic cavity; Ch, chromophile gland; R, rosette gland).

elongated pigmented bodies seen in the core structure. A cytoplasmic continuity between individual transparent sacculi and pigmented core bodies was indicated by a gradual transition in size and density of the vesicular profiles/granules present in both [**Fig. 3B, D**]. Further, cell nuclei were absent from both the pigmented core structure and the larger part of the radiating, transparent sacculi, and present only near the periphery of the rosette gland. We therefore suggest each peripheral sacculus to represent the basal part, and the yellow-pigmented bodies to represent the apical part of individual gland cells. In *T. helgolandica* each rosette gland appeared to consist of some 12 to 18 glandular cells.

In our SEM specimens of *T. helgolandica* both the anterior and posterior pinnal surface above the rosette glands appeared smooth and without any secretory pores [**Fig. 4A, C**]. In dry fractured material we regularly found a pit with cilia in the coelomic lining of the ramus where the underlying hidden rosette gland core structure was likely to be positioned [arrow in **Fig. 4D**].

Morphology of the hyaline glands

The hyaline glands, characteristic for all members of the subgenus *Tomopteris* (*stricto sensu*) are known to be difficult to identify in fixed material. However, in fresh, unfixed specimens we found every neuropodial pinna of *T. septentrionalis* and *T. planktonis* to reveal such a transparent gland with a yellow-pigmented core structure near the lateral border of the pinna [**Fig. 2D-H**].

In both species, these glands consisted of a bundle of some 6 to 12 more or less parallel, elongated, tubular sacks which traversed much of the pinnal diameter lateral and slightly dorsal to the apex of the coelomic ramus of the neuropodial pinnae [**Fig. 2D, G**]. In live specimens these tubular sacks converged laterally towards a core structure consisting of several elongated refractive and heavily yellow-pigmented bodies [**Fig. 2E, H**]. In *T. septentrionalis* the content of these tubular sacks was homogenous in some specimens [**Fig. 3E**] and vesicular with a granular content in others [**Fig. 3F**]. Shallow, transverse constrictions at fairly regular intervals were present along their walls [**Fig. 2G, 3E**]. At the lateral end, but still at a distance from the lateral border of the pinna, these tubular cells converged towards a lobed structure darkened by osmication [Hc in **Fig. 3E, F**]. Some of these osmicated lobes penetrated to the exterior of the posterior surface of the neuropodium [**Fig. 3F**].

In *T. carpenteri*, the hyaline glands were present from the third parapodium and onward. They shared structural similarities with both the hyaline glands of *T. planktonis* and *T. septentrionalis*, and the rosettes of *T. helgolandica* and *T. pacifica*. They were located in the neuropodial pinna only, on the top side, close to the apex of the coelomic ramus. However, the baso-apical axis of the gland is oriented perpendicularly to the plane of the pinna, so that they looked like a rosette in whole mount [**Fig. 2F**]. The gland cells converged and opened out posteriorly on the surface of the pinnae where the yellow-brown pigmented core structure was found in preserved material.

Morphology of the chromophile- and tubular glands

In addition to the rosette- and hyaline glands described above, well-defined chromophile glands were present in the neuropodial pinna of all our examined species [**Fig. 2, 4A, 5A**], except in *T. septentrionalis* [**Fig. 2G**]. These glands consisted of a large bundle of tubular cells that converged from a wide base at the ventral aspect of the coelomic ramus towards a narrow cluster of secretory pores. The latter emerged on the anterior surface of the pinna near its ventral edge [**Fig. 4B, 5B** (arrow)**, C**]. Such pores were seen by SEM and immunohistochemistry in parapodia of *T. helgolandica* (**Fig. 4B, 5B**), *T. pacifica* [**Fig. 5C**], *T. carpenteri* and *T. planktonis* (not shown). As shown in figure 2F the cells of these chromophile glands were usually well differentiated from the remainder tubular glandular cells of the parapodial pinnae. In *T. pacifica* the chromophile glands contained two distinct kinds of tubular cells: one containing granular and the other containing filamentous secretory material (not shown). These cell-types possibly correspond to the secretory pores of two different diameters observed by immunohistochemistry [**Fig. 5C**].

The more diffusely distributed slender tubular single-celled glands of the pinnal epithelium filled up the gap between the glands defined above. They appeared to be quite heterogenous glandular cells, at least with respect to the stainability of their secretory products. Staining by Alcian blue at pH 1 and 2.5 indicated the presence of much acidic mucopolysaccarides in many of these cells, especially the more lateral ones (not shown). Janus green, as applied to whole mounts of aldehyde-fixed pinnae from *T. planktonis*, revealed cell contents (unstained, clear blue and strongly metachromatic ones) of at least three distinct natures [**Fig. 2F**]. Clearly, also the most ventral tubular cells of *T. pacifica* revealed a texture quite distinct from that of the tubes in the rest of the pinnae [**Fig. 2C**]. In transverse sections through the pinnae of *T. helgolandica* the glands facing the anterior surface of the pinna were generally less stained and more rounded than the more elongated and deeper stained glands facing the posterior surface [**Fig. 3A**]. By SEM we also found more secretory pores scattered throughout the anterior pinnal surfaces of this species than on the posterior pinnal surfaces [**Fig. 4B, E**]. The highest density of secretory pores was found on the rim delineating the circumference of the pinnae. This rim also contained a number of short cilia [**Fig. 4E, 5H**].

The immunolabeling of whole parapodia with acetylated α-tubulin [**Fig. 5**] provided a view through the thickness of the pinna of at least some of the tubular cells. The apical part of these tubes were slightly bulbous and strongly immunolabeled around their entire circumference and terminated in a roundish pore at the surface of the pinna [**Fig. 5D-G**]. As suggested earlier, at the apical end of the chromophile glands, distinct populations of small and large pores probably represented two distinct cell types [**Fig. 5H**]. Deeper in the tissue the homogenous staining of the tubular wall often broke up to delicate immunolabeled bundles of cytoplasmic filaments separated by regions devoid of such labeling [**Fig. 5E**]. The course of neighbouring tubes appeared slightly curved and were separated from each other a by considerable space containing other, non-labeled cellular elements.

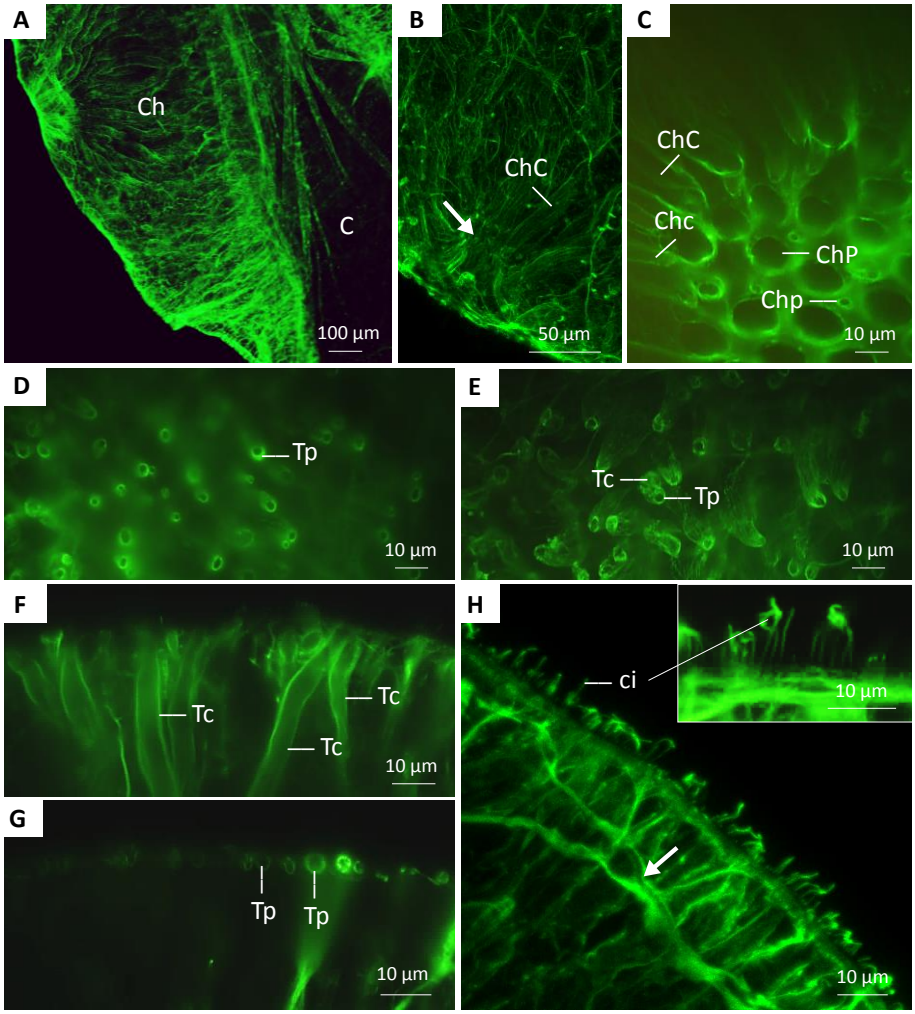
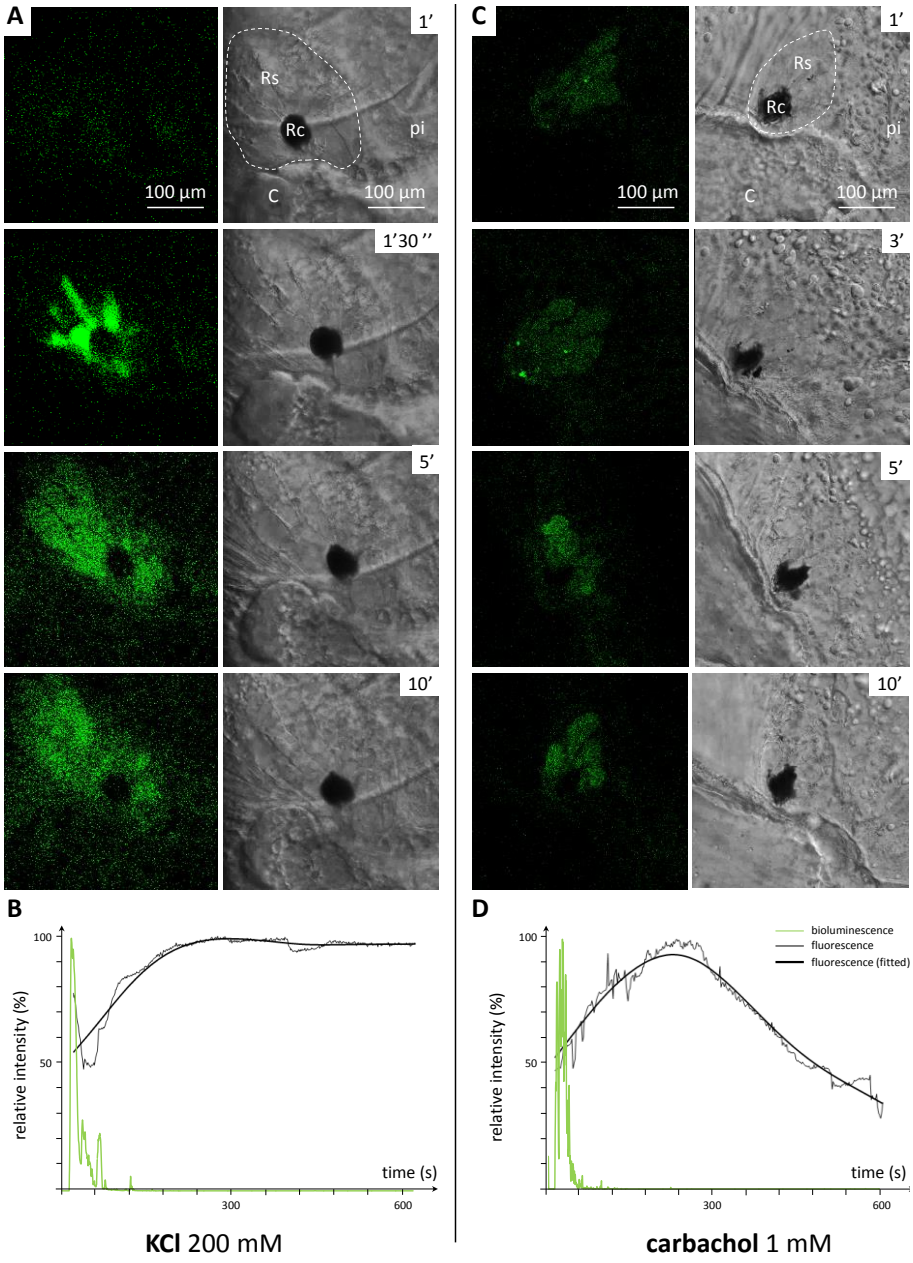


Fig. 5. Chromophile- and tubular mucus glands as revealed by immunofluorescence.
Whole mounts of aldehyde-fixed parapodia of *Tomopteris helgolandica* (A, C-G) and *T. pacifica* (B) incubated with FITC-conjugated anti-acetylated α-tubulin antibodies. **(A)** Whole view of the chromophile gland made of closely apposed, converging, tubular gland cells (posterior side of the neuropodial pinna). **(B)** The tubular cells converge to form the chromophile gland and open close to the edge of the pinna (anterior side of the neuropodial pinna). **(C)** Cluster of two different sized pores emerging from the chromophile gland in *T. pacifica*. **(D-E)** Tubular gland cells scattered in the pinna with focus on the pores **(D)** and on the thickness of the pinna **(E). (F-H)** Tubular cells **(F),** pores **(G)** and erected cilia **(H)** visible at the lateral margin of the pinna. (C, coelomic cavity; Ch, chromophile gland; Chc, chromophile small gland cells; ChC: chromophile large gland cells; Chp, small diameter pores of the chromophile gland; ChP, large diameter pores of the chromophile gland; ci, ciliated cells; T, tubular gland cell; Tp, pore of a tubular gland cell).

At the pinnal edge, strongly immunolabeled tufts of short erected cilia, probably sensory, were present between the tubular pores [**Fig. 5H**] as also evidenced by SEM [**Fig. 4E**]. Immunolabeled profiles running more or less parallel to the pinnal edge probably represented nervous network [arrow in **Fig. 5H**].



KCl 200 mM

carbachol 1 mM

Fig. 6. (previous page) Temporal changes in fluorescence emission in KCl- and carbachol-stimulated rosettes from *Tomopteris helgolandica*.
(A) Example of fluorescent emission (excitation wavelength 488 nm; maximal emission wavelength 536 nm) in a rosette observed 1, 1.5, 5 and 10 min after 200 mM KCl stimulation (light exposure time: 1.93 s per frame). The dashed line delineates the rosette tissue. On the right side are differential interference contrast images; **(B)** Bioluminescence (n = 3) and fluorescence (n = 3) emission kinetics in KCl-stimulated tissues. Bioluminescence and fluorescence measurements have been performed on distinct samples, using a luminometer and a cLSM respectively; **(C)** Example of fluorescent emission in a rosette observed 1, 3, 5 and 10 min after 1 mM carbachol stimulation; **(D)** Bioluminescence (n = 3) and fluorescence (n = 2) emission kinetics in 1 mM carbachol-stimulated tissues. (C, coelomic cavity; pi, pinna; Rc, core structure of rosette gland; Rs, sacculi of the rosette gland).

Fluorescence patterns

Endogenous fluorescence of the pinnal glands

The autofluorescence of the rosette glands of *T. helgolandica*, which was weak or absent in non-stimulated tissues, increased locally after KCl- or carbachol-stimulated light emission [**Fig. 6**]. About one min after the luminescence induction by KCl, the fluorescent material spread centrifugally and completely filled the periphery in two min [**Fig. 6A**] then the intensity continued to increase slightly until it reached a plateau. It is interesting to note that the fluorescence intensity increased in this peripheral layer after the peak or even after the extinction of bioluminescence emission [**Fig. 6B**]. Afterwards, the fluorescence persisted inside the rosette's cells while it tended to fade progressively after carbachol stimulation [**Fig. 6D**].

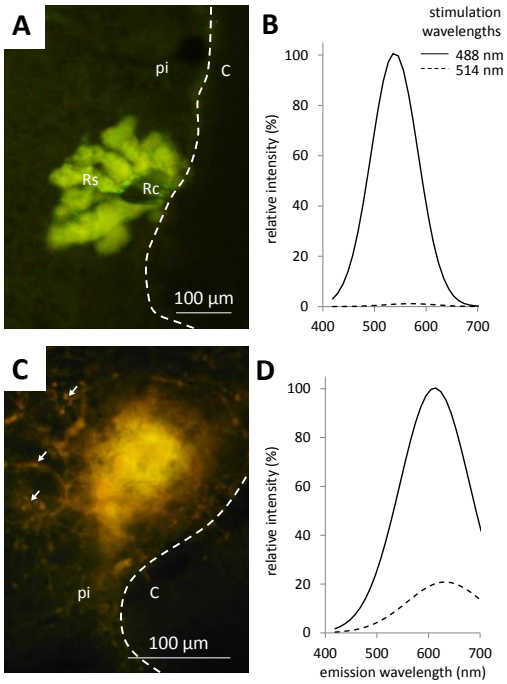
Responses to KCl and carbachol showed a similar pattern but the emission kinetic was slightly different [**Fig. 6B, D**]. The response to KCl tended to be faster, more contrasted and intense than the response to carbachol. However, the response to carbachol was usually accompanied by fluorescent flashes located .around the yellow-pigmented core structure [**Fig. 6C**, 3 min].

At the end of the reaction, the fluorescence clearly underlined the rosette shape of the gland and the granularities of the cells' content [**Fig. 7A**], also evidenced in semithin sections [**Fig. 3D**]. This autofluorescence had a specific spectral signature; it showed a unimodal distribution (maximal emission wavelength, λ_{max} = 539 nm, **Fig. 7B**) that was not modified by the bioluminescence emission [**Fig. SII**]. The yellow pigments contained in the core structure strongly absorbed the blue light (excitation wavelength 488 nm) [**Fig. 7A**]. Conversely, in *T. planktonis*, the lipoid pigmented substance produced an intense fluorescent signal. However, in both species the bioluminescence response to KCl and carbachol generated a centrifugal extension of the fluorescent area from the yellow-pigmented core to the peripheral layer of the gland (GOUVENEAUX *ET AL.*, in press).

Aldehyde-induced fluorescence

The use of aldehyde-based fixatives is common both on annelids (ROUSE AND PLEIJEL 2001) and on the gelatinous plankton (STEEDMAN 1976, HARRIS *ET AL.* 2000, JOHNSON AND ALLEN 2012), and was successfully used on *Tomopteris* (TERIO 1960, ÅKESSON 1962). However, the treatment of stimulated material by such fixatives was found to alter both the distribution and the spectral characteristics of the auto-fluorescence previously induced by the bioluminescence [**Fig. 7B, D**]. Indeed, the fluorescent signal was more diffuse, weaker in intensity (inferior by two orders of magnitude) and less specific (FWHM or full width at half maximum 50% higher, larger excitation range). The emission maximum under blue excitation light long-shifted of about 70 nm.

In some cases, fluorescent strands radiate from the rosette glands and bulb-like extensions [arrows in **Fig. 7C**].



E

	living material (A)		preserved material (B)	
excitation wavelength	488 nm	514 nm	488 nm	514 nm
λ_{max}	539.10	569.13	612.19	632.15
FWHM	106.80	116.24	159.40	173.88

Fig. 7. Fluorescence of rosette glands from fresh and formaldehyde-fixed stimulated parapodia of *Tomopteris helgolandica*.
(A) Autofluorescence of a freshly dissected, whole mounted, 200 mM KCl-stimulated parapodium and **(B)** the associated unimodal spectral distribution (Gaussian fitting). The yellow-pigmented bodies strongly absorbed the blue light excitation (488 nm) and appeared in dark; **(C)** Autofluorescence of a parapodium fixed in 4% formalin and (D) the associated spectral distribution; **(E)** Parameters of the spectra. (C, coelomic cavity; pi, pinna; Rc, core structure of rosette gland; Rs, sacculi of the rosette gland; λ_{max}, maximal emission wavelength; FWHM, full width at half maximum).

Discussion

Methodological discussion

Although the collected tomopterids were not fed, they kept swimming actively and retained their bioluminescent abilities for weeks after collection. Their physiological condition seemed good and their rosette-shaped organs appeared intact at the time of experimentations.

For our morphological studies only freshly collected specimens from plankton samples were used. A double fixation with glutaraldehyde followed by osmium tetroxide gave as expected the best cytological preservation of the rosette and hyaline glands (WEAKLEY 1981). However, most of the refractive yellow pigment, so characteristically present in these glands in their living state seemed to leak out. Fixation in 4% osmium tetroxide dissolved in the hydrophobic medium chloroform was therefore used to ensure rapid fixation without aqueous washout effects. Osmium tetroxide is known to react with unsaturated lipid bonds (PEARSE 1980) and gave a much better preservation of the pigment, although in a strongly blackened state caused by the osmium.

Rosette- *versus* hyaline glands

Our observations on both fresh and sectioned material also point to a strong homology between the rosette glands in tail-bearing tomopterid species and the hyaline glands of the tail-less species. The saccular shape and low contrast of the basal parts of the glandular cells as opposed to their strongly yellow-pigmented apical parts speak in favor of such a view, as previously held by Malaquin and Carin (1922).

However, in spite of this homology there also exists a clear-cut difference between the orientation of the rosette glands in *T. helgolandica* and the hyaline glands of *T. septentrionalis*. Whereas the apical part of rosette glands is oriented towards the coelomic cavity the apical part of the hyaline glands emerge through the cuticular surface towards the surrounding water on the posterior side of the neuropodium. The hyaline glands are therefore typical exocrine glands, whereas the rosette glands must be endocrine as its secretion cannot reach the exterior nor the gut. The rosette gland secretion may be limited to a confined extracellular space around the apical part of the gland, or it may gain access to the coelomic cavity through a small pore, in the thin coelomic lining. At present we cannot rule out that the extracellular space we see around the apex of these glands is an artifact caused by tissue shrinkage, nor can we exclude that a secretion into the coelomic cavity, if present, is a temporal event rather than a permanent pore in the coelomic lining.

Fluorescence versus bioluminescence

It is generally admitted that the endogenous fluorescence or autofluorescence of photogenic organs and cells in general is closely related to the biochemical nature of the intrinsic bioluminescent system (HASTINGS 1996). In this context, fluorescence

is a natural proxy of either the substrate, an intermediate or a final product of the luminescent reaction, which is commonly used to identify the light emitting structures (BASSOT AND BILBAUT 1977, SWEENEY 1980, ROBISON *ET AL*. 2003, RENWART *ET AL*. 2014).

Although we have not been able to combine direct observations of fluorescence and bioluminescence, our results show that an endogenous fluorescent signal, which is specific and dynamic during the bioluminescence emission, appears at the level of the rosette glands in *T. helgolandica*. This is in agreement with the involvement of these organs in the light emission (GREEFF 1882, 1885, MEYER 1930). However, given that [(1)] the glands are poorly or not fluorescent (at least in blue excitation light) before the bioluminescence induction and that [(2)] the fluorescence spreads from the core structure of the rosette to the transparent sacculi at the time the bioluminescence is going off, its distribution and its temporal dynamics can hardly reflect that of the bioluminescence itself. The fluorescence is rather produced by a breakdown product of this chemiluminescent reaction. Similarly, Bassot and Bilbaut (1977) demonstrated in the polynoid worm *Acholoe astericola* Delle Chiaje 1841 that electrically-stimulated elytra acquired a greenish fluorescence as the bioluminescence reaction occurs.

We still know little about the chemical bioluminescent system of the tomopterids, but several endogenous sources of autofluorescence are known apart from widespread cellular metabolites (*e.g.* flavins, NAD(P)H). Recently, Francis *et al.* (2014) isolated from *Tomopteris spp.* tissues a fluorescent anthraquinone, subject to spectral change, which may be involved as a final light-emitter. Indeed, the fluorescent properties of bioluminescent compounds can have, according to their reactional stage or their chemical environment, an important spectral plasticity (SHIMOMURA 2006). Thus, our green-yellow fluorescence could then be produced by the regenerated reduced form of this anthraquinone, which may be found in the elongated yellow-pigmented bodies as a non-fluorescent storage form.

Pattern of bioluminescence expression in *T. helgolandica*

The exudation of luminous material has been reported in several *Tomopteris* species (*e.g.* DALES 1971, ROBISON 1995, FRANCIS *ET AL.* 2014), but it has never been investigated any further. In *T. helgolandica* such an exudation is unlikely to occur as it has, to our knowledge, never been observed in living animals nor reproduced from isolated parapodia. Still, our morphological examination of the rosette glands strongly supports their secretory role, at least in the form of exocytosis of a vesicular material from their pigmented core to a surrounding extracellular space, which possibly communicates with the adjoining cavity of the coelomic ramus. Therefore, the secretory event may remain very localised and difficult to distinguish from a truly intracellular light emission. Thus it might be similar to a transient kiss-and-run exocytosis mode as opposed to full-collapse fusion (ALABI AND TSIEN 2013, LING-GANG *ET AL.* 2014). This hypothesis is in good agreement with the dynamics of the fluorescent reaction associated with the bioluminescent process of this species: The exocytosis of a potent (non-fluorescent) substrate by the rosette core structure may trigger a chemiluminescent reaction resulting in light emission

and accumulation of an autofluorescent breakdown product in the surrounding extracellular space. This breakdown product may gradually disappear or rather be reabsorbed in the more peripheral sacculi of the rosette gland to be regenerated to new luminescent material. The ionic changes associated with the exocytosis may be enough to trigger such a reaction. For example, distinct photoproteins are extremely sensitive to Ca^{2+}- and Fe^{2+}-ions (SHIMOMURA 2006). Alternatively, *T. helgolandica* might be, like other bioluminescent organisms (*e.g.* syllid worms: DEHEYN AND LATZ 2009; halocyprid ostracods: ANGEL 1968) able to emit graded luminescent responses including extra- and intraglandular processes and the conditions required for a complete exocytosis might not have been gathered here. This is consistent with the observation of changing emission patterns between 200 mM KCl or 1 mM carbachol and lower concentrations of carbachol (GOUVENEAUX AND MALLEFET 2013).

Salerno (1965) and Terio (1964) have suggested an alternative hypothesis merely based on the observation of fluorescence on fixed material, according to which a system of canaliculi conducts the luminous material from the rosette glands towards the edge of the pinnae. We observed similar fluorescent patterns in formaldehyde-fixed tissues [**Fig. 7C**]. However, given its diffuse distribution and its non-specific spectral signature, this fluorescence is most probably aldehyde-induced (JONES 1976, STEEDMAN 1976, BILLINTON AND KNIGHT 2001). Although it has been demonstrated in several luminous species that KCl depolarisation induced little or no morphological changes compared to light stimulation by physiological doses of neurotransmitters (GERMAIN AND ANCTIL 1988, DEHEYN *ET AL.* 2000), the maximal stimulation of tissues and the massive production of fluorescent breakdown products are likely to increase the diffusion of the latter, possibly between the closely apposed tubular gland cells evidenced by SEM and immunohistochemistry in most of the pinnae. These tubular single-celled glands and the chromophile pinnal glands being strongly stained by Alcian blue in *T. helgolandica*, they are rather involved in production and secretion of mucus. Particularly rich in tubulin, these cells could also play a role in maintaining the shape of the pinna.

A real exudation of bioluminescent material is more likely to occur in tomopterids with hyaline glands facing the external pinnal surface of the parapodia, like we have documented for *T. septentrionalis*. This conformation is consistent with the secretion of significant amounts of luminescent material to the surrounding water as is the case for *T. carpenteri* (GOUVENEAUX *ET AL.*, in press).

Bioluminescence diversity within the Tomopteridae

As already pointed out, the rosette- and hyaline glands obviously share structural features that can explain the occurrence of hybrid glands, difficult to classify in one or the other category, *e.g.* in *T. kefersteini* (GREEFF 1879) after Bonhomme (1954). However, it is interesting to note that they are distributed along a functional and migrational axis extending from endocrine secretion in *T. helgolandica* and *T. pacifica* to exocrine secretion in *T. planktonis*, with *T. carpenteri* and *T. septentrionalis* in intermediary positions [**Fig. 8**]. The transformation of the

embryonic coelomostomes into rosettes I described in *T. anadyomene* (MEYER 1929b) is accompanied by a similar migration phenomenon (MEYER 1930). Here, we suggest the organisational changes operated to have enhanced the diversification of modes of expression of bioluminescence and, possibly, of the functions associated to them.

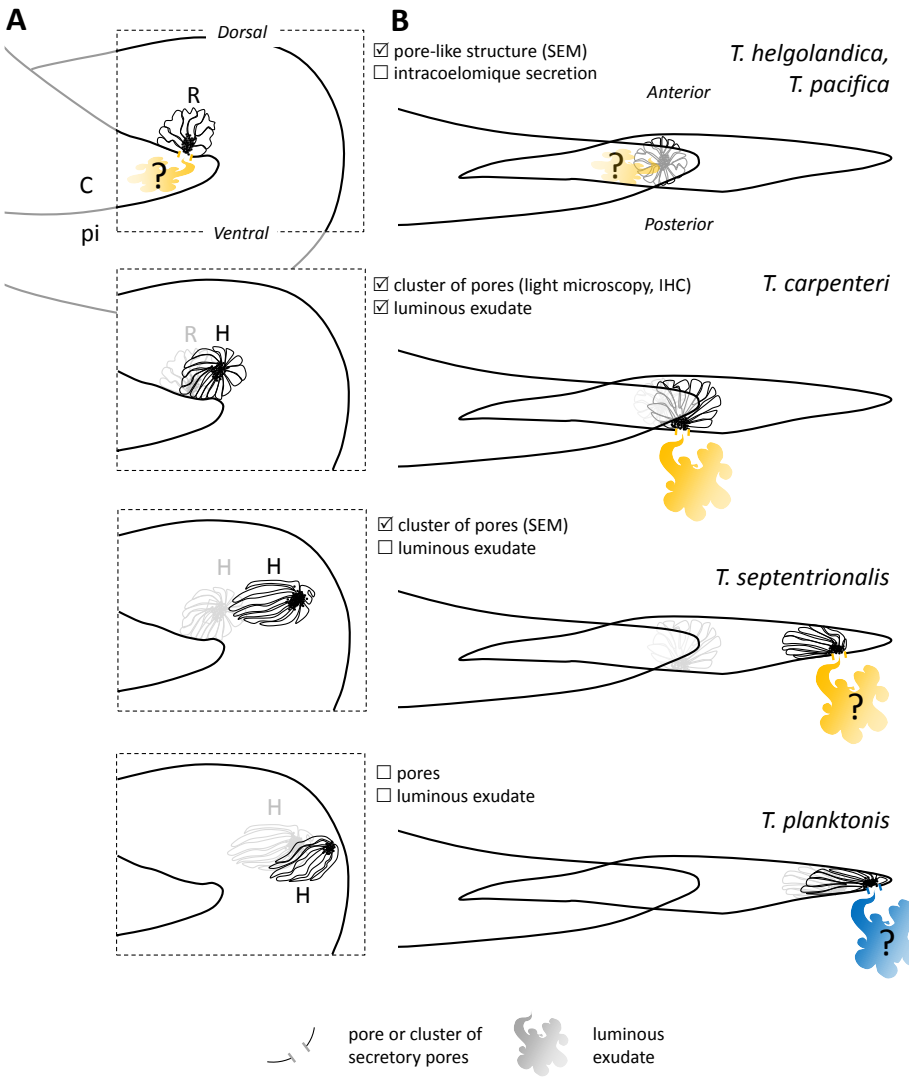


Fig. 8. Hypothesis of migration of the light glands to the distal part of the pinna.
Diagram of the neuropodia of each studied species showing the position and orientation of the rosette- or hyaline gland from posterior **(A)** and ventral views **(B).** The ticked boxes refer to results presented in this paper and the blank boxes are still open questions. (C, coelomic cavity; H, hyaline gland; pi, pinna; R, rosette gland).

Indeed, the diversity of bioluminescence patterns newly evidenced in tomopterids, including yellow and blue, secreted and localised emissions (GOUVENEAUX *ET AL.*, in press) reject the general assumption that all "errant" annelid produce localised luminous flashes whereas "sedentary" annelids secrete a luminous mucus (BONHOMME 1954, MORIN 1983). Comparisons between the patterns of emission within the various luminous annelid families (Accrocirridae, Chaetopteridae, Polynoidae, Syllidae, Terebellidae…) are inconclusive in that respect. Even within a single species, this localised/secreted duality may not represent a strict limit. In the syllid annelids, some mature specimens appear to produce both internal and secreted bioluminescence depending on the season (FISCHER AND FISCHER 1995, DEHEYN AND LATZ 2009). Thus, although the various emission patterns observed in our tested species could be linked to the stimulus intensity or the physiological state of the specimens (HERRING 1985) such variability can have a biological meaning and ecological value. The diversity of luminous patterns among this group therefore deserves to be considered and investigated further.

Since the appearance and locations of several types of parapodial pinnal gland represent key criteria for the identification of distinct species of Tomopteridae, it is tempting to interpret this migration pattern as reflecting the evolutionary history of this annelids' family. However, it is worth reminding that the genetic distance between the Tomopteridae as well as their relative positions are still entirely unknown (ROUSE AND PLEIJEL 2003, STRUCK *ET AL.* 2007).

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Authors' contributions

A.G, P.R.F. and J.M. designed the experiments, collected the samples and data. C.O. and J.L. provided support for immunohistochemistry. A.G. and P.R.F. wrote the manuscript. J.M., C.O. and J.L. revised the manuscript.

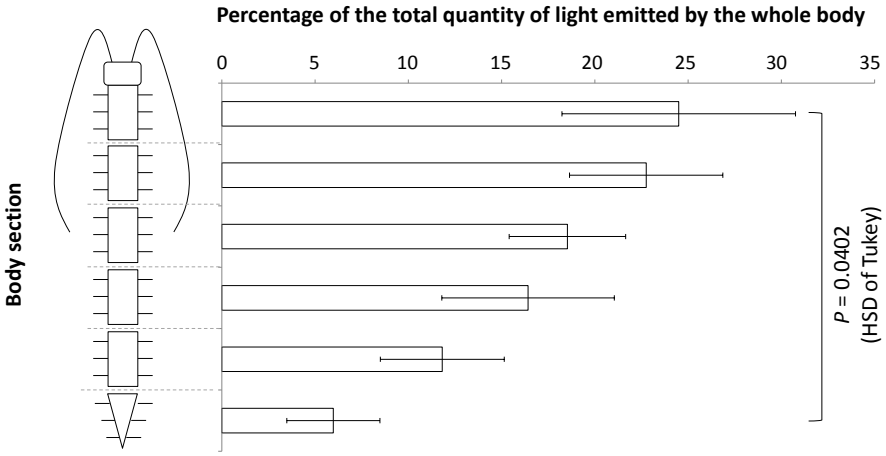

Fig. SI. Light emission potential along the body of *Tomopteris helgolandica*.

Light emissions were triggered by 200 mM KCl according to Gouveneaux and Mallefet (2013). The total quantity of light emitted (Mq) during 5 min by each preparation of three pairs of parapodia is expressed as percentage of the sum, for each individual (n = 6), of the values measured for all the preparations (mean ± s.e.m.).

The posterior sections have a bioluminescent potential around 5 times inferior to that of the most anterior sections. The progressive reduction of this potential from the head to the tail of *T. helgolandica* does not depend on the order in which the preparations were dissected and tested and could be explained by the progressive reduction in size of the rosette glands or by their maturity (the posterior segments being the last formed).

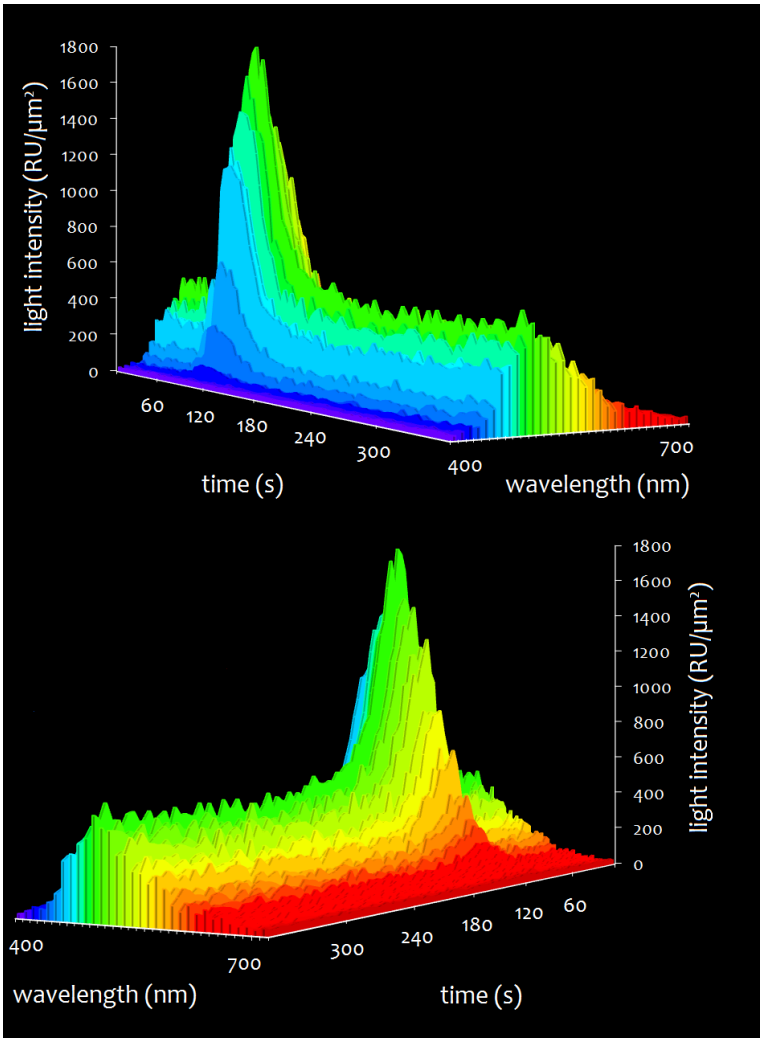

Fig. SII. Spectral analysis of a fluorescent microflash emitted by a rosette gland of *Tomopteris helgolandica*.

This microflash was emitted by a subset area (85 µm²) of the whole mounted rosette tissu (as shown in Fig. 6). The fluorescent spectrum is not altered during the emission.

4.2.2. Additional results and discussion

4.2.2.1. Endogenous fluorescence as a proxy of bioluminescent chemicals

Fluorescence and bioluminescence are distinct light emitting phenomena [**Box 1.1**]. However, biochemical evidence indicates that many photocytes are naturally sensitive to light excitation, the spectrum of which usually matches closely that of *in vivo* bioluminescence (*e.g.* in bacteria: ELEY *ET AL.* 1970, BALNY AND HASTINGS 1975; coelenterates: MORIN AND REYNOLDS 1970; fireflies: SUZUKI AND GOTO 1971). This endogenous- or autofluorescence is commonly due to a reactional intermediate or to the final light emitter. It is frequently the main or the only diagnostic feature used to identify and describe luminescent structures (BASSOT AND BILBAUT 1977b, BREHM AND MORIN 1977, JOHNSON *ET AL.* 1985, RENWART *ET AL.* 2014, ROBISON *ET AL.* 2003, SMALLEY 1980, SWEENEY 1980) [**see also the section 3.2.4**]. In the description of the early development of bioluminescence in *Porichthys notatus* (Actinopterygii) (ANCTIL 1977) and in *Etmopterus spinax* (Chondrichthyes) (CLAES AND MALLEFET 2008), the fluorescence of photocytes reflects their functional or maturation state. Indeed, bioluminescent organisms differ from each other in the temporal distribution and stability of the fluorescence. For example, the autofluorescence of *Gonyaulax polyedra* (dinoflagellate) luciferin is lost after exhaustive stimulation of bioluminescence (JOHNSON *ET AL.* 1985). In *Ophiopsila californica* (Echinodermata), fluorescence appears in response to bioluminescence induction and is retained even after the tissue has been appropriately fixed and sectioned (BREHM AND MORIN 1977). In polynoid worms, the fluorescence of unstimulated active sites – the intensity of which varies by a factor of 1 to 100 depending on the elytra – is highly sensitive to the phenomenon of fading. It develops locally during the light emission and proportionally to the bioluminescent activity (BASSOT AND BILBAUT 1977b).

A similar situation appeared in *T. helgolandica*: the rosette glands from fresh non-stimulated tissues were usually not or weakly autofluorescent. The signal, maximally excited by light of 488 nm, intensified during the light emission and centrifugally spread from the rosette core structure to the peripheral layer, consisting of transparent, wide and irregular sacculi. After the bioluminescent emission, the fluorescence persisted inside these sacculi (KCl stimulation) or progressively faded (especially under carbachol stimulation, - 0.2% of the maximum light emission per second). Hence, (1) our observations are in agreement with the involvement of these organs in the light emission (Greeff, 1882, 1885; Meyer, 1930) and (2) the autofluorescence is likely to be produced by a breakdown product of the chemiluminescent reaction. This product may be gradually reinternalised to be regenerated to a new non-fluorescent luminescent substrate [**paper III**]. This is consistent with the fact that the spectral signature of the fluorescent signal

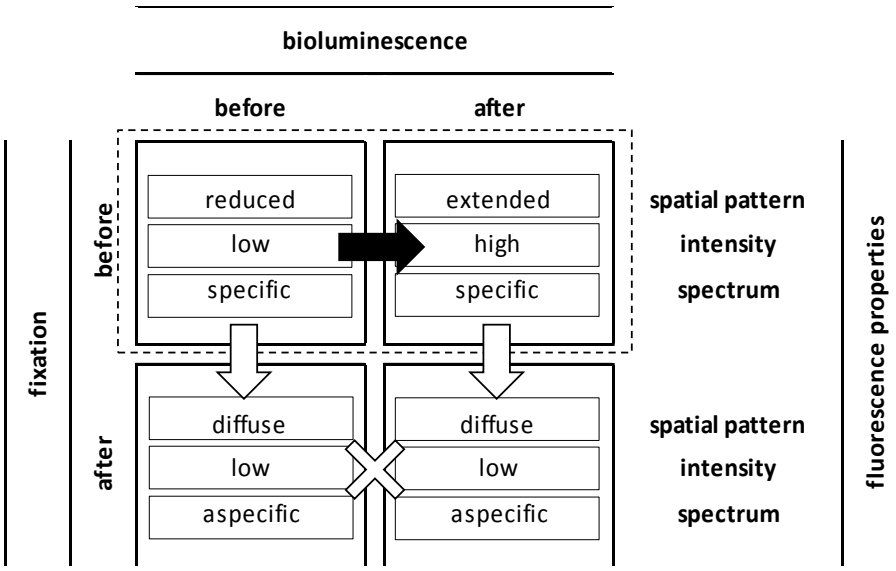

Fig. 4.3. Alteration of the fluorescence pattern of *T. helgolandica* rosette glands.
Bioluminescence induction (black arrow). Fixation by aldehyde-based fixators (white arrows). The light emission can not be induced from fixed tissues (white cross).

(λ_{max} = 540 nm under blue excitation, 570 nm under green excitation) remains unchanged during the luminous reaction [**paper III: Fig. SII**]. Such is the case, for instance, when the luminescent reaction produces several intermediates of different spectral properties (*e.g.* flavins and derived species in bacterian isolated system: BALNY AND HASTINGS 1975, MATHESON *ET AL.* 1981, HASTINGS 1996). A detailed model of the light emission process in *Tomopteris* is proposed in the next section.

As already indicated in the methodological section, both the spectral and spatial distributions of autofluorescence were found to be altered by aldehyde-based fixatives. The signal was more diffuse, weaker in intensity and less specific [**Fig. 4.3, see also paper III: Fig. 7**]. The emission maximum shifted from 539.10 nm to 612.19 nm under blue excitation light. This effect was also observed in *T. planktonis*: the distribution pattern of fluorescence in living parapodia [**paper II**] was not observable anymore in preserved material. These results underline the extreme need to observe the natural endogenous fluorescence on living tissues, or to develop a fixation protocol that does not alter or interfere with the signal of interest.

4.2.2.2. Structural homology, mechanistic diversity

Having faced methodological difficulties [**see the section 3.2.4 for details, and paper III**] in the preservation of the integrity of certain essential components of the putative light glands, especially the yellow-pigmented lipoid substance, the results presented in paper III are the result of a close collaboration with Per R. Flood, who furnished semithin sections and scanning electron micrographs, as well as insightful contributions to the interpretation of our results.

The discrimination of rosette and hyaline glands is originally a taxonomic criterion based on osmic differential staining (*supra*, p. 61). The first comparative histology of these glands in five species of *Tomopteris* provided evidence of their structural homology [**paper III**]. Both are composed of large transparent cells arranged in the form of a rosette and containing in their apical part a core structure of closely apposed, sausage-shaped, yellow-pigmented sacculi. This conformation strongly supports the secretory role of these cells. However, whereas the rosette glands and their pigmented core structure were found to point towards the coelomic ramus of the pinna and possibly open towards this cavity, the hyaline glands were found to point in the opposite direction towards the posterior surface of the pinna and there open towards the surrounding water. Consequently, an exudation of bioluminescent material, like in *T. carpenteri* [**paper II: Fig. 2d**], is more likely to occur in species with hyaline glands. Of the cases studied *T. planktonis* and *T. septentrionalis* would also be concerned. By contrast, the hypothesis of intracoelomic emission in *T. helgolandica*, which was already suggested by Meyer in 1929, does not correlate with our observations of an emission event very localised and difficult to distinguish from a truly intracellular light emission. Since we did not observe what could be distinct steps of a classical secretory cycle (such as gland cells with variable amounts of the pigmented substance), we speculated that the emission process might be similar to a transient kiss-and-run exocytosis mode, as opposed to full-collapse fusion (ALABI AND TSIEN 2013, LING-GANG *ET AL.* 2014). In the benthic polychaete *Odontosyllis phosphorea*, the fine line between intra- and extracellular mechanisms would be related to a compound mode of exocytosis: Deheyn and Latz (2009) described a coalescence of fluorescent small vesicles upon light excitation, then the release of fluorescent mucus by the larger vesicles. Based on the structural and fluorescent features, the emission dynamics of the rosette glands could include 3 stages: (1) the release of a non-fluorescent substrate by exocytosis, (2) the chemiluminescent reaction in the coelomic cavity, and, last but not least, (3) the almost instantaneous reabsorption (and possibly the recycling) of a reactional fluorescent product by glandular cells. This model could be a promising starting point for more extensive morphological and biochemical investigations. In any case, although the emission dynamics can be

influenced by the stimulus strength or by the physiological condition of the animals, the morphological analysis does not support, at least in rosette bearing species, the hypothesis of a direct release of luminescent products into sea water.

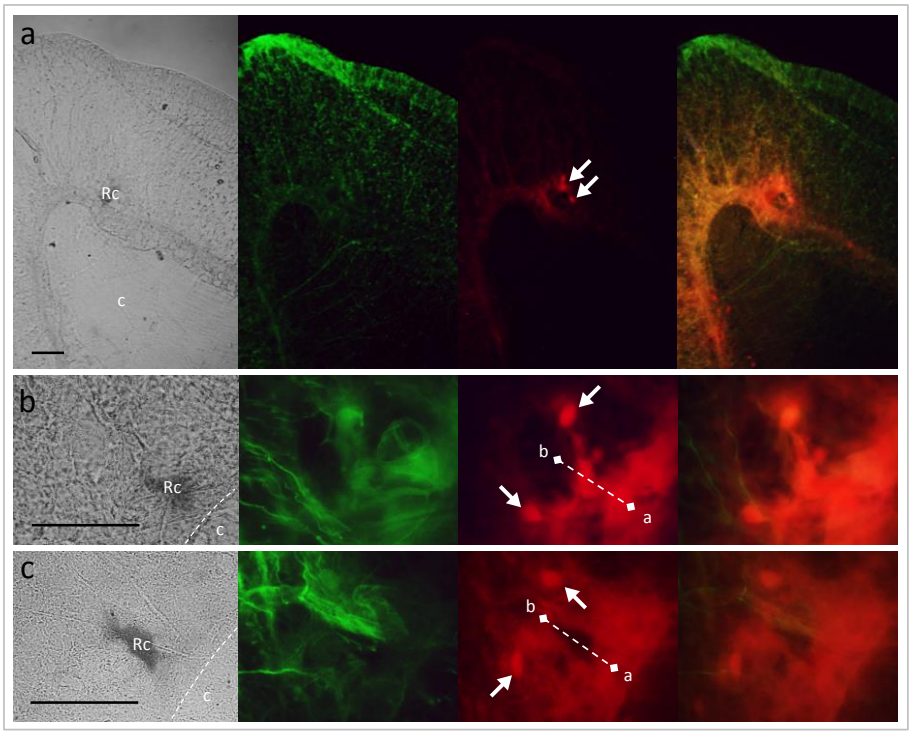

Fig. 4.4. Immunofluorescence detection of nerve fibers in *T. helgolandica* tissues.
(a-c) Whole mounts of aldehyde-fixed parapodia incubated with FITC- (donkey anti-mouse 715-095-150, 1:100, Jackson ImmunoResearch Laboratories, West Groce, PA, USA) conjugated anti-α-acetylated tubulin antibodies (T-6793, mouse, 1:100-1:1000, Sigma-Aldrich Company, St Louis, MO, USA) in green, and CY3- (donkey anti-mouse 715-165-150, 1:1000, Jackson ImmunoResearch Laboratories) conjugated anti-choline acetyltransferase antibodies (ChAT AB144 P, goat, 1:100, Chemicon International Inc., Temecula, CA, USA) in red. Epifluorescence microscopy; **(a)** Double-stained ramus and pinna with choline acetyltransferase positive ganglion cells (arrows); **(b)** The ganglion cells (arrows) are localised on both sides of the apico-basal axis of the rosette glands (a ⬥---⬥ b). c: coelomic cavity; Rc: core structure of the rosette gland. Scale bars 100 µm.

Finally, we suggest that both gland types evolved from a common light emitting structure and differentiated along a functional and migrational axis extending from endocrine secretion, close to the coelomic ramus, to exocrine secretion, close to the lateral margin of the pinna, or conversely [**paper III: Fig. 8**]. Indeed, it must be assumed that the possibility of reabsorption and recycling of secretion products by the luminous glands itself represents an interesting innovation from an energical point of view. Thus, a purely intraglandular emission

could constitute an additional evolutive stage towards this economy, and could be realistically represented in some *Tomopteris* species, as reported by Harvey (1952). Based on a comparative histology of various mesopelagic fishes, Bassot (1966) highlighted a range of homologous glandular light organs including, for some of them, closed or missing ducts.

4.2.2.3. Innervation

Having proven that bioluminescence in *T. helgolandica* and *T. planktonis* was under cholinergic nervous control [**papers I and II**], we aimed to investigate the morphology of the parapodial nervous system with a focus on the innervation pattern of the photocytes.

Two immunolabeling have been tried using anti-α-acetylated tubulin antibodies (T-6793, mouse, 1:100-1:1000, Sigma-Aldrich Company, St Louis, MO, USA) and anti-choline acetyltransferase antibodies (ChAT AB144 P, goat, 1:100, Chemicon International Inc., Temecula, CA, USA). Since the tubulin is present in a large spectrum of cellular types, the anti-α-acetylated tubulin antibodies not only stained the nervous fibres [**Fig. 4.5**], but also the cytoskeleton of the tubular cells, which form the greatest part of the pinnae [**paper III: Fig. 5**]. Therefore, it could be tricky to distinguish true nerve fibres from the remaining labelling, especially in the pinnal tissue. In addition, the description of the rosette gland labelling pattern in whole mounts was constrained by a blurred area that may be due to a lens effect created by the superficial layer of wide transparent sacculi [**Fig. 4.4a**]. Confocal laser scanning microscopy usually allowed us to make better observations [**Fig. 4.5**].

The anti-choline acetyltransferase labelling was poorly contrasted. Although they were demonstrated to react in zebrafish (CLEMENTE *ET AL.* 2004), AB144P antibodies are raised against human ChAT thus its specificity may be insufficient to detect the choline acetyltransferase in polychaetes. This could be ascertained by blasting the RNA ChAT sequence in the newly acquired transcriptome of *T. helgolandica* [**see the section 4.3.2**]. However, ChAT remains a much more specific marker of cholinergic neurons than acetylcholinesterase which is widely distributed even in non-cholinergic tissues (ELDEFRAWI AND ELDEFRAWI 1988). Despite this technical constraint, a pair of ganglion cells was repeatedly revealed in the vicinity of the rosette glands [**Fig. 4.4**]. As shown in figure 4.4b, these masses appeared to be attached to the pigmented area or core structure of the rosette gland through two branches. In tubulin positive whole mounts, this core structure appeared surrounded on the pinnae side by flame-shaped bundles of fibres [arrows in **Fig. 4.5c,d**]. The thin end of these structures sometimes wrapped around what might be the two ganglion cells previously observed [**Fig. 4.4 and 4.5e**].

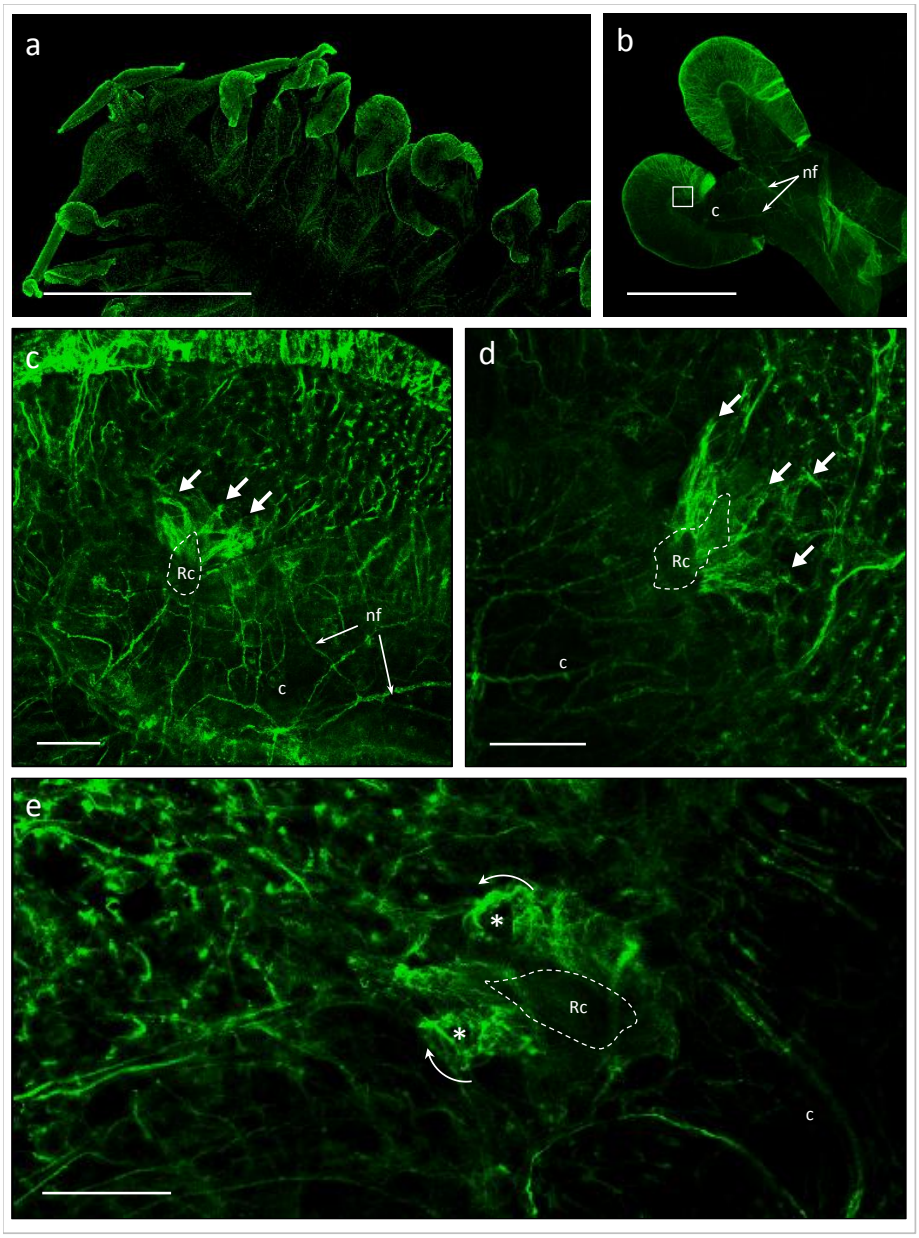

Fig. 4.5. Immunofluorescence detection of nerve fibers in *T. helgolandica* tissues.
Whole mounts of aldehyde-fixed parapodia incubated with FITC- (donkey anti-mouse 715-095-150, 1:100, Jackson ImmunoResearch Laboratories, West Groce, PA, USA) conjugated anti-α-acetylated tubulin antibodies (T-6793, mouse, 1:100-1:1000, Sigma-Aldrich Company, St Louis, MO, USA). Confocal laser scanning microscopy (Zeiss LSM 710). **(a)** Whole worm; **(b)** Isolated parapodium; **(c-d)** Rosette glands with bundles of fibers (arrows) emerging from their core structure; **(e)** Same image with fibers wound arround two nodal masses (*). c: coelomic cavity; Rc: core structure of the rosette gland; nf: nerve fibers. Scale bars 1000 µm (a-b), 100 µm (c-e).

In 1885, Greeff distinguished two cellular types in the rosette (yellow-pigmented photocytes and transparent accessory cells) and reported the presence of a nervous ganglion at its base. According to his model [**Fig. 1.13b**], nervous fibres penetrate the gland between the transparent cells, thus connecting the ganglion to the base of the photocytes. Amongst polynoid worms, the removal of the elytral ganglion, which is the only nervous centre remaining in the autonomised scales, does not seem to alter the excitability of the photogenic tissues (ANCTIL 1987). The functional role of the two ganglionic cells and of the nervous fibres associated that were observed in *T. helgolandica* remains to be determined. At this stage, they seem to be too intimately associated with the rosette not to play one. However, although these first results of whole mount stainings are encouraging, complementary neuronal labellings should first be implemented to confirm the identity of these structures. Several collaborative projects in progress should allow us to precisely and thoroughly describe the innervation pattern of the rosettes.

4.2.2.4. Other pinnal glands

In addition to the photogenic organs, chromophile and tubular pinnal glands were usually observed [**paper III: Fig. 4, 5**]. The well-defined chromophile glands consisted of a bundle of tubular cells that converged towards a narrow cluster of secretory pores emerging on the anterior surface of the pinna near its ventral edge. Highlighted by anti-α-acetylated tubulin immunostaining, the tubular single-celled glands were to be found diffusely distributed in the remaining part of the pinnal epithelium. Although we suggested these glands to be involved in the production of mucus, they seem to combine distinct cellular types, at least with respect to the stainability of their secretory products (FLOOD, P.R., unpublished data). This mucus could enhance the effect of bioluminescence. Indeed, mucus and light emission being both produced upon mechanical stimulation, the biochemical nature of the mucus (*e.g.* sex pheromone, repellent chemical) is possibly an indicator of the function fulfilled by the bioluminescence (sexual communication, aposematism) [**see the section 1.3.5**].

The alternative hypothesis according to which these tubules are part of a system of canaliculi conducting the luminous material from the rosette glands towards the edge of the pinnae (TERIO 1964, SALERNO 1965) probably results from the misinterpretation of a distorted (aldehyde-induced) fluorescent pattern [**for details, see the section 3.2.4.1**].

4.3. Light perception of *T. helgolandica*

The study of bioluminescence as communication signal implies taking the photoreception abilities of the receivers into consideration. The hypotheses of intraspecific recognition in tomopterids (DALES 1971), assume that their spectral sensitivity coevolved towards long-shifted wavelengths. To emcompass these two questions, we focused on a behavioural approach of light perception of *T. helgolandica*.

Merely based on the structure and distribution of light organs (ROBISON 2004), most functional hypotheses lack supportive behavioural data. Until recently, the behavioural study of plankton was mainly concerned with quantitative aspects of feeding ecology and bioenergetics rather than with behaviour *per se* (HAMNER 1985). Today, almost every behavioural pattern shown by the most complex, supposedly evolved, higher phyla is recognised to the planktonic invertebrates. However, the behaviour of Tomopteridae is only known through field observations of shape change in *T. nisseni* and *T. elegans* (ROBISON 1999) and a study in laboratory by Buskey and Swift (1985), who reported a sharp increase in the swimming speed of *T. septentrionalis* in response to a simulated copepod flash. Adapted from this work, our experiments consisted of video tracking and motion analysis designed to quantify the swimming pattern of isolated worms to photic stimuli simulating intra- (yellow light) or interspecific (blue light) interactions. The manuscript presented here constitutes a preliminary work.

Additionally, transcriptomic data regarding the differential expression of opsin genes in the cephalic and body parts of *T. helgolandica* are dicussed.

4.3.1. Behavioural responses of the planktonic worm *Tomopteris helgolandica* to photic stimuli (Paper IV)

(*in preparation*)

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Abstract

In contrast to most mesopelagic bioluminescent organisms highly specialised in the emission and reception of blue light, the planktonic annelid *Tomopteris helgolandica* is able to produce a bright yellow light. This unusual feature has long been suggested to serve for intraspecific private communication. Yet, this virtually admitted hypothesis has never been tested. In this behavioural approach of light perception in this species, we first present an illustrated repertoire of the postures and action patterns described by captive specimens, undisturbed or under various mechanical stimuli. Then video tracking and motion analysis are used to quantify the behavioural responses of isolated worms to photic stimuli imitating intra- (yellow light) or extraspecific (blue light) bioluminescent signals. Based on these new data, we show the ability of *T. helgolandica* to perceive and to contrast its responses to bioluminescent-like light signals. In particular, the variation of angular velocity observed according to the type of yellow stimuli (flashes *versus* continuous light signal) supports the intraspecific communication hypothesis. However, as suggested by the mechanically induced light responses in this species, the possibility that bioluminescence may fulfill multiple functions, especially of defence against predation, should remain an open question. The startle effect, aposematism and smoke screen are three strategies which should primarily be investigated in the future.

Introduction

Few organisms in the world are not somehow influenced by light. In the clearest waters, at approximately 150 m depth, more than 99% of the sunlight penetrating the surface has been scattered and absorbed (ROBISON 2004, JOHNSEN 2014) while below 1000 m, there is no longer enough light for the most sensitive visual system (WARRANT AND LOCKET 2004). For organisms living in midwater, bioluminescence is the primary source of light and a major form of communication (LLOYD 1977, HERRING 1990, 2000, WIDDER 2002, 2010, HADDOCK *ET AL.* 2010). The vast majority of them only have a single visual pigment whose absorption maximum (450-500 nm) matches both the most common bioluminescent colours (HERRING 1983, WIDDER *ET AL.* 1983) and the wavelengths that most easily penetrate the water column (DOUGLAS AND PARTRIDGE 1997, WARRANT AND LOCKET 2004). *Tomopteris helgolandica* Greeff 1879 is a free-swimming holoplanktonic annelid, widely distributed in North Atlantic, sampled from the surface down to 300 m (OBIS 2016). Transparent, almost invisible under natural lighting conditions, it is also able to produce a bright yellow light emission (GOUVENEAUX AND MALLEFET 2013). The adaptive value of luminescence in gelatinous plankton (gelata) has not been clearly evidenced. However, light emissions are often elicited in these soft-bodied organisms by physical contact, possibly as a means to deter predation (*e.g.* ROBISON 1992, HERRING AND WIDDER 2004). Indeed, as suggested by Haddock and Case (1999), the bioluminescence produced by supposedly poorly light-sensitive organisms probably evolved under the influence of interactions with species from higher taxa. However, given the taxonomic heterogeneity of gelata, one can expect a significant diversity of strategies for defence. The glowing fluid released by some ctenophores, siphonophores and chaetognaths may act as a smoke screen making the escaping prey difficult to track (HADDOCK AND CASE 1999, HADDOCK *ET AL.* 2010). Bioluminescence could also be an aposematic signal, especially in organisms with chemical defences such as cnidarians (ROBISON 2004). Regarding the unusual light emission maxima, they are commonly thought to be used as private visual channels for intraspecific communication (HERRING 1990). This hypothesis was supported in particular by the case of stomiatoid fishes whose visual spectral sensitivity and emission spectrum substantially overlap in the red range (DOUGLAS AND PARTRIDGE 1997, DOUGLAS *ET AL.* 1999). As a counter-example, the spectral sensitivity of the amphipod *Scina crassicornis* (Fabricius, 1775) (wavelength at the maximum emission intensity or λ_{max} = 435-444 nm, after HERRING 1983, WIDDER *ET AL.* 1983, LATZ *ET AL.* 1988) does not exactly match its emission wavelengths (λ_{max} = 472 nm) so the question of its ecological function and evolution remains open (BOWLBY AND CASE 1991, COHEN AND FRANK 2007).

In order to explore these functional hypotheses in *T. helgolandica*, one of the key steps is to investigate whether it is able to perceive its own light and that of conspecifics. In fact, little is known about the visual system and the sensory ecology of tomopterids. The adults are endowed with antenna or palps, nuchal epaulettes and a pair of setigerous cirri reaching two-thirds of the body length [**Fig. 1**] (ROSA 1908, FAUCHALD AND ROUSE 1997). Two eyes, consisting of seven large retinal cells and a three celled-lens, are located dorsally between the basis of the antennas and the insertion zones of the cirri (ÅKESSON 1964). In addition, almost nothing is known about their reproductive physiology and behaviour. Although spawning maximum was reported in this species in June in Gullmar Fjord, North sea (ÅKESSON 1962) and between May and August in Ireland (KIRKEGAARD 1992), they are believed to have a continuous reproduction cycle (ÅKESSON 1962, SIMMONS 2009). Lastly, there is still debate whether the fertilisation is internal or not (CARPENTER AND CLAPARÈDE 1860, FULLARTON 1895, HACHFELD 1926, ÅKESSON 1962).

In this fundamentally behavioural approach of light perception in *T. helgolandica*, we first present an illustrated repertoire of the postures and action patterns exhibited by captive specimens, undisturbed or under various mechanical stimuli. Then video tracking and motion analysis were used to experimentally investigate and quantify the behavioural responses of isolated worms to photic stimuli involved in intra- (yellow light) or interspecific (blue light) encounters. Indeed, the photic environment of *T. helgolandica*, in addition to the residual daylight, is also characterised by luminous signals emitted by potential preys or predators, or by blue emitting tomopterids (GOUVENEAUX *ET AL.*, in press). The observations are finally integrated and possible function(s) of bioluminescence in *T. helgolandica* are discussed.

Material and methods

Specimen collection and maintenance

The results reported here are based on laboratory observations of *Tomopteris helgolandica* specimens. They were collected in korsfjorden (60°12'49.4"N, 5°12'16.6"E), Western Norway, in October 2013 aboard the R/V Hans Brattström (University of Bergen). Oblique tows were made using the Isaacs-Kidd midwater trawl (1.75 m wide × 1.30 m high mouth, 6.5 m long, 500 µm mesh aperture) brought down to 200 m. A speed of < 1 knot was maintained during the collection in order to prevent any damage to the plankton. The sample was immediately diluted and sorted aboard. The cumulative duration of light exposure (solar irradiance and 1 watt-LED headlamp) did not exceed a few minutes. The specimens were subsequently handled under red or infra-red light, which are significantly less disruptive than white light (RAYMOND AND WIDDER 2007). The collected specimens

were housed separately in small aquaria (50 x 50 x 50 mm) in lighting (intensity, wavelength) and temperature conditions similar to natural (8°C). A recovery period of minimum 24 h prior to testing was observed that is reportedly sufficient to allow substantial recovery of luminescence abilities (LATZ ET AL. 1990, BOWLBY *ET AL.* 1991) in many other planktonic organisms. In addition, as revealed by successful chemical and mechanical inductions of light emission, they appeared to retain their bioluminescent abilities during several weeks after collection. Despite the lack of physiological data, the housing conditions seem to preserve at least part of their photosensitivity since they react to a brief photic stimulation. The specimens used, ranging from 15 to 50 mm in length, appeared to be in good condition at the time of the experiments, *i.e.* they were highly transparent, active, their parapodia and appendices were undamaged.

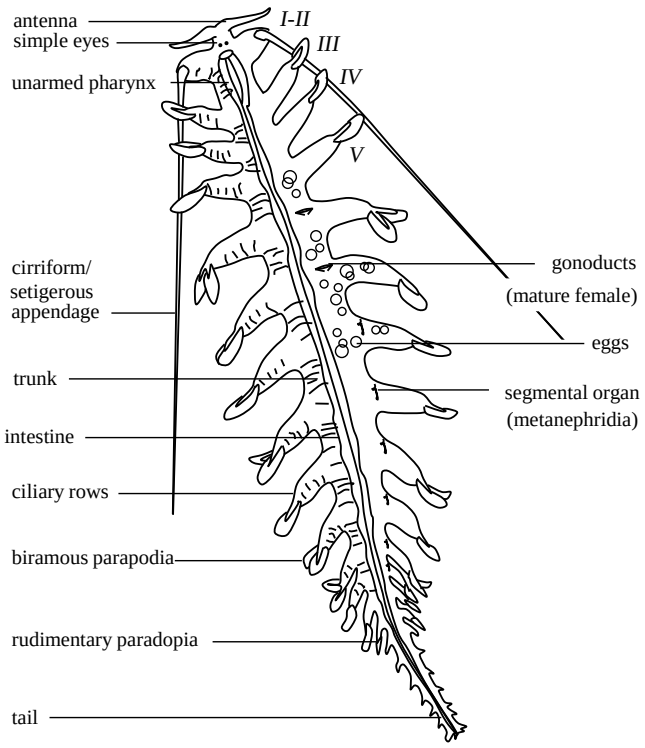


Fig. 1. External anatomy of *Tomopteris helgolandica*.
The terminology used is that employed in the descriptions of the behaviour. Redrawn from Meyer 1929a, with permission.

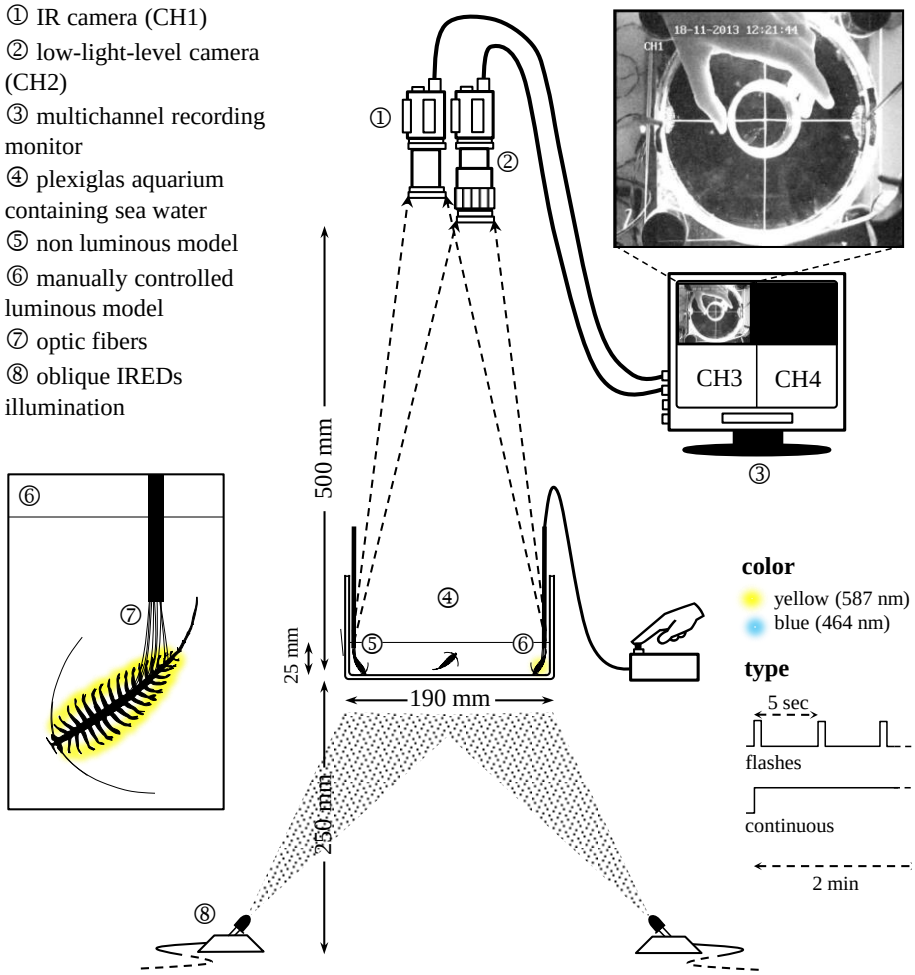


Fig. 2. Diagram of the experimental set-up used to record the swimming behaviour of *Tomopteris helgolandica* in reaction to various photic stimuli.

Behavioural observations and mechanical stimulation

The various behaviours of *T. helgolandica* described in this work are exclusively based on observations of isolated specimens maintained in aquariums under the conditions described above. The worms were either left undisturbed or exposed to three levels of mechanical constraints. (1) Hydromechanical stimulation was created by manually generating water motions with a pipette that simulated the presence of an organism and (2) brief or (3) prolonged direct mechanical stimulation were applied using forceps. Although these stimuli strength was not quantified, we reasonably assume that a direct contact is more intense and stressful than hydromechanical stimuli.

Video tracking

Imaging system

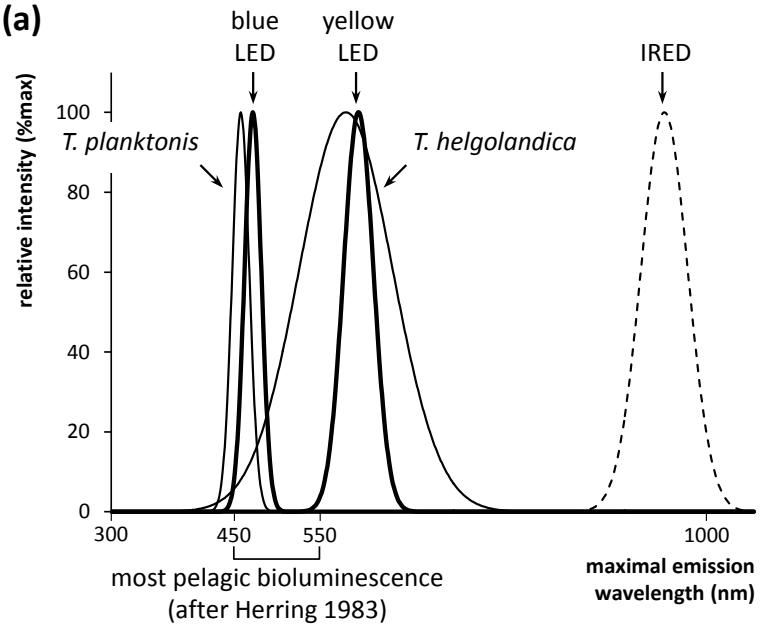
The recording system, illustrated in figure 2, consisted of two video cameras: an infra-red (IR) sensitive camera (Watec 902H ultimate, lens 5-50 mm, F/1.4, Watec cameras, Japan) for recording the swimming behaviour of the IR illuminated worms and a low-light-level camera (Watec 902H ultimate, Watec cameras, Japan) for simultaneous recording of low-intensity bioluminescence events. This camera was coupled to an auto-iris lens (HZ848DC, F/1.0 6X high-speed manual zoom lens, SPACE inc., Tokyo, Japan) which allowed the camera to adjust to sharp changes of light conditions due to bioluminescence events. Its efficiency has been validated by the observation of a light emission during preliminary tests. Both were positioned 50 cm above the bottom of a round aquarium (Crystal extruded acrylic, 190 mm diameter) which was obliquely lighted with eight infra-red light emitting diodes (IREDs) with a wavelength of maximal emission at 945 nm and a spectral bandwidth or full width at half maximum (FWHM) of 60 nm. In this way, the transparent plankter *Tomopteris helgolandica*, minimally disturbed, appeared brilliantly illuminated against a dark background (BATTY 1983, WIDDER 1992, WIDDER *ET AL.* 2005, RAYMOND AND WIDDER 2007). Indeed, the low illumination power used in these experiments (300 mW), combined with the narrow spectral width of IR (λ_{max} = 702 nm, FWHM = 95 nm) [**Fig. 3**], is well below the IR sensitivity threshold of most marine visual organisms.

The video signals were displayed separately but simultaneously on a monitor with an integrated multi-channels hard disk recorder (SEC-DVRMON30 4 channels, König electronic, The Netherlands). The experiments were video recorded in 2D at 30 fps (frame per second). The set-up was shielded from any extraneous light (*e.g.* light from the screen).

Experimental design

The aquarium was filled with fresh filtered sea water which was renewed between two experimental sessions. Two fake worms of 40 mm long, made of Sylgard transparent silicon and 0.2-0.5 mm diameter optic fibres [**Fig. 2**], were positioned at opposite sides of the aquarium. The photic stimulation was applied through one of these models which was designed to mimic the bioluminescence pattern of *Tomopteris helgolandica*. Indeed, the optic fibres have been arranged in order to reproduce the parapodial distribution of the light sources. The parameters of light stimulation (λ_{max} = 587 nm, FWHM = 36 nm, intensity 15 Mq.s^{-1}) have also been tuned to match the light emissions induced by physiological concentrations of carbachol: λ_{max} = 573 nm, FWHM = 130 nm, 0.5-2 Mq.s^{-1} per pair of parapodia (after GOUVENEAUX AND MALLEFET 2013) [**Fig. 3**]. These conditions which simulate

(a)



(b)

		colour	
		blue LED (λ_{max} = 464 nm)	**yellow LED** (λ_{max} = 573 nm)
type	**continuous**	ambiant daylight (surface waters above 100m) bacteria (free living or symbiotic)[2(3)], counterilluminating fish[3]... *T. planktonis*[1,2] (λ_{max} = 450 nm)	*T. helgolandica* (λ_{max} = 573 nm) *Aliivibrio Fisherii* Y-1 (λ_{max} = 545 nm)
	flashes (0.2 s^{-1})	dinoflagellates[2], ostracods[2], copepods[2]... *T. planktonis*[1,2] (λ_{max} = 450 nm)	*T. helgolandica* (λ_{max} = 573 nm) other yellow emitting *Tomopteris* (*T. nisseni*, *T. pacifica*, *T. carpenteri*...)

[1] sympatric species of *T. helgolandica*, [2] potential prey, [3] potential predator

Fig. 3. Independent variables and related light events *in natura*.
(a) Spectra of the light emitting diodes (LEDs) used as stimuli (blue and yellow) and as background illumination (infra red) compared with spectra from the sympatric species *Tomopteris helgolandica* and *Tomopteris planktonis* (*n* = 3). **(b)** Correspondance between the independent variables (colour and type of signal) and natural light events.

the presence of a conspecific allow us to test the intraspecific communication hypothesis, namely the main functional hypothesis argued in tomopterids (DALES 1971). In addition, a blue light (λ_{max} = 464 nm, FWHM = 22 nm) was used as *T. helgolandica* naturally faces blue bioluminescence displayed by potential preys (*e.g.* dinoflagellates, siphonophores, chaetognaths, crustaceans) (LEBOUR 1923, ÅKESSON 1962, RAKUSA-SUSZCZEWSKI 1968) or predators, and by the sympatric species *Tomopteris planktonis* Apstein, 1900 (λ_{max} = 450 nm, FWHM = 23 nm) (GOUVENEAUX *ET AL.*, in press). For each spectral condition, the light was applied *via* manual remote control, continuously or, as observed under carbachol stimulation (GOUVENEAUX AND MALLEFET 2013), in subsequent flashes every 5 seconds.

Combinations of two independent variables (type of signal and colour), each of which includes two levels (continuous/flash and yellow/blue, respectively), were applied through the silicon model and tested during a 2 min experimental session [**Fig. 2**]. The other silicon model was non-luminous in order to distinguish the effect due to the presence of a worm-like object from that of the photic stimulation. Finally, their respective positions were interchanged to prevent any bias due to, for instance, a heterogeneous IR illumination of the aquarium. Each of the 12 tested worms has therefore been tested twice, once with the luminous model located on the right-hand side and one with the luminous model on the left-hand side, for a total of n = 24. During a recording session, each tested specimen was initially isolated in a 50 mm diameter tube placed in the centre of the aquarium, equidistantly from the models. The five experimental conditions (four light conditions: blue flash, blue continuous, yellow flash, yellow continuous, and a dark control) were successively applied to each worm. A 5 min break for recovery in darkness interrupted each trial.

Video analysis

The IR illuminated images of the organisms were analysed with the automated video tracking software and motion analysis system Ethovision XT 10.0 (Noldus Information Technology 2013). The subject detection is based on its contrast relatively to the background which was increased by IR illumination.

The basic settings include the definition and calibration of tracking areas, the choice of tracked feature (centre-point of the body) and that of the detection method (static or dynamic subtraction: *i.e.* the comparison of the live image with a fixed or updated reference image without subject). The detailed settings optimised for this study are summarised in table SI (supplementary material). The data (coordinates) indicating the position of the subject were extracted and transposed into a series of dependent variables quantifying the swimming behaviour of each worm [**Table I**]. The arena was virtually divided in two zones, each one containing

one or the other model, in order to assess the time spent in each area and to identify possible effects due to the position of the animal.

In addition, the occurrence of typical behaviours (loopings, backwards swimming) were visually identified and reported. Similarly, the images recorded by the low-light-level camera were examined in real time and a second time later by the experimenter in order to identify luminescent emissions.

Statistical analysis

Data extracted from Ethovision (clustered per arena zone and per minute) were statistically analysed using the software package R v. 3.1.2. R (R Development Core Team 2014). Six dependent variables have been tested [**Table I**] in order to analyse the proximity of the worms to the models (taxis) and characterise their swimming pattern (kinesis).

To find evidence of a preference for one or the other zone of the arena as a function of the type and colour of the photic stimuli, we compared for each condition the total time spent in each zone and on the one hand, and the distance (mean, minimum) to the luminous model on the other hand. Then to find evidence of a swimming pattern change (velocity, angular velocity) as a function of the various photic stimuli and of the occupied zone, mean and maximum values calculated over the first and second minute periods were analysed.

We performed a linear mixed effects analysis of the relationship between each dependent variable and each of the independent variables: colour (yellow/blue), type of signal (continuous/flash) and the interaction variable

dependent variables	description	descriptive statistics
Taxis		
in zone cumulative duration [s]	the total duration spent by the center-point of the worm in a given zone of the arena (see above the three designs tested)	total
distance to model [mm]	the shortest distance from the center-point of the worm to each model	mean minimum
Kinesis		
linear velocity [mm/s and body length/s]	the distance moved by the center-point of the worm per second	mean maximum
angular velocity [deg/s]	the change in direction of movement of the center-point of the worm between two consecutive tracking points	mean maximum
backward [nb occurrences]	the number of sudden, quick and straight rearward movements of the worm	total maximum
Loopings [nb occurrences]	the number of 360° turns achieved by the worm while swimming	total maximum

Table I. Dependent variables and arena designs

between both. This model [(1)] takes into account the non-independence between repeated observations for each individual by adding intercepts for subjects as random effect, [(2)] allows the inequality of variances, and [(3)] is unaffected by randomly missing data (non-visited zones). We used the *lme4* package (BATES *ET AL.* 2012) as a tool for estimation of model parameters (restricted maximum likelihood) and model selection based on the Akaike Information Criterion and Bayesian Information Criterion. When visual inspection of residual plots revealed deviations from normality, cube root or logarithmic transformations were applied in order to satisfy this assumption. Then all possible single fixed-effect terms from the mixed effect model were identified using a likelihood ratio. When significativity was evidenced for a given independent variable, pairwise comparisons were tested using a *post hoc* Tukey's t-test ($\alpha_{single} < 0.05$; α_{global} limited to 0.05) to adjust the *P* value, which is less powerful but reduces the risk of Type I error.

The inferential statistics were produced only with the data extracted from the first minute period of observation (all the corrected *P*-values are available in the supplementary material, **Table SII**). Few results being significant and the data from the second minute period showing higher dispersion, the latter were used for descriptive statistics only.

Results

Postures and action patterns

We identified eleven postures and action patterns (a-k) including seven avoidance behaviours (e-k) induced by various mechanical stimuli [**Fig. 4**]. Since we were limited to laboratory observations of isolated individuals, reproductive and feeding patterns could hardly be elicited.

a. Swimming

Tomopterids are part of the Phyllodocida, also called "paddle-worms" because of parapodia transformed as swimming appendages. Incompletely described by Foxon (1936), the locomotion of *Tomopteris helgolandica* involved not only the parapodia, but also the body wall musculature and probably the coelomic fluid as hydrostatic skeleton. Lateral body undulations were produced by the antagonistic action of longitudinal muscles (more developed than the circular muscle layer) on opposite sides of the body in each segment [**Fig. 4a**]. The wave progressed along the body in a caudal direction and pushed the animal forwards. As suggested by Åkesson (1962), the elongated cirri probably act as sensory, balancing and floating organs. In darkness, the mean linear velocity is 0.15 body length.s^{-1} with a

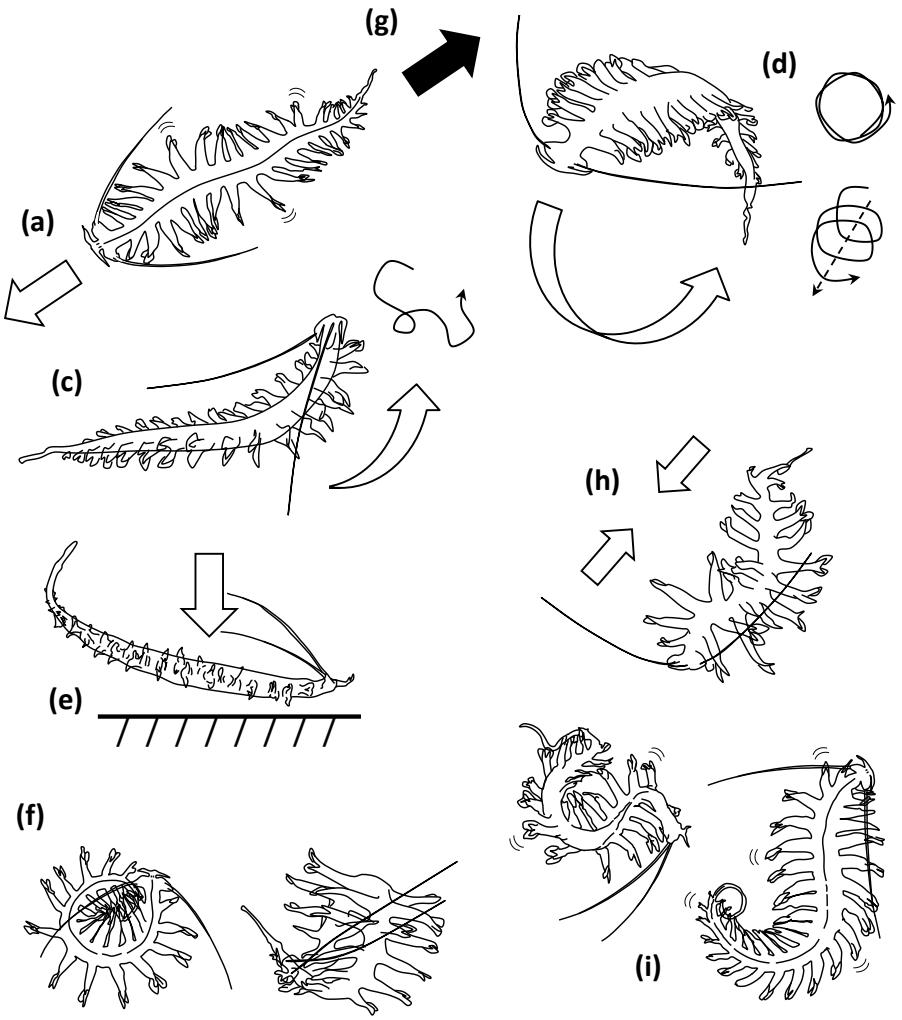


Fig. 4. Posture and action patterns of *Tomopteris helgolandica*.
The letters labelling each diagram also refer to those in the text. **(a)** Swimming; **(c)** Turning; **(d)** Looping; **(e)** Static; **(f)** Balled-up; **(g)** Backwards swimming; **(h)** Body shortening and thickening ; **(i)** Wriggling. The patterns (b), (j) and (k) are not illustrated.

maximum of 0.40 bl.s^{-1} which is not particularly fast compared to other planktonic organisms (*e.g.* 2 mm ostracod crustaceans can reach swimming speeds of 7 cm.s^{-1} or 350 bl.s^{-1}, after RIVERS AND MORIN 2009).

Although some specimens were maintaining a contact with the aquarium bottom while moving, crawling is unlikely to occur under natural conditions. This

typically holopelagic species lacks parapodial features such as chaeta or cirri, which allow benthic species to anchor to the bottom.

b. Undulating/waving

The movements of parapodia were similar to the swimming movements but did not generate motive force. The animals that exhibited this behaviour were not disabled. They generally alternated active swimming, total immobility and undulatory movements. The undulation amplitude was variable. We suggest this behaviour, *via* the muscular contractions of the body walls, to increase the coelomic circulation and oxygen transport while remaining still.

c. Turning

Tomopterids naturally live in a tridimensional environment. Their parapods, their long cirri and the flexibility of their body certainly helped them to orientate in every direction [**Fig. 4c**].

d. Looping

Loopings were generated at constant speed, in both horizontal and vertical planes, but always in the sagittal plane of the animal. Loopings could be associated to a displacement or remain in the same area [**Fig. 4d**].

e. Static

When the worms did not move, they could either sink until they reach the bottom of their aquarium or be maintained in the water volume. The first situation occurred when the aquarium water was stagnant or when the floating capacities of the worm were altered (as suggested by ÅKESSON 1962). The animal's body was slightly arched and touched the bottom at the level of the first third of its body length [**Fig. 4e**]. If the animal was housed in a circulating water aquarium (Kreisel tank type), it was maintained in the water mass and moved thanks to the stream, making little use of its parapodia. This static/sinking behaviour could also be induced in a specimen swimming in calm water by generating a sudden water motion (hydromechanical stimulation) that simulated the presence of an organism.

f. Balled-up

In response to hydromechanical (*i.e.* water motion induced with a pipette) or tactile stimulation, certain worms rolled themselves into a ball [**Fig. 4f**]. A disturbed worm, when it was in this position, kept it until a given threshold – *i.e.* a stimulation of great intensity (pinching with forceps, aspiration with a pipette) – was necessary for him to respond by one of the avoidance behaviours described below (g-k). Balled-up behaviour has been repeatedly noticed in other *Tomopteris* species during field observations (ROBISON 1999).

g. Backwards swimming

When encountering an obstacle or a stimulus coming from ahead, the worms rarely turned around but swam backwards. The direction of body undulation as reported in (a) was inverted.

h. Body shortening and thickening

When the worm was retained with forceps (mechanichal stimulation), its body contracted by shortening and thickening for a few seconds [**Fig. 4h**]. This also occurred during dissection. In that case, part of the coelomic fluid was lost and the worm did not recover its initial size. If the body wall was not damaged, the coelome quickly recovered its initial turgescence.

i. Wriggling

If the mechanical constraint was prolonged, the worm wriggled and rolled up on itself [**Fig. 4i**]. After the end of the stimulus, the worm generally remained rolled up and immobile. It is only after a few minutes that it started to extend and swim again.

j. Production of mucus

When mechanically disturbed, *T. helgolandica* could produce great amounts of extremely viscous mucus that fully coated the animal. This phenomenon was even more spectacular during dissections.

k. Light emission

Although rarely observed in the laboratory, light emissions could occur in response to rough handling (pinching, emersion). All the parapodia emitted light simultaneously. The intensity rapidly decreased and ended after a few seconds. The temporal dynamics was similar to the monophasic response induced in this species by 200 mM KCl or 10 mM carbachol (GOUVENEAUX AND MALLEFET 2013). The emission of *T. helgolandica* is not secreted in contrast to what was observed in other *Tomopteris* species (FRANCIS *ET AL.* 2014) and it was not responsive to photic stimulation (low-light-level video recordings).

Phototaxis

The potential attractant or repellent effect of the luminous model was assessed in terms of time spent in each zone (containing or not the luminous model) and distance to the model.

The analysis revealed no significant differences or trends in term of time spent in each half arena [**Table SII**]. However, when a photic stimulus (regardless of

its type and colour) was applied, the worms tended to swim closer to the luminous model than to the non-luminous one [**Fig. 5**].

Photokinesis

In experimental ethology, a given stimulus sometimes does not induce taxis, but rather affect the activity level of the organism. Analysing other parameters like the linear and angular velocities thus enabled to characterise and quantify non-directional responses to the photic stimuli.

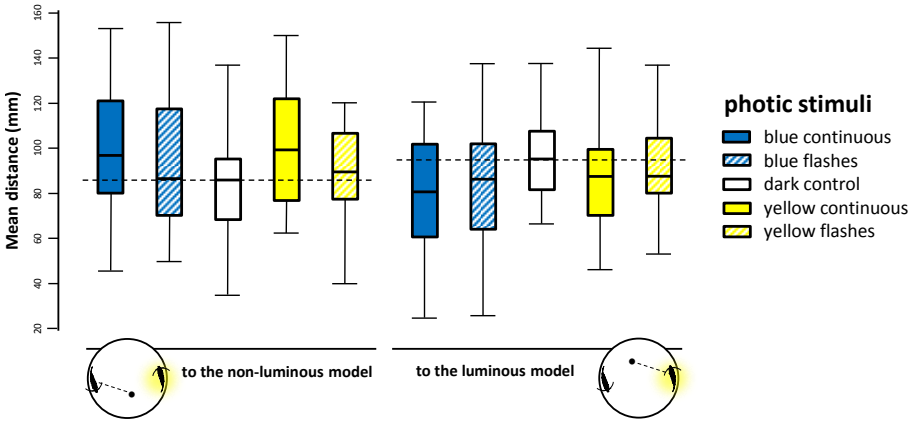


Fig. 5. Boxplot of mean distances to the non-luminous and luminous models during the first minute of observation.
No significant difference was revealed but, under photic stimuli, the worms tended to get closer to the luminous model compared to the dark control.

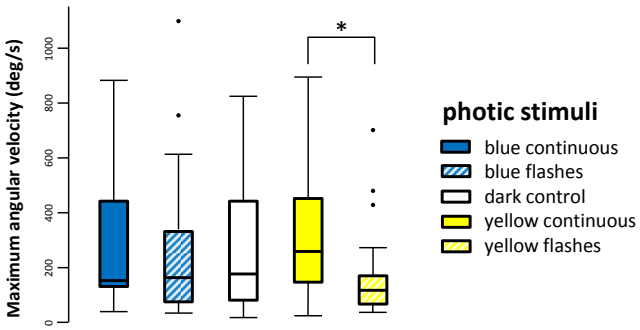


Fig. 6. Boxplot of maximum angular velocity during the first minute of observation.
No significant difference appeared between the control and the various photic stimuli.
* $P = 0.0433$.

Yellow light stimuli triggered significantly different responses (P = 0.0433) in term of maximum angular velocity depending on whether they were applied by flashes or continuously [**Fig. 6**]. It is a case of klinokinesis according to which the stimulus affects the frequency at which the animal turns or changes direction. The maximum angular velocity was higher under continuous stimulation than under flashes stimulation during the first minute but this effect seemed attenuated during the second minute period of observation (not shown).

Regarding the mean and minimum linear velocities, the difference statistically revealed between the colours for the first minute [**Table SII**] was not highlighted by *post hoc* comparisons. This may be the result of the reduced power of the test after correction of the *P*-values or, as suggested by the boxplot representation [**Fig. 7**], may indicate that it was significant by chance. However, a contrast tended to appear between the two half arenas during the second minute of observation with a slight increase of the velocity in the zone containing the luminous model [white arrows in **Fig. 7**]. Looking at the four photic conditions, this effect seemed particularly pronounced under blue flashes stimulation [white arrow in **Fig. SI**].

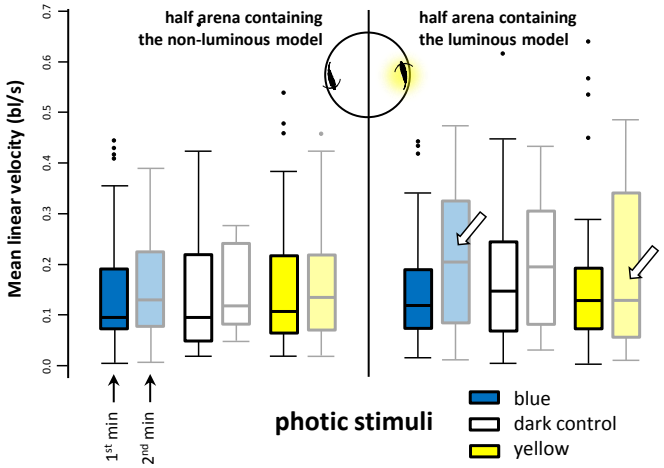


Fig. 7. Boxplot of mean linear velocities per zone.
The mean statistics for the first and second minutes of trial are represented by the bright- and faded-colored boxes respectively. The "blue" and "yellow" conditions each combine the "continuous" and "flashes" levels. No significant difference was revealed. Tendencies appeared more contrasted between the two half arenas during the second minute of observation with a slight increase of the velocity (boxplots stretched to higher values) in the zone containing the luminous model.

Finally, the data concerning the occurrences of looping and backwards swim behaviours were quite homogeneous eventhough their values were too weak and little variable to highlight meaningful differences or to perform statistical analysis [**Tables II and SII**]. Indeed, a total of only 1 to 7 observations of backwards movements were noted per condition, all tested trials combined, but none when the worm stayed in the zone containing the yellow or blue flashes emitting model.

dependant variables	arena settings	simulated light signal				
		blue		control	yellow	
		continuous	flashes		continuous	flashes
total number of loopings	**2 zones**					
	non-luminous model	2 ; 2	10 ; 12	11 ; 6	9 ; 16	13 ; 14
	luminous model	7 ; 5	3 ; 8	8 ; 8	9 ; 4	7 ; 8
total number of backward swimming	**2 zones**					
	non-luminous model	3 ; 2	0 ; 1	1 ; 3	1 ; 3	4 ; 1
	luminous model	0 ; 5	0 ; 0	1 ; 3	1 ; 1	0 ; 0

Table II. Occurrence of backwards swimming and looping behaviours.
The two values reported for each experimental condition refer to the number of observations made from the n = 24 trials available per condition, during the first and second minutes (1^{st} min ; 2^{nd} min).

Discussion

The experimental use of lures as visual stimuli is well known in ethology and has already proved its efficiency in the study of bioluminescence: *e.g.* predatory avoidance of luminescence was demonstrated with luminescent and non-luminescent clay model millipedes (MAREK *ET AL.* 2011), the "e-jellyfish" model made of blue LEDs epoxy encapsulated, was designed to emulate four burglar alarm displays of the deep-sea cnidarian *Atolla wyvillei* Haeckel 1880 and succeeded in luring large squids in their natural habitat (RAYMOND 2008). In this work, we analysed the swimming pattern of *Tomopteris helgolandica* when brought together with a light-emitting silicone model. This first behavioural approach of light perception in this taxon presents few significant effects but several meaningful tendencies.

Photosensitivity

The tested specimens generally tended to swim closer to the model when it produced light, showing they were sensitive to it. This tendency was not influenced by the colour or type of the photic stimuli, but might have been affected by their intensity, the duration of their application or their punctual character. Indeed, the attractive effect of light (the downward irradiance as well as the bioluminescence)

on zooplanktonic organisms is known to be intensity-dependent, with a preferency for weaker intensities (SPOONER 1933). The variations in solar luminosity are agreed to be the main exogenous factor regulating the diel vertical migrations (FORWARDS 1976). In terms of bioluminescence, glowing signals are generally considered as attractive whereas brief signals are found to be repulsive (NICOL 1959, MORIN 1983). However, a thorough study is necessary in order to deconvolve the effect of those various parameters and to precise whether the motivation for attraction is sexual or predatory.

Versatility of bioluminescence function in *Tomopteris*

Because of its unusual spectral characteristics, the bioluminescence of tomopterids is generally thought to be used for intraspecific communication rather than for defence purpose (HADDOCK *ET AL.* 2010). The species-specific distribution of light glands was suggested to produce different intensities and/or spatial patterns of luminescence that might act as recognition signals for mating (HARVEY 1952, DALES 1971). However, as in the case of sexual dimorphism, this argument is valid only if the signals are discriminating, *i.e.* if the differences are likely to be detected by conspecifics, which remains largely unproven in marine organisms (HERRING 2007). On the other hand, given the differential response to flash and continuous yellow stimuli evidenced in this study and the very small separation of the light glands, of about several hundred micrometers, a possible sexual significance of luminescence in *T. helgolandica* is more likely to be related to differences in emission kinetics or associated behaviours than to morphological differences. However, although the tested specimens appeared to modulate their activity according to the type of yellow signal, this latter induced neither original behaviour nor luminescent response supporting the hypothesis of luminescent courtship display in *Tomopteris*. In this context, it is worth reminding that the experimental isolation of the "bioluminescence" variable, accomplished in this study by using of a silicon model, aimed to highlight its effects in a broad sense. More precise questionings, especially those related to bioluminescent courtship displays, need other intrinsic (physiological condition, pheromone production…) and extrinsic (lunar, seasonal and diurnal variations of light and temperature, presence of conspecifics…) factors to be taken into account. Indeed, mate-signalling and mate-finding behaviours in pelagic organisms clearly result from interactions and interpretation of various visual and chemical cues.

A variety of pelagic species, including ostracods, copepods, pyrosomes and ctenophores, are known to emit light in response to photic stimulation (*e.g.* TSUJI *ET AL.* 1970, MACKIE AND BONE 1978, LAPOTA *ET AL.* 1986). Here, attempts to stimulate light-induced luminous responses from *T. helgolandica* failed. Only a few

specimens were repeatedly observed to produce light in a defensive context (*i.e.* when mechanically disturbed). Thus, it may be that yellow light emission rather plays a defensive role.

To ensure their protection against visually-cued predators, the tomopterids primarily rely on crypsis and mimicry. Crypsis, served by transparency, is probably helped by the fact they usually remain still in the water mass. Additionally, when we applied hydromechanical stimuli, some specimens rolled themselves into a ball and remained static instead of escaping. This behaviour, also observed in *Tomopteris nisseni* (Rosa, 1908) in its natural environment, as well as in a variety of taxa (ROBISON 1999), was interpreted as a case of protective mimicry that could also be described as Batesian mimicry. Since *Tomopteris* has no known harmful defence against predators, it might take advantage of the abundance of organisms whose form it imitates. In the pelagic environment, the ideal models could be jellyfish, which are unpalatable preys that some predators have learned to avoid. To some predators, this posture may look like the bell margin of a jellyfish, where, moreover, photogenic sites can be found. There must be a trade-off between optimising their transparency (thinner tissues) and their locomotion and, thus, to flee predators (JOHNSEN 2014). However, the measurement of high lactate dehydrogenase activities in several *Tomopteris* species suggests an adaptation for sustained swimming effort (THUESEN AND CHILDRESS 1993).

Earlier work by Buskey and Swift (1985) revealed sharp changes in direction in the yellow emitter *Tomopteris septentrionalis* (Steenstrup, 1849) when exposed to simulated copepod flashes. This behaviour was then interpreted as a reaction to copepod antipredatory luminescence. According to our results, only a slight increase of linear velocity appeared after one minute of blue flashes stimulation [**Fig. SII**], which does not resemble a reflexive response.

Given the comparatively high threshold of bioluminescence excitability, this reaction, which is energetically demanding, might be regarded as a secondary response to predation. The figure 8 illustrates this hypothesis as well as the possible scenarios and outcomes of an interaction between *T. helgolandica* and one of its predators. In this model, its behavioural response is suggested to depend on the risk of predation or level of encounter between the interactants that was experimentally simulated by the stimulation intensity in our experiments. That being said, bioluminescent defensive signal may act in a variety of way as suggested by the recently evidenced diversity of emission patterns of tomopterid species (GOUVENEAUX *ET AL.*, in press). The light emission of *T. helgolandica*, intracoelomic according to Meyer (1929a) and as recently supported by histological observations (GOUVENEAUX *ET AL.*, unpublished results), could induce a startle effect while the secreted luminescence of *T. carpenteri* (Quatrefages, 1866) and other

species (FRANCIS *ET AL.* 2014) would rather act as a smoke screen confusing the predator while the worm escapes. Also, the abundant production of mucus by mechanically disturbed specimens, possibly induced by stress or as a mean to facilitate the escape, could, depending on its chemical properties, serve an aposematic function.

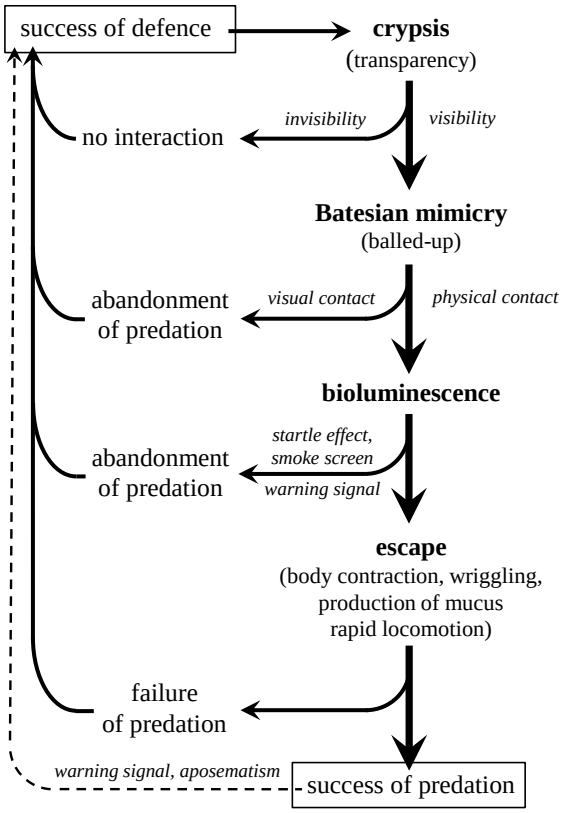


Fig. 8. Flowchart showing the possible outcomes of an interaction *between T. helgolandica* and a predator.
In this model, crypsis, mimicry and bioluminescence are three adaptations structuring a hierarchy of decision-making. Even if predation is a success, the strategies of aposematisms and warning signals can have a benefit at population level.

Conclusion and perspectives

The capacities for locomotion and decision-making in plankton are today acknowledged. However, they remain little studied, despite the fact that they constitute an important factor of structuration of midwater communities.

In this work, we evidenced the ability of *Tomopteris helgolandica* to perceive and to react to bioluminescent-like light signals. Based on our observation of a contrasted response to yellow continuous signals and yellow flashes, it is tempting to encourage further research into the already advocated hypothesis of intraspecific communication. However, the possibility that bioluminescence may fulfill multiple functions, especially of defence against predation, should remain an open question.

To predict the natural behaviour of *Tomopteris helgolandica* in the field from its behaviour in the laboratory only is an incomplete approach. This work merely provides a starting point for more extensive behavioural work, including field observations and stimulating in-depth studies about tomopterids biology and reproduction.

Supplementary material

Ethovision settings	Description	Settings values
Setup		
Experiment Settings	To specify the aspects of the experiment that remain constant during the entire course of the experiment	**Video source**: from video file
		Tracked features : center-point detection
		Units: distance = mm, time = s, rotation= deg
Arena Settings	To specify the region on the screen (i.e. the arena) in which EthoVision XT detects the subject, as well as its possible subdivisions in zones	**Arena** : circular, delimited by the edge of the aquarium viewed from the top
		Calibration of the arena: 95 mm in radius
		Zones: subdivision in 8, 3 and 2 zones
Control Trial Settings	To automate the start and/or stop of data acquisition, and to set a maximum duration for our trials	**Start:** directly after having manually pressed the start button
		Stop: after a delay of 3 minutes
Detection Settings	To select a method to distinguish the subject from the background, to specify how contrasted the subject is from the background, to specify how many images per second we want EthoVision XT to analyze, and to set the average subject size.	**Detection method**: static substraction
		Detection settings: subject is brighter than the background, bright contrast adjusted for each video file, reference image created by the "start learning" method
		Sample rate: 5 or 6,25 samples per second
		Subject size: min = 30 pixels, max = 6000 pixels
Trial List	To pre-define which video files and settings EthoVision XT will use to track the subjects in each trial, and to indicate the levels of the independent variables as well as additional information for each trials	**Video file** : the one corresponding to the trial
		Arena settings: the one corresponding to the trial
		Control trial setting: the unique one for all trials
		Detection settings: the one corresponding to the trial
		Independent variables: Individual N°, color, type of signal
		Additional information: position of the fake worms, position of the luminous fake worm, size of the worms
Acquisition		
Acquisition	The animal is tracked by Ethovision while it is moving in the arena	**Start:** manual, after having correctly positioned the worm in the center of the aquarium
		Stop: automatic, after a delay of 3 minutes
Track Editor	To rectify the position of the tracking points when EthoVision XT tracked objects other than the subject, or failed to detect the subject (missing sample)	Manual rectification with the mouse pointer
Track Smoothing Profile	To eliminate small movements in the tracking path which might affect the measurement of the dependent variables	**Smoothing:** based on 10 samples before and after every sample point
		Minimal distance moved: 0,5mm
Analysis		
Data Profile	To filter and select our data for analysis	**"Per time bin":** tracking data nested over time intervals of 1 minute

Ethovision settings	Description	Settings values
Setup		
Analysis		
Analysis Profile	To specify what dependent variables and what descriptive statistics for these variables to calculate	**"Per time bin**": in zone frequency and cumulative duration; mean and minimal distance to the fake worms; total distance moved; mean and maximum velocity; mean and maximum angular velocity; mean and maximum meander; backward behavior; and rotation behavior
		"Per time bin per zone": mean and maximum velocity; mean and maximum angular velocity; mean and maximum meander; backward behavior; and rotation behavior
Results	To calculate for each trial the dependent variables and their summarized statistics, based on the tracking points acquired	Results calculated based on the specified data profile, analysis profile and track smoothing profile

Table SI. Ethovision XT settings

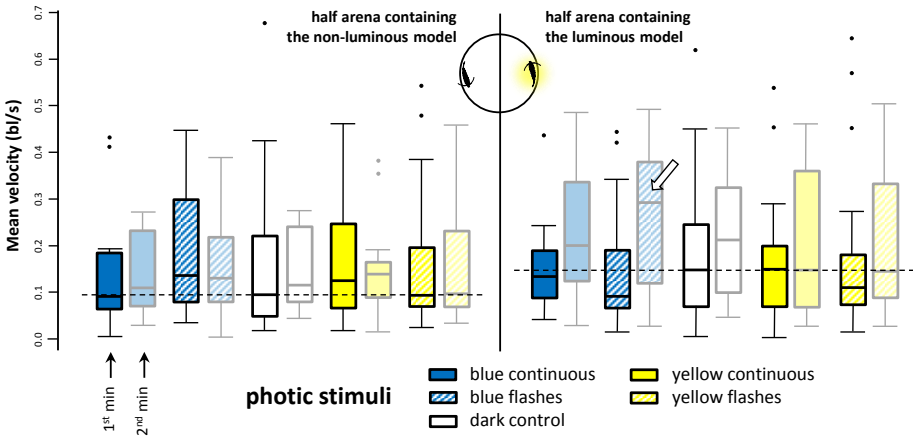


Fig. SI. Boxplot of mean linear velocities per zone.
The mean statistics for the first and second minutes of observation are represented by the bright- and faded-colored boxes respectively. No significant difference was revealed. Tendencies appeared more contrasted between the two half arenas during the second minute of observation with a slight increase of the velocity especially under blue flashes stimuli, in the zone containing the luminous model (arrow).

Dependent variables	1st minute		
	Colour * Type of signal	Colour	Type of signal
Phototaxis			
In zone cumulative duration	n.t. [1]	n.t. [1]	n.t. [1]
Mean distance to fake worm	0.9565	0.7995	0.9093
Minimum distance to fake worm	0.9786	0.8472	0.8759
Photokinesis			
Mean velocity [mm/s]	0.6774	0.5726	0.7094
Mean velocity [bl/s]	0.6874	0.5726	0.7094
Maximum velocity [mm/s]	0.8405	0.8699	0.8678
Maximum velocity [bl/s]	0.8405	0.8699	0.8678
Mean angular velocity	0.2261	0.7227	0.5045
Maximum angular velocity	0.05756 (yellow flashes-yellow continuous: 0.0433*)	0.8061	0.0354* (flashes-continuous: 0.0286*)
In zone Mean velocity [mm/s]	0.0738	0.0195*	0.0886
In zone Mean velocity [bl/s]	0.0772	0.0206*	0.0928
In zone Maximum velocity [mm/s]	0.2380	0.1084	0.1976
In zone Maximum velocity [bl/s]	0.2467	0.1123	0.2038
In zone Mean angular velocity	0.9958	0.9514	0.9173
In zone Maximum angular velocity	0.6282	0.306	0.2934
Total backward	n.t. [2]	n.t. [2]	n.t. [2]
In zone Total backward	n.t. [2]	n.t. [2]	n.t. [2]
Total rotation	0.4083	0.8074	0.1883
In zone Total rotation	n.t. [3]	0.5524	0.3258

Table SII: Summary of the corrected *P*-values from the statistical tests (linear mixed-model) carried out for the first minute of recording.
"In zone" refers to data nested per zone (half arena). * *P*-value < 0.05, the significant *P*-values resulting from the multiple comparisons (*post hoc* Tukey's t-test) are presented in brackets . n.t = not tested for various reasons: [1] no data transformation possible to normality, [2] too small values, [3] no convergence.

4.3.2. Additional results and discussion

Inducing the natural behaviour of *T. helgolandica* in the field from observations in the laboratory is undoubtedly an incomplete approach. The work reported in the paper IV merely aimed to provide a starting point and emphasised the need for more extensive behavioural investigations. In this context, it clearly appears that fondamental knowledge of tomopterids' visual sensitivity should equally be improved and valued.

As part of the project FRFC-FNRS (grant n°2.4590.11), transcriptomic data were used to investigate the extraocular photoreception in several bioluminescent species including *T. helgolandica*. Using a blast approach, we searched and identified the sequences of nine opsin-based proteins: four rhabdomeric opsins, four neuropsins and one peropsin (DELROISSE *ET AL.* 2016). All the corresponding mRNA were found to be more intensely expressed in the cephalic part than in the rest of the body, except the r-opsin 4 or melanopsin [**Fig. 4.6a**]. This photosensitive pigment, originally isolated from the dermal melanophores of *Xenopus laevis* (Amphibia) (PROVENCIO *ET AL.* 1998), has recently appeared to be expressed in both retinal ganglion cells and extraocular tissues (pineal organ, deep brain, iridophores/melanophores), modulating a broad range of behavioural and physiological responses to light such as the control of circadian rhythms, of pupillary constriction or skin pigmentation (HANKINS *ET AL.* 2007, PEIRSON *ET AL.* 2009). Phylogenetic and biochemical evidence clearly support the affiliation of this opsin family with invertebrate photopigments, especially with regard to their signalling pathway (PEIRSON AND FOSTER 2006, KOYANAGI *ET AL.* 2007).

The first immunostainings performed with anti-melanopsin antibodies (OPN4 GTX87627, rabbit, 1:500-1:1000, GeneTex Inc., Irvine, CA, USA) revealed the presence of this opsin in well-defined more or less elongated structure, situated along the apico-basal axis of the rosette glands [**Fig. 4.6b**] (DELROISSE *ET AL.* 2016). The fluorescence outlined the contours of the yellow-pigmented sacculi and stretched as a large bundle of fibres until it reached two deeply labelled cell bodies. Given their location, these two bodies seem to be those outlined by the anti-choline acetyltransferase marking [**Fig. 4.5**].

Leaving aside the demonstrated photogenic function of the rosette glands, this newly-revealed structure may appear as a bicellular photosensitive system embedded in the extracellular cavity formed by accessory cells, namely the photocytes. Interestingly, this hypothesis goes back to Vejdovský's first interpretation of the rosettes as eyes in 1878. In fact, many polycheates are known to possess ectopic ocelli (segmental, branchial or pygidial occelli) in addition to their cerebral eyes (PURSCHKE *ET AL.* 2006). Cerebral eyes are usually composed of

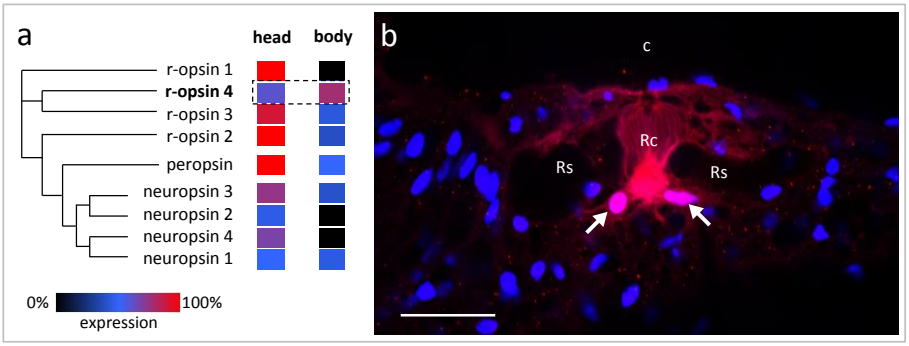


Fig. 4.6. Screening and immunofluorescence detection of opsins in non stimulated *T. helgolandica* tissues (Modified from DELROISSE *ET AL.* 2016 with permission).
(a) Expression of opsin transcripts from RNA extracts from the head and the body of *T. helgolandica*; **(b)** Whole mounts of aldehyde-fixed parapodia incubated with Alexa Fluor® 594- (A-11037, goat, 1:500, Thermo Fisher Scientific, Waltham, MA, USA) conjugated anti-melanopsin antibodies (OPN4 GTX87627, rabbit, 1:500-1:1000, GeneTex Inc., Irvine, CA, USA) and DAPI to stain the nuclei. The bundle of fibers connecting the core structure of the rosettes to the ganglion cells (arrows) are clearly outlined. c: coelomic cavity; Rc: core structure of the rosette gland; Rs: transparent sacculi of the rosette gland. Scale bar 100 µm.

rhabdomeric photoreceptor cells while ciliary photoreceptor cells are present in ectopic structures. The large expanses of their apical membranes, supported by microfilaments, can be observed by cLSM from immunohistochemical preparation (PURSCHKE *ET AL.* 2006). The emergence of these two cell lines from a primitive two-celled eye is associated by Arendt (2008) to the concept of functional segregation (*i.e.* one sister cellular type specifically loses a function that is retained in the other) is to be found within polychaetes with cerebral eyes adapted for vision and ectopic ocelli involved in non-directional photoresponses (ARENDT *ET AL.* 2004). However, given the specific expression of melanopsin in the two cells revealed by immunostaining, these latter would rather be rhabdomeric in nature. This structurally diverse cellular type is believed to outperform the ciliary photoreceptors of invertebrates by supporting vision in both dim and bright light conditions with high sensitivity and versatility (FAIN *ET AL.* 2010). This feature might confer a selective advantage to species that emit or use bioluminescence. Since we failed to induce the light emission in *T. helgolandica* by photic stimuli [**section 4.3.1**], and the two cells observed with cLSM being oriented, like the whole rosette, towards the coelomic cavity, they are probably not used to the perception of extraneous light signals but may rather be involved in a feedback control of light production by the rosette glands. These cells could even be effectors of the activating pathway(s) (cholinergic, calcium-dependent) solicited during the pharmacological tests [**section 4.1.3.1**]: (1) the choline acetyltransferase seemed to be detected in the same cells [**Fig. 4.5**] and (2) calcium is known to make an

important contribution to the transduction cascade of the rhabdomeric cells of invertebrates (FAIN *ET AL.* 2010).

The close anatomical, and possibly functional relationship between the photocytes and what could be photoreceptors (even the dual function of the rosette cells previously described as photocytes and photoreceptor's accessory cells or lens) could represent a case of exaptation: initially, the organ may have evolved for light perception, but later was adapted for light emission. Indeed, since several works on the squid *Euprymna scolopes* (TONG *ET AL.* 2009), the ctenophore *Mnemiopsis leidyi* (SCHNITZLER *ET AL.* 2012) and the brittle star *Amphiura filiformis* (DELROISSE *ET AL.* 2014), there is a growing belief in the dual role of light organs in both bioluminescence and photoreception. The substantial overlap of the emission spectra of purified bioluminescent photoproteins ($\lambda_{max} \approx$ 490-496 nm) and the absorption spectra of the isolated photopigments (λ_{max} = 501 nm) in *M. leidyi* supports the idea that light production and light perception probably co-evolved. The maximal absorption wavelength of the melanopsin expressed by mammalian retinal ganglion cells is around 480 nm while, as a reminder, the emission maximum of *T. helgolandica* is 573 nm [**see paper II**]. However, the melanopsin can reach a second stable state upon blue irradiance, increasing its absorbance up to 520 nm (KOYANAGI *ET AL.* 2007). Interestingly, these two stable states, if they acted through distinct signalling transduction pathways, could provide a means for discriminating different spectral signatures (PEIRSON *ET AL.* 2009).

5. General discussion and perspectives

While the majority of pelagic organisms emit and perceive blue light, some tomopterid annelids have been found to emit yellow light. This singular biological trait is addressed in this general discussion through the conceptual framework proposed by Tinbergen (1963) including both a proximate (the "how" question) and ultimate (the "why" question) analysis of it. Put another way, the discussion first focuses on how tomopterids' light emission is produced and controlled before going into evolutionary questions or, in a broad sense, why they evolve such adaptation.

By extending the field of reflection developed in this thesis, this synthesis intends to inspire further research on the bioluminescence of *Tomopteris*.

5.1. Physiology of light production in *T. helgolandica*

Originated from a mechanism of detoxification of deleterious oxygen derivatives (McElroy and Seliger 1962, Seliger 1975, McCapra and Hart 1980, Rees *et al.* 1998, Timmins *et al.* 2001), bioluminescence is, for many marine species, more than the byproduct of a vestigial metabolic process. It is a controlled, adaptable and ecologically functional cellular response (Lloyd 1977, Haddock *et al.* 2010). This ubiquitous and energetically costly function is ensured by a developed effector system, which can be highly specialised.

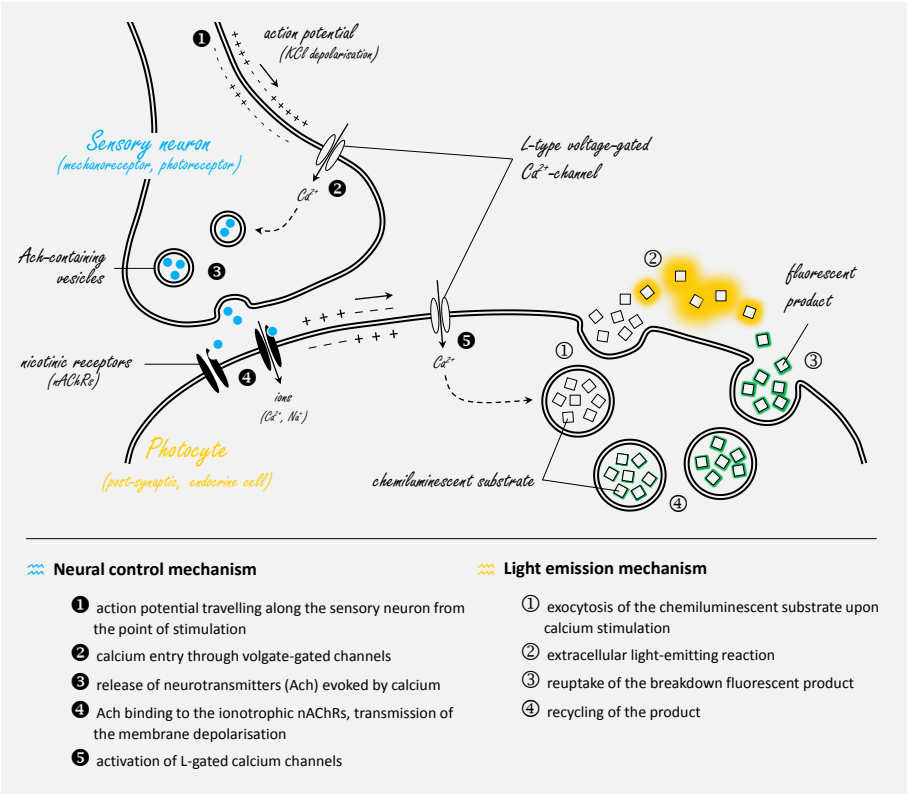
5.1.1. Light emission mechanisms

As we have evidenced, the photogenic structures of *T. helgolandica* are made of multicellular glandular organs distributed by pairs on each parapodium. Contrary to what Greeff (1885) suggested in his model [**Fig. 1.13b**], these rosette-shaped glands seem to be made of only one type of large photogenic cells accumulating yellow-pigmented secretory products in their apical parts. These photocytes converge towards a single opening, which suggests the clearance of light emitting products in the coelomic cavity. The close apposition of yellow-pigmented vesicles forms what we have named the "core structure" of the gland, as opposed to the peripheral and transparent layer of the gland where the nuclei are to be found [**paper III**]. It is, therefore, a polarised structure, but the propagation of light is probably non-directional because of high tissues transparency. However, the peripheral transparent part of the rosettes could play the role of a lens, even if it does not form a differentiated structure. Similarly, the yellow-pigmented core structure may act as a filter involved either in light emission or light reception.

The comparative study of photogenic glands in four other bioluminescent species, *T. planktonis*, *T. carpenteri*, *T. pacifica* and *T. septentrionalis*, confirmed

the structural homology between rosette and hyaline glands (MALAQUIN AND CARIN 1922), the main macroscopic differences between both being their location and orientation, as well as the nature of their secretory products. The rosette glands point towards the coelomic cavity inside the parapodial rami, whereas the hyaline glands point towards the surrounding water and open at the pinnal surface on the posterior side of the parapodia. Consequently, a real exudation of bioluminescent material like we have documented for *T. carpenteri* [**paper II**], is more likely to occur in tomopterids with hyaline glands (*i.e*. the worms belonging to the subgenus *Tomopteris*) which communicate with the sea water through pores piercing the cuticule that covers their pinnules. In contrast, the histological examination of *T. helgolandica*'s rosette glands suggests that its light emission would be intracoelomic, as originally reported by Meyer (1929a). However, both spontaneous and induced light emissions observed all along these years of research remained localised and difficult to distinguish from a truly intracellular event. Two hypotheses could be investigated in the future: according to the first one, which is suggested in the paper III, the exocytosis mechanism might be similar to a transient kiss-and-run exocytosis, as opposed to full-collapse fusion (ALABI AND TSIEN 2013, LING-GANG *ET AL.* 2014). This hypothesis is in agreement with the dynamics of fluorescence associated with the bioluminescent emission of this species [**paper III: Fig. 6**]. We interprete the late increase of intracellular fluorescence as resulting from the exocytosis of a potent non-fluorescent substrate by the rosette core structure followed by the light emitting reaction in the extracellular space. Then, a breakdown fluorescent product might be reabsorbed by the more peripheral sacculi of the rosette gland to be regenerated to a new luminescent material [**Box 5.1**]. Alternatively, *T. helgolandica* might be, like other bioluminescent organisms (*e.g.* syllid worms: DEHEYN AND LATZ 2009, halocyprid ostracods: ANGEL 1968) able to emit graded luminescent responses including extra- and intraglandular processes and the conditions required for a complete exocytosis might not have been gathered here. However, the hypothesis according to which the worms exhausted their light emitting potential during the collection by trawling is unlikely, since *T. helgolandica* and *T. carpenteri* were collected, maintained and treated in the same way, but only *T. carpenteri* was observed to produce a luminous exudate.

Given the variety of chemical (lipoid, osmiophile…), biochemical and fluorescent properties of the yellow-pigmented granular substance depending on species and on the stage of photocyte emission (*e.g.* BONHOMME 1952b, TERIO 1960, 1964, SALERNO 1965), there is currently no consensus regarding the nature and the role of this material in the light-emitting process. Similar cases of undetermined vesicles containing a lipoid substance were reported and suggested as support for light emission in various bioluminescent species (BONHOMME 1952a, SWEENEY 1980). If so in *T. helgolandica*, the yellow-pigmented vesicles probably contain the non-



Box 5.1. Model for bioluminescence emission and control mechanisms based on the results of this work.

fluorescent substrate (possibly a storage form) of the luminous reaction. According to currently available data, its biochemical system is not a luciferin-luciferase system, but rather involves a coelenterazine-based photoprotein (HARVEY 1952, THOMSON *ET AL.* 1997, SHIMOMURA 2006) which, according to us, might be associated with a yellow fluorescent protein, possibly absent or not functional in the blue emitter *T. planktonis* [**paper II**]. For future work, several aspects of this luminous system should be investigated, especially the ultrastructural changes (synthesis and storage organelles or microsources; exocytosis mode) occurring in the photocytes over the light emission process, as well as the biochemical reaction responsible for blue- and yellow emission displays.

As a conclusion, two scenarios about the evolution of the mechanism of the photogenic glands emissions within the Tomopteridae are proposed and illustrated in the **box 5.2**. The first model suggests a distoproximal migration of the gland from a single hyaline-like ancestral gland located next to the pinna, towards rosette glands located at the interface between the pinnal tissue and the coelomic

cavity. The system progresses towards a higher complexity (adding one YFP and a second gland) and an energetical economy (recycling the reactional products that are maintained in the worms' internal environment). However, this model remains difficult to unify with Meyer's hypothesis (1929b) according to which the rosettes I derived from the coelomostomes located on the trunk of the parapodia. The second model suggests a succession of species in a reversed order, with a proximodistal migration of the glands and a simplification of the system: deactivation or loss of the YFP, loss of a gland and loss of the tail. Assuming that a YFP catalyses an energy transfer from the chemiluminescent reaction, we speculate that the yellow emitting system was nonetheless preceded by a blue emitting system. The strong selective pressure in favour of blue colour in the pelagic environment also supports this hypothesis. However, the apparition *de novo* of a yellow emitting system, later modified towards the blue colour, should not be excluded. In section 5.2, we discuss the various factors that may have influenced the evolutive direction of these emission mechanisms as communication systems.

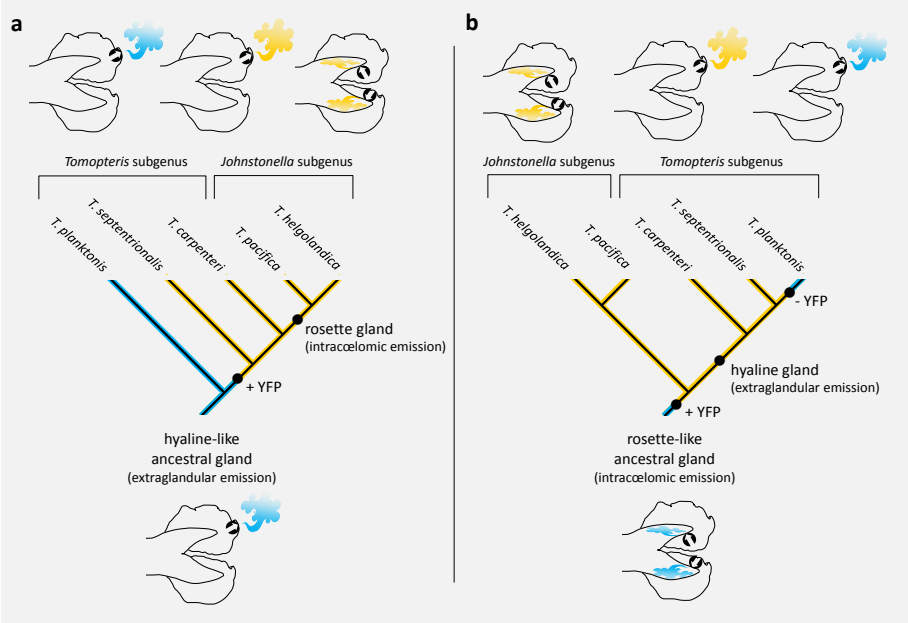
5.1.2. Neural control mechanisms

In the papers I and II, the bioluminescence of *T. helgolandica* and *T. planktonis* was found to depend on the cholinergic nervous system. The involvement of this nervous pathway was already highlighted in other bioluminescent annelids, including the Polynoidae and Chaetopteridae (NICOLAS *ET AL.* 1978, GARDNER AND WALKER 1982, ANCTIL 1987). However, the cholinergic control mechanisms are generally widespread amongst annelids (GARDNER AND WALKER 1982, WALKER *ET AL.* 1996). In this regard, Anctil (1987) stressed that there is no need for novel or distinct nerve pathways to account for bioluminescence control since the photogenic structures of annelids are derived from pre-existing effectors (epithelio-muscular, mucus cells…) already associated with nervous control systems.

At this stage, the triggering pathway to light emission is still incompletely characterised but we are able to propose a first functional model including some of the identified effectors [**Box 5.1**]. Assuming the existence of direct neuro-photocyte synapses[3], the light emission would be induced by the activation of nicotinic receptors located in the photocytes' membrane. This would allow a fast response without having to mobilise a transduction chain. The depolarisation transmitted by the receptors could possibly be transmitted to L-type calcium channels, which would amplify the signal and modulate the cellular response. These channels might also be located on the surface of sensory neurons (mechano-receptors or photoreceptors) to activate the release of neurotransmitters.

[3] This is not always the case in bioluminescent systems controlled by nervous pathway. It remains to be proven in the case of *Tomopteris*.

However, calcium, which is to be found in all excitable cells including the photocytes (CASE AND STRAUSE 1978), can have multiple functions, and the role of the nucleotidic second messenger (cAMP, cGMP) that are not encompassed in the model presented here remains to be determined. Cytological analyses should also be implemented in order to highlight more precisely the innervation pattern of photogenic glands, and the role of the two hypothetical photosensory cells observed inside the rosettes of *T. helgolandica* which stained positively to anti-melanopsin antibodies [**see section 4.3.2**]. Given the differential opsin gene expression between cerebral and ectopic photoreceptors, the latter might be involved in a feedback mechanism of control of the light emission rather than in visual, image-forming reception. Such mechanism would allow adjustment of the emission features that were proven to be versatile [**paper I**]. In any case, the role of extraocular photoreception in bioluminescent invertebrates (TONG *ET AL.* 2009, SCHNITZLER *ET AL.* 2012, DELROISSE *ET AL.* 2014), but also in vertebrates (DUCHATELET 2014), remains unknown, but it is likely to be determinant both in the design of light signals and in the evolution of the light emitting organs.



Box 5.2. Evolution scenarios of the light emission mechanisms within Tomopteridae (biochemical and dynamic aspects).
(a) Distoproximal migration of light organs; **(b)** Proximodistal migration of light organs. Only the five studied species are included in these trees and might not be representative of the evolutionary history of the tomopterid family.

5.2. Evolution and diversification of bioluminescence in tomopterids

When it comes to dealing with the evolution of bioluminescence, almost all attention is paid to the multiple origins of this phenomenon with emphasis on the diversity of light emitting biochemical mechanisms (HASTINGS 1983, MCCAPRA 1990, CAMPBELL 2012), but less and less to its evolution as a major communication system. In *Tomopteris*, the nature and properties of their bioluminescence, as they seem to contradict the idea of an efficient communication system, suggest an evolutionary history made of trade-offs (YOUNG 1981). In this sense, they are a valuable case study which reminds us that there is usually more than one evolutionary response to a particular environmental challenge (SOUTHWOOD 1988).

The pelagic environment, as opposed to coastal and terrestrial habitats, is considered as chemically stable and optically homogeneous (WIDDER 2002, JOHNSEN 2014). As appointed in the introduction, this last feature could explain certain convergent and almost exclusively marine adaptations such as crypsis by transparency or counterillumination by bioluminescence (MCFALL-NGAI 1990). However, it does not explain the impressive diversity of bioluminescence communication systems exhibited by most species living in this environment. Indeed, the environmental constraints mainly affect one intermediary stage of the communication process, between an emitter generating the signal and a receiver perceiving and interpreting it. Many other biophysical, physiological, neurocognitive and behavioural factors actually influence the stages that precede and follow the signal transmission through sea water and thus affect the direction of the communication system evolution. According to the "sensory drive" hypothesis, any change in the cycle of interactions between signals, transmission properties, sensory systems and signalling behaviours alter the entire system of communication (ENDLER 1992, 1993, ENDLER AND BASOLO 1998). This model is an interesting tool to describe the evolution of communication systems and to figure out how each component of the system can shape it.

This work, as many others dealing with the adaptive value of bioluminescence (*e.g.* MESINGER AND CASE 1992, MAREK *ET AL.* 2011, JONES AND MALLEFET 2013), focused on the structure of the signal, its mechanism and its organs of production (summed up in the previous section) but gave only a secondary importance to the receiver. According to our behavioural observations, this latter might be a conspecific, a predator, or alternatively one or the other according to the situation at hand [**paper IV**]. This section is provided with two models of communication systems [**boxes 5.1 and 5.2**] that synthesise and illustrate the

various discussed hypothesis. The readers are invited to refer to those throughout their reading of this section.

5.2.1. Constraints of the pelagic environment

Of all the long-distance systems of communication beneath the sea, acoustic systems are the best (TYACK 1998). However, although the speed of sound is affected by the temperature, salinity, and pressure of the seawater, they diffuse freely and omnidirectionally[4] in the pelagic environment and can thus easily be intercepted by predators or animals other than the intended recipient. In addition, it appears that only three groups use this mode of communication, namely the crustaceans, fishes and marine mammals. Conversely, bioluminescence seems to be a communication system accessible to a broader spectrum of phyla, including unicellular organisms (HERRING 1983, WIDDER *ET AL.* 1983, HADDOCK *ET AL.* 2010).

Although the open ocean is considered as a homogeneous environment, the distribution of pelagic life is well-known to be highly structured by light [**Fig. 1.2**]. In pure water, an increased distance from the light sources induces a logarithmic decrease of light intensity, while the absorbance of short, ultraviolet and long visible wavelengths of the spectrum results in a predominance of blue light at depth (HERRING 1990, MCFALL-NGAI 1990). In contrast, nearshore pelagic environments could be much more variable than the open ocean, especially because of the seasonal inflow of soluble and suspended, organic and inorganic, light-absorbing material in coastal waters (JERLOV 1976, MCFALL-NGAI 1990).

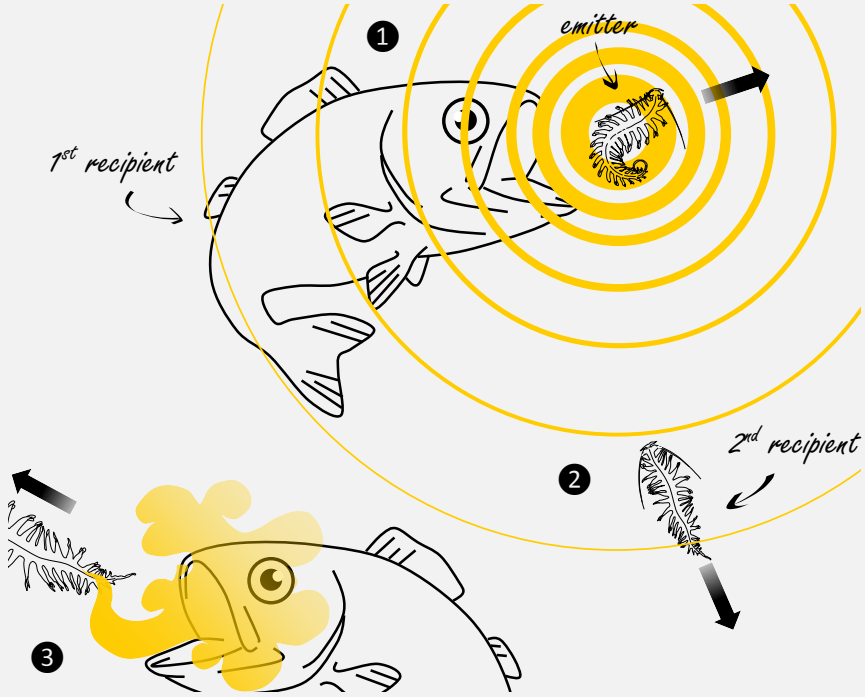
This being said, natural selection should logically favour signals, receptors, and signaling behaviour that maximise transmission to the recipient and minimise signal degradation (ENDLER 1992, 1993). As predicted, the pelagic environment clearly favours the evolution of blue bioluminescence (λ_{max} = 450-490 nm) which is the most efficient colour for long-distance communication (HERRING 1983, WIDDER *ET AL.* 1983, HASTINGS 1996). A spectrally long-shifted signal as observed in *Tomopteris* implies more strict propagation limits and theoretically reduces the viewing distance. However, (1) at short distance, signal loss is squarely proportional to the distance from the light source, regardless spectral parameters, so short-range signals favour more diverse emission wavelengths (HERRING 1990) and (2) in order to be visible underwater, a visual signal must above all contrast with the background light field by showing a significant signal-to-noise ratio (DENTON 1990, JOHNSEN

[4] With the exception of the SOFAR channel (Sound Fixing and Ranging channel) or DSC (Deep Sound Channel) which "traps" and effectively carries acoustic signals horizontally on very long distances. This phenomenon occurs because of the combined effect of the thermo-, pycno- and haloclines, creating a layer inside which the sound propagates by refraction on the upper and deeper fronts. At low and middle latitudes, the SOFAR channel axis oscillates between 600 and 1200 m deep (OER 2010).

2014). The yellow colour happens to be the one which best contrasts with the oceanic blue waters since the one emits in the spectrum where the other does not and *vice versa* (MARSHALL 2000a, 2000b). This is probably the reason why many reef fish exhibit skin patterns combining yellow and blue colours. Indeed, this complementary combination is believed to simultaneously provide a conspicuous signal to close observers and an efficient camouflage to the potential predators at a greater distance (MARSHALL 2000b). As a conclusion, the yellow light signals displayed by some tomopterids are likely to have evolved as a short-distance signal, mainly influenced by close interactions with visually skilled organisms. The question of the photosensitivity of the recipient, which is of crucial importance, is addressed in the next section.

Signalling behaviours can also evolve to favour the times and locations that would best transmit the signal. In fact, the enhanced turbidity of nearshore waters at certain times of the year increases the light absorption. On the surface or in deep-sea, night or day, the contrast and therefore the visibility of a luminous signal are also highly affected. In the long run, the periodicity of signalling behaviours can find itself deeply inscribed in the biology of certain species that, for instance, exhibit circadian cycles of responsiveness to stimulation (*e.g.* in dinoflagellates: JOHNSON *ET AL.* 1985), or a synchronous development of reproductive and photogenic organs (*e.g.* in leiognathid fish: IKEJIMA *ET AL.* 2008, SEBASTIAN AND INASU 2012). These are good illustrations of the interdependency relation between different stages and effectors of bioluminescence communication. Salerno (1965) reports a visible increase in the bioluminescence of *Tomopteris* associated with sexual activity, which, at these latitudes, seems to occur in March, although this has never been documented. The importance of photosecretory systems in benthic environments, compared to pelagic environments, appear to depend on the life-history strategies of the species, especially on their mobility (MORIN 1983). Given that Tomopteridae are a strictly holopelagic family, the diversification of their emission dynamics [**papers II and III**] probably reflects the evolution of an extraglandular emission system towards an intracoelomic, or intraglandular, production, *i.e.* allowing the reabsorption and recycling of reactional products (HERRING 1985b).

It is doubtless in the interest of *Tomopteris* to report their presence by emitting visible signals, especially during reproduction. Yet, as cryptic and transparent organisms, discretion remains an absolute priority. In this context, bioluminescence presents the double advantage of being optional (as opposed to permanent pigmentation) and exclusive (as opposed to an acoustic signal reaching many unintended receivers). Tomopterids can thus produce a luminous response at a precise moment and direct it towards a close target, whether it be a conspecific



〰 When *Tomopteris* is confronted with a predator, its bioluminescence (probably in the form of bright pulses) may act as a short-distance deterrent signal (❶) and simultaneously as a short/mid-distance warning signal to conspecifics (❷).

〰 The emission of an extraglandular exudate like in *T. carpenteri* and, most likely, in many *Tomopteris* with hyaline glands (❸) may act as a confusing signal (*i.e.* smoke screen strategy) or, if the emission was associated with a repulsive mucus, as an aposematic signal.

〰 The short-range propagation of the luminous signal being independent of its wavelength (HERRING 1990), its contrast with ambient light and its intensity could be sufficient for at least part of the signal to be perceived by predators with diverse visual capacities.

〰 The predator's visual system is not likely to co-evolve under the influence of this type of interaction, but the development of an exploiter behaviour could lead towards this evolutive direction.

Box 5.3. Model for short-distance defensive function

or a predator, probably without risking to be detected by a distant predator. Consequently, if environmental factors are highly important in the evolution of bioluminescence in *Tomopteris*, the receivers implied in this communication system have arguably contributed to the development of the emission patterns observed. In this sense, Lloyd (1977) speculated that luminescence might have evolved in the mesopelagic zone from pre-existing communicative signals depending on reflected light and the occurrence of well-developed photoreceptors. This would explain the prevalence of bioluminescent species in this environment.

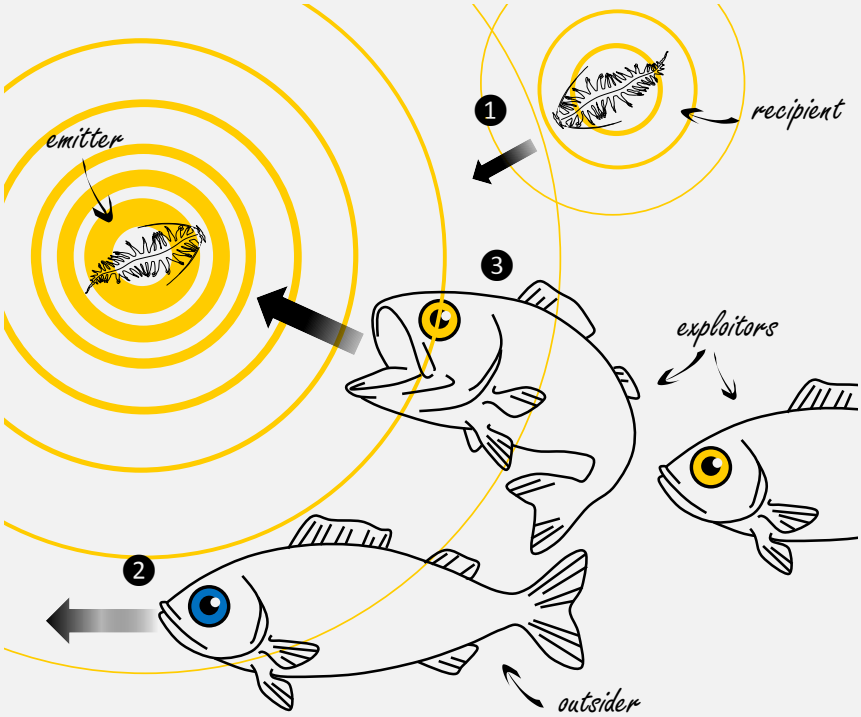
5.2.2. Perceptual tuning and spectral matching

Obviously, bioluminescent signals can have an evolutionary advantage only if they are received (HERRING 1990, LLOYD 1977). In fact, the receiver's capacities for perception and information processing contained in the signal are certainly the main selective forces for the evolution and maintenance of bioluminescent systems of communication. Endler and Basolo (1998) distinguish five stages between the reception of the signal *via* a sensory system, and the receiver's response: transduction, coding, perception, classification and assessment. Each of these stages can influence the evolutionary direction of the system, depending on diverse factors.

The marine visual systems are remarkably diversified, but they have mostly been studied in fishes, squids and crustaceans (DENTON 1990, WARRANT AND LOCKET 2004). For a review of photoreceptive structures in annelids, see Purschke *et al.* (2006) and Suschenko and Purschke (2006). As might be expected, we observe that the majority of known visual systems are only sensitive to a narrow range of wavelengths corresponding to the blue submarine daylight. However, because of local adaptations in photoreception, all marine organisms are not equal regarding their capacities to receive bioluminescent signals. The changing nature of the light environment with depth – from the background daylight to bioluminescent point sources – affect both the acuity, the spatial resolution and the spectral sensitivity of the visual systems (DOUGLAS *ET AL.* 1998, WARRANT AND LOCKET 2004). Thus, it is believed that the visual pigments of deep-sea predators may be under significant selective pressure for maximising the sensitivity to bioluminescence (DOUGLAS *ET AL.* 1998). Interestingly, some mesopelagic and deep-sea fishes and cephalopods were even found to possess yellow-pigmented lenses acting as shortwave cut-off filters (MUNTZ 1976, DOUGLAS AND THORPE 1992). The function of these lenses may be to discriminate bioluminescence from the residual surface light and, especially, to break the camouflage of counterilluminating species. We will come back to this adaptation later. According to Herring (1990), a finer tuning of visual and bioluminescent adaptations might be expected for intraspecific communication and might contribute to reproductive isolation and speciation (BOUGHMAN 2002,

CARLETON 2014). Substantial overlap between absorption and emission spectra were evidenced in several bioluminescent species, including alciopid and syllid annelids (WILKENS AND WOLKEN 1981, WALD AND RAYPORT 2009), but data on the spectral sensitivity of tomopterids are currently unavailable. Although the alternative behavioural approach developed in this work [**paper IV**] did provide encouraging results, it remains inconclusive with respect to the intraspecific function of yellow light and reinforces the idea that direct assessment of their spectral sensitivity through microphotospectrometry or electrophysiology should be made. However, the efficiency of the signal is not solely in the receiver's sensorial capacity, but also in its capacity to process the signal, to extract and to interpret its content (*i.e.* the encoded information). This field of study especially resorts to neurocognitive biology notions that have been left aside here. Instead, the next paragraph puts particular stress on the subjectivity of the criteria used to discriminate the informations carried by a given signal.

The analysis and interpretation of a given signal are likely to vary according to whether the receiver is a conspecific, a prey or a predator, each of these being able to identify and select different coding parameters (such as colour, intensity or frequency). According to our results, the bioluminescence of *T. helgolandica* is likely to be versatile [**papers I and II**] and might be involved in various interaction contexts [**paper IV**]. In response to a specimen emitting trains of yellow flashes, conspecifics could give importance to the signal's colour, distinguish different frequencies (as suggested by our video analysis) or spatial patterns, whereas a predator would rather be influenced on the signal's intensity. On the other hand, one could imagine a brief and intense light emission to be informative for both predators and conspecifics, thus respectively acting as a deterrent and warning signal [**Box 5.3**]. In this regard, Herring (1990) rightly pointed out that the criteria experimentally used to characterise and distinguish bioluminescent displays do not necessarily reflect those that the receivers pay attention to. Their attentiveness could also be affected by their physiological condition, activity and psychology (GUILFORD AND DAWKINS 1991, ENDLER 1993). For instance, a hungry, foraging receiver may be less available and attentive to sexual signals than a sated one. The idea of "psychological landscape" as developed by Guilford and Dawkins (1991), supposes that the brain of any animal, even relatively simple organisms has its own history of ancestral innovations, changes in capacity and efficiency compromises, resulting in a system with original distinctive characteristics. Thus, a receiver may find certain features of a signal particularly attention-getting or memorable for reasons independent of the signal design or its sensory system. Often neglected, this could considerably contribute to the signal's evolution. Surprisingly, the features of the receivers' psychological landscape, namely what he finds easy to detect, to discriminate and to remember, may have contributed to the selection of



〰 Long-distance communication might be possible between yellow-emitting *Tomopteris* conspecifics (❶). The sensitivity of tomopterids to yellow light is still to be demonstrated.

〰 At a distance, the intensity of the remaining yellow light is too weak to be detected predators (typically endowed with short-wavelength-biased visual systems) (❷), except by a visually adapted exploiter (❸).

Box 5.4. Model for intraspecific private communication

what Young (1981) called “exotic” bioluminescence colours. Mesopelagic receivers usually having one, sometimes two or three visual pigments, they might be better at detecting fine contrasts (including the conspicuous yellow and blue combination). The discriminability and memorability of the bioluminescence of *Tomopteris*, compared to other signals, probably takes advantage from the extreme scarcity of yellow light in this environment. Although *Tomopteris* tries to mimic other organisms by changing its shape [**paper IV**], its spectral signature gives to it a strong identity. These characteristics are particularly interesting for species recognition, but also for aposematism. Many animals are predisposed to avoid contrasted or novel signals, which, additionally, are unlikely to be associated with those of palatable preys.

Some unintended receivers, called exploiters, can also intercept and take advantage of the emitted signal. Their intrusion in the communication system influences its evolution at the same level as the main actors do. This problem can be limited by quantitatively or qualitatively reducing the emission bandwidth: light flashes are energetically less costly to produce than a long signal, thus allowing to minimise the reception window. Such a tuning induces a trade-off on signal efficacy. In comparison, the yellow emission of tomopterids would be a more exclusive and selective innovation that presents numerous advantages. If we assume that they have adapted perception capacities, *Tomopteris* of the same species benefit from a private communication channel that most organisms (especially predators) cannot reach [**Box 5.4**]. As pointed out by Endler (2000), signal degradation (here, absorption of long wavelengths by sea water) is not necessarily a disadvantage and can be used to mitigate the trade-off between intended and unintended receivers. In addition, the uniqueness of this signal in the pelagic environment does not *a priori* favour the coevolution of the potential benefiter's sensory systems. Indeed, *Tomopteris* would have to be a major, if not the only, trophic resource of a predator for this latter to develop a countermeasure (yellow visual sensitivity). The system of red illumination and red sensitivity used by the stomiatoid fishes to reveal the presence of red-pigmented preys was probably favoured by the prominence of this system of camouflage in deep-sea invertebrates (JOHNSEN 2002, 2014). To date, they are the only known to have long-shifted visual pigments (*ca.* 510-600 nm), compared to other mesopelagic and deep-sea animals (DOUGLAS *ET AL.* 1998, 1999). In addition, it should be remembered that some visual predators are endowed with yellow lenses (*supra*, p. 176) that are expected to make the long-shifted type of bioluminescence more conspicuous. However, yellow lenses [(1)] are exceptions and [(2)] might have primarily evolved in response to counterillumination which is more widespread than yellow-light emission (Muntz 1976, DOUGLAS AND THORPE 1992). At best, these adaptations can help predators feeding on Tomopteridae, but they probably do not form a specifically coevolved countermeasure to their yellow bioluminescence. Finally, the major constraint to the emergence of new detection systems in potential exploiters, and, paradoxically, the major asset of the bioluminescence of *Tomopteris*, remains the low diffusivity of long wavelengths on long distances.

5.2.3. Species recognition and speciation

Species recognition mechanisms are usually suspected in bioluminescent organisms showing interspecific or sexual differences in the emission patterns or in photophores distribution (HERRING 2000). Based on the species-specific patterns of rosettes distribution, it has been suggested that such communication mechanisms occur in tomopterids (DALES 1971). However, as Herring rightly pointed out (2007),

these differences could be functional only if they are perceived and discriminated by the conspecifics. Thus, if a gap of a few hundreds of micrometers between the position of the light glands of *T. helgolandica* and those of related species is certainly insignificant, the difference of emission dynamics (intra- or extraglandular) associated with their respective location [**paper III**] possibly have a significant effect on specific recognition. In that respect, it is known that the organisms with a limited morphological plasticity or subject to a strong selective pressure (*e.g.* competition for mating) can develop or extend their semiotic repertoire by modulating the emitted signals or by using various postures and action patterns. However, the idea of behaviours associated with bioluminescence does not necessarily reflect the evolutionary direction of the communication system, since, according to Lloyd (1977), luminescence might have amplified or highlighted already-existing signals such as postures or movements. Finally, these signalling behaviours result from the coevolution of both a behavioural potential and light emitting structures. Thus, an important morphological and distributional variety of photophores would indicate a significant diversity of behaviours (HERRING 1990).

In general, the need for intraspecific recognition makes sense, especially during reproduction. In fact, the evolution of recognition system is particularly sensitive to sexual selection and bioluminescent courtship displays are most likely a sexually selected trait. The mating system of ostracods, which is doubtless the most studied and the best known system involving bioluminescent behaviours, is characterised by a strong competitive pressure between males. Consequently, they try to stand out by exploring a wide range of signalling behaviours and developing a different bioluminescent identity (RIVERS AND MORIN 2008, 2009). Like also hypothesised for deep sea fishes (CLAES *ET AL.* 2015, DAVIS *ET AL.* 2014, 2016) and recently highlighted for various luminous courting lineages (ALFARO 2016, ELLIS AND OAKLEY 2016), this process is likely to contribute, *in fine*, to genetic isolation and to significant increase of speciation rate (PALUMBI 1994).

Although an increase of bioluminescence associated with periods of sexual activity was reported in *T. nationalis* (SALERNO 1965), there is currently no evidence of bioluminescence involvement in a possible courtship display. However, the discovery of a blue emitting species, *T. planktonis*, which occurs in sympatry with the yellow emitter *T. helgolandica* [**paper II**], may indicate that, by creating distinct semiotic niches, the bioluminescence participates in maintaining a reproductive barrier. In order to explore these interesting questions, I hope this work will stimulate in-depth studies about the general biology, ecology and reproduction of tomopterids, and that it will inspire researchers to contribute to establishing a resolved phylogeny, without which the evolutionary history narrated by the Tomopteridae will remain as inscrutable as a drop of yellow light in a blue ocean.

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Appendices

Appendix A: Polychaete annelids

The phylum name Annelida (from the latin *anulus* meaning little ring and the greek *εἶδος/eidos* meaning form, shape) was coined by J.B. de Lamarck in 1801 to define the segmented worms such as sand worms, tube worms and leeches. The taxon currently comprises about 17,000 described species, including over 10,000 polychaetes. They have successfully invaded all habitats where water is available. Errant, burrowing, tube-dwelling, interstitial or planktonic, the polychaetes took advantage of a large diversity of life history strategies making them particularly abundant in the Ocean (FAUCHALD AND ROUSE 1997, BRUSCA AND BRUSCA 2003).

Here are proposed some additional information about annelids (including bibliographic and terminological elements) useful to the general understanding of this work. As a comparison, the original characteristics of tomopterids are recalled at the end of each paragraph.

General anatomy

Polychaetes are triploblastic schizocoelous coelomates. Their body is usually divided into three fundamental regions: (1) the presegmental region, composed of the prostomium and the peristomium, (2) the segmented region, or metastomium, forming the bulk of the body, and (3) the posterior non-segmental region, named pygidium. The segments develop from a prepygidial growing zone. Based on this general scheme, a large diversity of forms is represented in this polyphyletic class (GRASSÉ 1959, FAUCHALD AND ROUSE 1997, ROUSE AND PLEIJEL 2001).

Anterior region

Polychaete anterior region exhibits a variety of appendages, including sensory organs (antennae, palps and cirri) and extensive feeding and gas exchange tentacular structures. The prostomium, carrying photosensitive structures and containing at least a part of the brain, is more or less fused with the peristomium, where the oral orifice is located.

The visual systems of annelids are highly diversified (reviewed by PURSCHKE *ET AL.* 2006). Three types of photosensitive cells are represented: rhabdomeric, ciliary and phaosomous cells, forming various visual structures. Some of them are only present during the larval phase and either replaced by the adult eyes or completely reduced during postlarval and adult stages.

→ *The eyes of* T. helgolandica *are composed of seven large rhabdomeric cells and do not show close resemblance with those of other polychaetes. (ÅKESSON 1964).*

The foregut is often modified as eversible stomodeal pharynx (proboscis) and is sometimes armed with chitinous jaws. Depending on the feeding method (deposit, raptorial, filter feeding), the food intake can be facilitated by a number of accessory structures (FAUCHALD AND JUMARS 1979, ROUSE AND PLEIJEL 2001, BRUSCA AND BRUSCA 2003).

→ *Tomopterids are believed to grasp preys using their unarmed, strongly muscularised eversible proboscis (LEBOUR 1923, RAKUSA-SUSZCZEWSKI 1968).*

Segmentation, parapodia and chaetae

Each segment usually carries a pair of paddle-like and highly vascularised parapodia, in addition to various segmentally arranged internal organs. Parapodia often consist of two rami, a dorsal notopodium and a ventral neuropodium. They are basically used for locomotion, but, in many species, also act as the worm's primary respiratory surfaces.

A major external feature of virtually all polychaetes is the presence of chaetae (or setae) projecting from the parapodia. An exceptional diversity of forms, distribution patterns and functions has evolved. For instance, capillary chaetae, which are found in most modern families of polychaetes, are important in locomotion, stabilisation, mechano-reception and protection. Hooked chaetae are generally found in worms that need to anchor such as tube-dwelling, symbiotic or interstitial species (reviewed by MERZ AND WOODIN 2006).

→ Tomopteris *do not bear chaetal structure other than the tentacular cirri of the first and second segments. The first pair can be observed in larvae but usually regresses in adults (FAUCHALD AND ROUSE 1997).*

Segments are separated from each other by septa and the coelom is divided vertically into two parts. The external and internal anatomical organisation follow a repetitive pattern, except some parts of the body, which can have specialised structures and functions (regionation). Some species have a fixed number of segments while some others continue to develop new segments throughout their life. One or several segments can autotomise and regenerate another individual (architomy).

→ Tomopteris *do not have intersegmental septa nor gular membrane, so the coelomic cavity forms a continuous space divided by the dorsal and ventral mesentery of the gut only (MEYER 1929a, FAUCHALD AND ROUSE 1997).*

Physiological anatomy

The physiological systems of polychaetes include the circulatory system, the respiratory system, the digestive system, the excretory system, the reproductive system and the nervous system. This taxon being highly diversified, numerous degrees of complexity are represented.

→ *In tomopterids, which lack both vascular system and respiratory organs, the circulatory and respiratory functions are fulfilled by the circulation of the coelomic system (MEYER 1929a). The coelomic cavity is also a passage zone for excretion and sexual products. Their gut is a straight tube without differentiated region apart from the muscular pharynx.*

Segmental organs

The terms segmental organs and segmental ducts have nothing to do the segmentation process, but rather refer to the segmentally arranged ducts used for both waste products excretion (nephridia) and gametes emission (gonoducts). Nephridia can have the coelomic end closed (protonephridia) or an open funnel connecting the coelomic cavity to the external environment (metanephridia) ROUSE AND PLEIJEL 2001. According to Bartolomaeus (1999), segmental organs originally function as excretory organs and, after having been modified, may also act as gonoducts during maturity. Consequently, these structures are likely to arise from a single tissue source, but they are not the result of the fusion of two kinds of ducts, as stated by Goodrich (1945). However, some polychaetes have additional dedicated genital ducts which are probably non-homologous to the nephridia. Finally, if none of these structures is differentiated, the genital products can be released outside by rupture of the body wall (*BARTOLOMAEUS 1999*).

→ T. helgolandica *has paired metanephridial organs, which open to the exterior ventrally and close to the frontal parapodial base. These nephridia can be found from segment VIII onwards in each segment. In male specimens, these nephridia store the sperms before they are released, whereas in females additional gonoducts are developed later in ontogeny in segments VI and VII (BARTOLOMAEUS 1997, MEYER 1924, 1926). According to Meyer (1929a), the rosette glands are derived from the coelomostomes.*

Nervous system

The fundamental plan of the nervous system in annelids consists of a dorsal cerebral ganglion connected to one or several ventral longitudinal nerve cords by two circumoesophageal connectives. The typical rope-ladder-like nerve cord consists of paired segmental ganglia connected intersegmentally by connectives

and intrasegmentally by transverse commissures. The connectives and then the ganglia tend to fuse to form a single longitudinal cord. Lateral nerves emerge from each ganglion to the body wall, muscle and gut and pedal ganglia constitute the central nervous system of the parapodia (reviewed by ORRHAGE AND MÜLLER 2005, MÜLLER 2006, HEUER *ET AL.* 2010). In Annelida as in protostomes in general, the nervous system is considered to be amongst the most conservative physiological systems.

→ *The brain of* T. helgolandica*, situated between its eyes, consists of an undifferentiated central neuropil dorsally covered by neuronal somata. At the posterior margin of the brain, two protrusions of the neuropil are surrounded by clusters of small globuli cells (HEUER ET AL. 2010). Its double nerve cord tends to fuse during the development (FAUVEL 1923, GRASSÉ 1959, ÅKESSON 1962). As opposed to the majority of polycheates, the transverse commissures retain their unsegmented appearance in all stages of development (ÅKESSON 1962).*

Glossary

(After MIKKELSEN AND VINSTEIN 1982)

ABDOMEN	The posterior region of the body behind the thorax and sometimes followed by a caudal region or "tail" (used primarily with sedentary polychaetes).
ACHAETOUS	Small, palmately arranged finger-like lobes located behind the notopodial lamellae.
ANTENNA	A sensory projection arising from the anterior end or dorsal surface of the prostomium.
APODOUS SEGMENT	A segment without parapodia.
ASETIGEROUS/ ACHETOUS	Without saete; see also: SETIGER.
ATOKE/ATOKOUS	A polychaete not in reproductive state; see also: EPITOKE.
BIRAMOUS	Having two rami or forks; with two branches (usually referring to parapoida having both notopodia and neuropodia present); see also: UNIRAMOUS.
CAPILLIFORM	Capillary-like.
CHAETA/SETA	A chitinous rod projecting from, and forming the sarmature of the parapodia; see also: SETIGER.
CIRRUS	A sensory projection, usually tapered and derived from the superior part of the notopodium or the inferior part of the neuropodium.
ELYTRAE/ELYTRON/ ELYTRA	A dorsal, scale-like structure (modified cirrostyle) in scaleworms; see also: ELYTROPHORE.
ELYTROPHORE	A projection bearing an elytron or scale.
EPITOKE	Modified reproductive stage, often swarming; see also: ATOKE.
ERRANTIA	A convenient, non-taxonomic grouping (formerly an order) of the Polychaeta, generally characterised by a relatively undifferentiated body metamerism with fully developed intersegmental septa, a muscular proboscis generally armed with chitinous teeth of jaws, and frequently with well-developed parapodia; see also: SEDENTARIA.
EVERSIBLE PROBOSCIS	A proboscis capable of being extended by turning the inner part outwards; see also: PHARYNX, PROBOSCIS.

FRONTAL/PROSTOMIAL HORN	A prominent, digitiform, lateral process projecting from the anterior margin of the prostomium.
HEAD	The combination of the prostomium and the peristomium, and other highly modified segments (not considered a "region" of the body).
HYALINE GLAND	A gland which occurs in the pinnules of certain species of *Tomopteris* and appears relatively transparent, sometimes with a yellow spot in the centre.
MEMBRANOUS	Thin and flattened; sheet-like; skin-like.
METAMERISM	Having the body segmented, with each segment being replicates of each other and possessing a similar internal and external morphology.
METANEPHRIDIUM	A nephridium that originates in a ciliated coelomic funnel; see also: NEPHRIDIUM; PROTONEPHRIDIUM.
METASTOMIUM	The segmented portion of the body of an annelid worm; that portion between the prostomium and pygidium, but including neither.
NATATORY	Swimming; used for purposes of swimming.
NEPHRIDIUM	An excretory organ that is characteristic of various coelomic invertebrates, occurring paired in each body segment or as a single pair serving the whole body, and is often lengthened and convoluted and has glandular walls, at maturity partly functioning as gonopores; see also: METANEPHRIDIUM; PROTONEPHRIDIUM.
NEUROPODIUM	The lower or ventral ramus of a biramous parapodium; see also: NOTOPODIUM.
NOTOPODIUM	The upper or dorsal ramus of a biramous parapodium; see also: NEUROPODIUM.
NUCHAL EPAULETTE	A raised and elongated sensory organ projecting posterolateral to the prostomium.
NUCHAL ORGAN	A sensory organ on the prostomium or extending back from it, usually in the form of a groove or ciliated ridge.
PALP	One of a set of paired projections growing from the sides of the head; in errant polychaetes they arise from the ventral surface of the prostomium and have a gustatory function, but in sedentary polychaetes they arise from the peristomium and are usually grooved, ciliated and adhesive, and pass food to the mouth.

PARAPODIAL TRUNK	The proximal, undivided part of an elongate parapodium.
PARAPODIUM/ PARAPODIA	A segmental, foot-like projection bearing setae; see also: RAMUS.
PERISTOMIUM	The segment behind the prostomium which is modified to form part of the head and may surround the mouth; only the first segment forms the true peristomium, but in the families Nereididae, Hesionidae and others, the possession of more than two pairs of tentacular cirri shows that two or more segments have fused to form the head; see also: PROSTOMIUM.
PHARYNX	The posterior part of the mouth cavity leading to the oesophagus; the anterior part of the digestive tract, often eversible, always modified for feeding purposes and sometimes used for burrowing; see also: EVERSIBLE PROBOSCIS.
PINNULES	Foliaceous, fin-like tissue bordering the rami in *Tomopteris*.
PROBOSCIS	The anterior part of the alimentary canal, derived from the stomodeum, which can usually be everted to project forwards for feeding and burrowing; see also: EVERSIBLE PROBOSCIS; PHARYNX.
PROSTOMIUM	The anteriormost, presegmental lobe of a polychaete, in front of the mouth, bearing eyes, antennae, and palps, and at least the anterior part of the brain; see also: METASTOMIUM; PERISTOMIUM.
PROTONEPHRIDIUM	A nephridium equipped with a solenocyte, found in the Phyllodocidae, Nephtyidae, Glyceridae and trochophore larvae; see also: NEPHRIDIUM; METANEPHRIDIUM; SOLENOCYTE.
PYGIDIUM	The anal or terminal part of the body (not a true or modified segment).
RAMUS/RAMI	A branch or prong, *e.g.*, the neuropodium and notopodium are rami of the parapodium; see also: UNIRAMOUS; BIRAMOUS.
ROSETTE GLAND	The yellowish, star-shaped gland in the pinnules of *Tomopteris spp.* (Tomopteridae), situated next to the apices of the parapodial rami; see also: SPUR GLAND.
SCALEWORM	Polychaetes bearing elytra (*e.g.*, the Aphroditidae, Polynoidae, Sigalionidae and Polyodontidae).
SEDENTARIA	A convenient, non-taxonomic grouping (formerly an order) of the Polychaeta, generally characterised by the reduction or loss of body metamerism, tendency towards loss of intersegmental septa and corresponding differentiation of

	body into thorax and abdomen, absence of horny or chitinous proboscidial teeth or jaws, and typically with reduced parapodia and simple setae; see also: ERRANTIA.
SETAE	A chitinous rod projecting from, and forming the armature of the parapodia; see also: SETIGER.
SETIGER	A segment with setae; see also: ASETIGEROUS.
SOLENOCYTE	Any of various modified tubular, flagellated cells occurring in the nephridia of the larvae of some annelids, molluscs, rotifers and a few lancelets; see also: PROTONEPHRIDIUM.
SPUR GLAND	A gland in some species of Tomopteris whose pointed end projects from the edge of the pinnule, usually next to the chromatophil gland; see also: ROSETTE GLAND.
TAIL/CAUDA	A posterior region of modified segments which lack parapodia; a tail; see also: PYGIDIUM (not a true or modified segment).
TENTACLE	A slender outgrowth of sensory function, emanating from the head.
TENTACULAR CIRRUS	A cirrus arising from the peristome, or a tentacular segment, which is elongated to act as a tactile organ; tentacular cirri may arise from cephalised segments, in which case they are considered homologous with the dorsal and ventral cirri of postcephalic parapodia.
THORAX	Term used primarily with sedentary polychaetes to designate the anterior region of the body, posterior to the prostomium or possibly the head, and anterior to the abdomen, differentiated exteriorly from the thorax by a change in setal position on the body; see also: ABDOMEN; HEAD; METASTOMIUM.
TROCHOPHORE	The larval stage of an annelid or mollusc which develops from the gastrula.
TUBICULOUS	Forming and living within its own tube (*e.g.*, the Chaetopteridae, Pectinariidae, Maldanidae, Serpu-lidae, etc.).
UNIRAMOUS	With a single setigerous, acicular lobe or ramus (as in the parapodia of the Syllidae and Pontodoridae); see also: BIRAMOUS.

Appendix B

Oral communications

- GOUVENEAUX A. & MALLEFET J. First description of neural control mechanisms in bioluminescence of *Tomopteris helgolandica* (Annelida, Polychaeta). 17th International Symposium on Bioluminescence and Chemiluminescence, Guelph, Canada (May 28 - June 2, 2012)

- GOUVENEAUX A. & MALLEFET J. Study of bioluminescence from the transparent planktonic annelid *Tomopteris helgolandica* Greeff 1879 CIBIM meeting (Interuniversitary Centre for Marine Biology), Mons, Belgium (January 4, 2013)

- GOUVENEAUX A. & MALLEFET J. Characterization of photogenic sites in deep-sea planktonic worms: a comparative approach within Tomopteridae. 48th European Marine Biology Symposium, Galway, Ireland (August 19-23, 2013)

- GOUVENEAUX A. & MALLEFET J. Yellow lights in the deep-sea: Multidisciplinary approach of tomopterids' bioluminescence. Seminar at the Department of Biological and Environmental Sciences (University of Gothenburg), Gothenburg, Sweden (January 30, 2014)

- GOUVENEAUX A. & MALLEFET J. Yellow lights in the deep-sea: Morphological approach of tomopterids' bioluminescence. CIBIM meeting (Interuniversitary Centre for Marine Biology), Brussels, Belgium (February 21, 2014)

- GOUVENEAUX A. & MALLEFET J. Characterization of photogenic sites in deep-sea planktonic worms: a comparative approach within Tomopteridae. 18th International Symposium on Bioluminescence and Chemiluminescence, Uppsala, Sweden (June 23-28, 2014)

Posters

- GOUVENEAUX A. & MALLEFET J. Comparative morphology of photogenic structures in deep-sea planktonic worms (Polychaeta: Tomopteridae). "Living Light: Uniting biology and photonics", memorial meeting for Jean-Pol Vigneron, Namur, Belgium (April 10-12, 2014)

- GOUVENEAUX A., GIELEN M.-C. & MALLEFET J. Conversation with a bioluminescent planktonic worm. 18th International Symposium on Bioluminescence and Chemiluminescence, Uppsala, Sweden (June 23-28, 2014)

- DELROISSE, J., DUCHATELET L., GOUVENEAUX, A., FLAMMANG P., MALLEFET J. Opsin diversity in a luminous marine worm. 19th International Symposium on Bioluminescence and Chemiluminescence, Tsukuba, Japan (May 29 - June 2, 2016)

Abstracts

First description of neural control mechanisms in bioluminescence of Tomopteris helgolandica (Annelida, Polychaeta)

Gouveneaux A. and Mallefet J.

Luminescence **2012**; 27(2): 118–119
Abstracts of the 17th International Symposium on Bioluminescence and Chemiluminescence, May 28-June 2 2012, Guelph, Canada

Transparency is a common passive cryptic adaptation in pelagic environment.[1] In fact, by reducing light reflection and light scattering, biological transparency makes organism invisible. Paradoxally, many gelatinous zooplankton species are able to produce visible light.[2] So, what ecological advantage(s) bioluminescence could provide to such organisms? The model studied here is *Tomopteris helgolandica*, a transparent planktonic worm collected by trawling between 250 and 300 m depth in fjords near Bergen (Norway). The specimens were picked out and maintained during few days in sea water containers placed in a dark cold room. The bioluminescence potential was measured on pieces of three pairs of parapods from anaesthetised organisms. Each preparation was stocked in cold artificial sea water up to chemical induction of bioluminescence response.

Available data about tomopterids bioluminescence were limited to anecdotic observations and morphological descriptions, poorly documented.[3] In way of having a global view of its bioluminescent potential, different aspects of the light emission process are currently explored, including neural basis of physiological control. After a screening of major pharmacological components, investigation has been oriented in cholinergic control hypothesis. It has been confirmed by dose-dependent emission of light in response to carbachol stimulation [**Fig. 1**]. Inhibitory effect of tubocurarine on light emission suggested that nicotinic receptors were involved in the signal transmission pathway leading to light emission [**Fig. 2**]. These results constitute the first data about neural control mechanisms in bioluminescence of a pelagic worm.

Two annelid families have been previously studied and both concerned benthic species.[4] Only pharmacological investigations have been conducted so far but these results will be completed by electrophysiological and immunohistochemical approaches.

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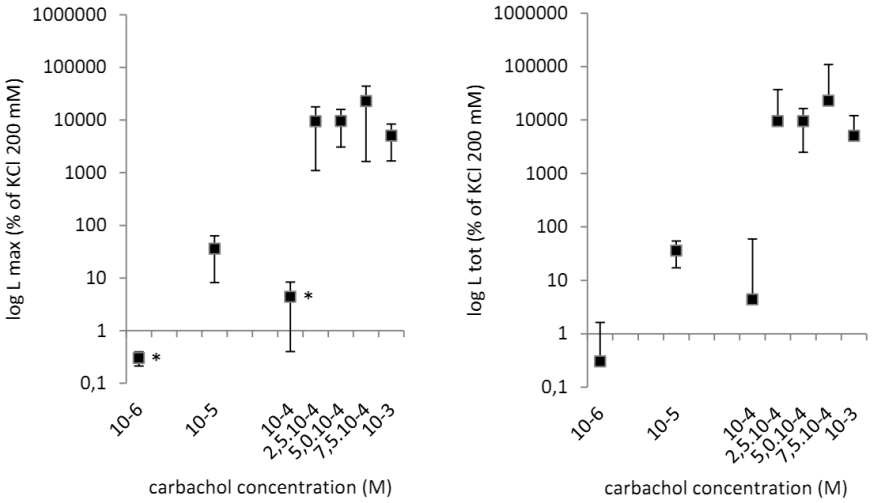


Fig. 1. Effect of carbachol concentration on luminescence of parapods preparations from *Tomopteris helgolandica*. Intensities of light emitted are expressed as a percentage of those measured in control preparation, stimulated by 200mM KCl solution. Mean SEM, n=6, * indicates a difference between this response and the higher carbachol-induced one (10^3 M) (P<0,05).

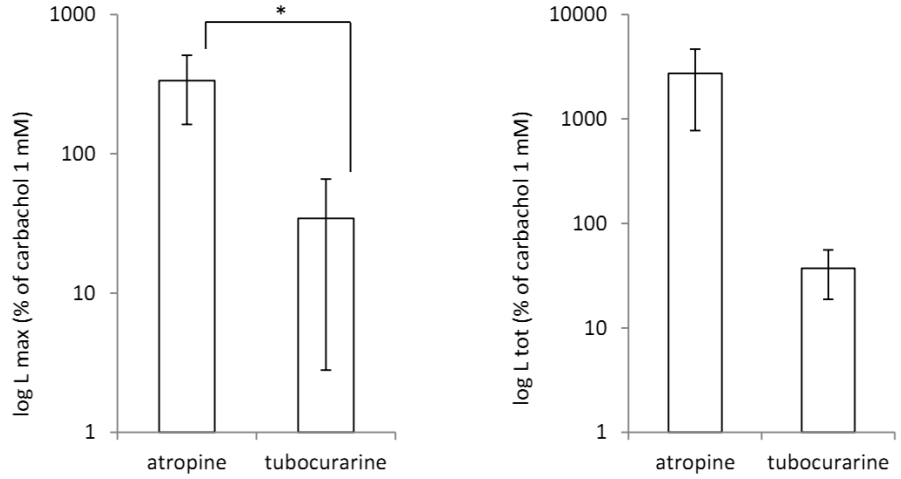
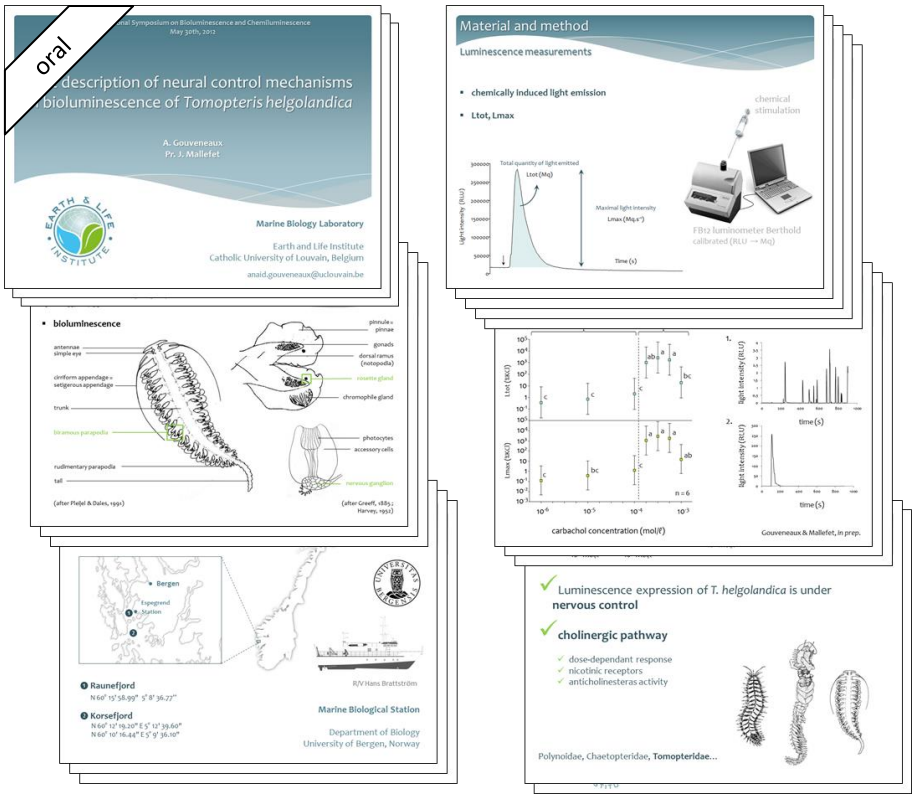


Fig. 2. Inhibitory effect of tubocurarine on carbachol-induced luminescence of parapods preparations from *Tomopteris helgolandica*. Intensities of light emitted are expressed as a percentage of those measured in control preparations not treated with cholinergic antagonists. Mean SEM, n=6, *P=0,0553.


oral
description of neural control mechanisms
bioluminescence of Tomopteris helgolandica
A. Gouveneaux
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Material and method
Luminescence measurements
chemically induced light emission
Ltot, Lmax
chemical stimulation
FB12 luminometer Berthold
calibrated (RLU → Mq)
bioluminescence
antennae
simple eye
cirriform appendage
setigerous appendage
trunk
biramous parapodia
rudimentary parapodia
tail
pinnule
pinnae
gonads
dorsal ramus (notopodia)
rosette gland
chromophile gland
photocytes
accessory cells
nervous ganglion
(after Pleijel & Dales, 1991)
(after Greeff, 1885 ; Harvey, 1952)
carbachol concentration (mol/l)
Gouveneaux & Mallefet, in prep.
Bergen
Espegrend
Station
Raunefjord
N 60° 15' 58.99" 5° 8' 36.27"
Korsefjord
N 60° 12' 19.20" E 5° 12' 39.60"
N 60° 10' 16.44" E 5° 9' 36.10"
R/V Hans Brattström
Marine Biological Station
Department of Biology
University of Bergen, Norway
Luminescence expression of T. helgolandica is under nervous control
cholinergic pathway
dose-dependant response
nicotinic receptors
anticholinesteras activity
Polynoidae, Chaetopteridae, Tomopteridae...

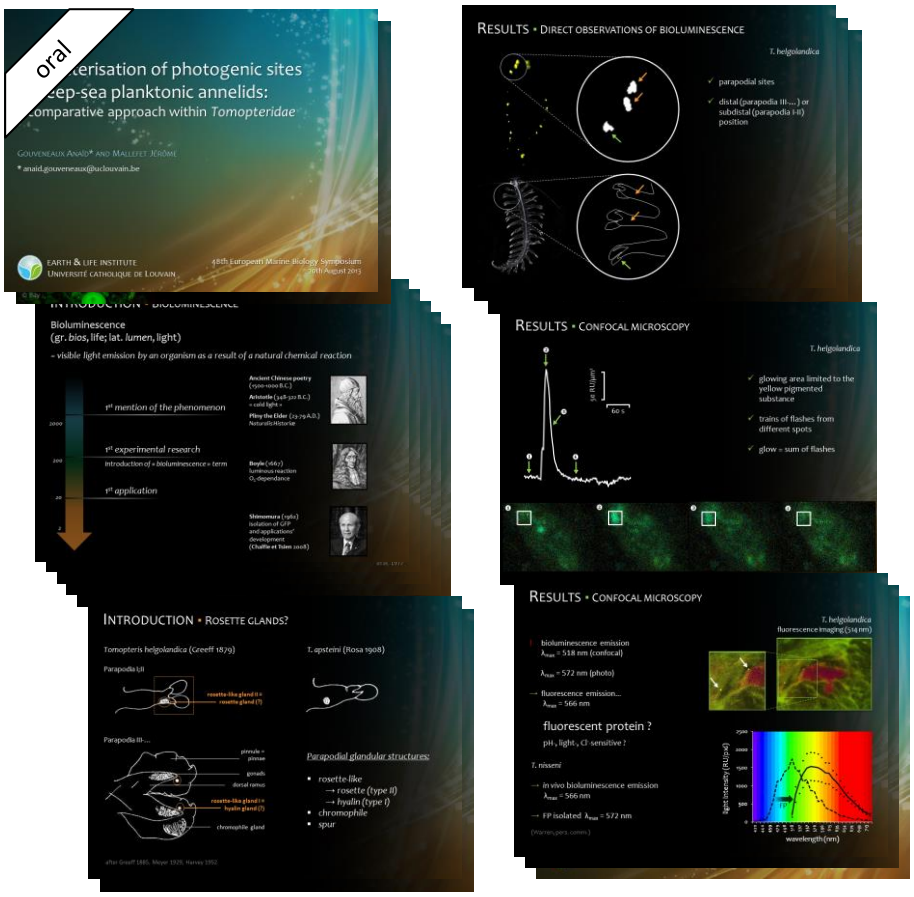
Characterization of photogenic sites in deep-sea planktonic worms: a comparative approach within Tomopteridae

Gouveneaux A. and Mallefet J.

Abstract booklet of the 48th European Marine Biology Symposium, August 19-23, 2013, Galway, Ireland

It is currently estimated that bioluminescence has evolved independently between 40 and 50 times among known organisms. Mostly distributed in marine species, this remarkable ability to emit visible light is the source of a wide diversity of structures, emission patterns and associated functions. Except for sophisticated photophores, the characterization and discrimination of photogenic units, including cells and intracellular sources, remain generally hypothetic for lack of specific marker. Thus, because they must be conducted on a case-by-case basis, morphological studies are rarely detailed.

The yellow-light emission known in the planktonic Tomopterid worms is a very atypical characteristic since most of the pelagic marine organisms emits a blue-green light. Although the spectral distribution has been measured in only two species (λ_{max} = 565 nm *Tomopteris nisseni* Rosa, 1908, λ_{max} = 570 nm *T. septentrionalis* Quatrefages, 1865) amongst twelve described as bioluminescent, these observations are often generalised to the family. However, the recent advances in the study of these deep-sea planktonic annelids' bioluminescence have revealed an unexpected diversity of light emission patterns. Here we compare photogenic structures from *T. helgolandica* Greeff, 1879 and *T. nisseni* Rosa, 1908 which are East Atlantic and Pacific species respectively. Through combined optic, fluorescent, electronic and confocal microscopy techniques, we provide the first experimental data for the clarification of the ambiguous status of the parapodial rosette glands suspected as specific sites of light production in Tomopteridae.


oral
terisation of photogenic sites
eep-sea planktonic annelids:
omparative approach within Tomopteridae
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Earth & Life Institute
Université catholique de Louvain
48th European Marine Biology Symposium
RESULTS • Direct observations of bioluminescence
T. helgolandica
parapodial sites
distal (parapodia III-...) or subdistal (parapodia I-II) position
Bioluminescence
(gr. bios, life; lat. lumen, light)
« visible light emission by an organism as a result of a natural chemical reaction
1st mention of the phenomenon
1st experimental research
introduction of « bioluminescence » term
1st application
RESULTS • Confocal microscopy
T. helgolandica
glowing area limited to the yellow pigmented substance
trains of flashes from different spots
glow = sum of flashes
INTRODUCTION • Rosette glands?
Tomopteris helgolandica (Greeff 1879)
T. apsteini (Rosa 1908)
Parapodia I,II
Parapodia III-...
Parapodial glandular structures:
rosette-like
→ rosette (type II)
→ hyalin (type I)
chromophile
spur
RESULTS • Confocal microscopy
T. helgolandica
fluorescence imaging (514 nm)
bioluminescence emission
λmax = 518 nm (confocal)
λmax = 572 nm (photo)
→ fluorescence emission...
λmax = 566 nm
fluorescent protein ?
pH, light, Cl- sensitive ?
T. nisseni
→ in vivo bioluminescence emission
λmax = 566 nm
→ FP isolated λmax = 572 nm
wavelength (nm)

Comparative morphology of photogenic structures in deep-sea planktonic worms (Polychaeta: Tomopteridae)

Gouveneaux A. and Mallefet J.

Abstract booklet of "Living Light: Uniting biology and photonics", memorial meeting for Jean-Pol Vigneron, April 10-12 2014, Namur, Belgium

We currently estimate that the bioluminescence has evolved independently between 40 and 50 times among living organisms. Mostly represented in marine species, this remarkable ability to emit visible light conceals a diversity of photogenic structures, emission patterns and associated functions which is still widely unknown.[1] Thus, the characterization of photogenic structures - from the intracellular microsources to the most sophisticated photophores - in some cases may require the development of original approaches using new criteria.
Some tomopterid polychaetes are able to produce yellow light emissions while the majority of bioluminescent pelagic organisms are blue emitters.[2][3] Despite many reports of this spectral distinctive feature – often generalizing anecdotal observations – the bioluminescence of the tomopterids remains poorly documented. After Greeff described in 1880 the parapodial rosette glands as the photogenic organs [4], many species have been reported as bioluminescent referring on this morphological criterion only.[5] However, recent observations have revealed an unexpected diversity of light emission patterns within this family, including intracellular and secreted bioluminescence.
Here we compare the bioluminescent signals and structures from *T. helgolandica* Greeff 1879, *T. nisseni* Rosa 1908 and *T. carpenteri* Quatrefages 1866 which are East Atlantic, Pacific and Antarctic species respectively. Through combined optic, fluorescent, confocal microscopy techniques, we provide the first experimental data for the clarification of the ambiguous status of the rosette glands in Tomopteridae.

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poster

Comparative morphology of photogenic structures in deep-sea planktonic worms (Polychaeta: Tomopteridae)

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Introduction

After Greeff described in 1880 the parapodial rosette gland of *Tomopteris mariana*, containing yellow-pigmented cells, as the photogenic organ[1], many other species has been reported as bioluminescent referring on this morpho-logical criteria only.
However, although this hypothesis seems consistent, the structure-function coupling has never be demonstrated.

Material and methods

Three yellow-emitting species: ***Tomopteris helgolandica*** Greeff 1879 (North Atlantic), ***T. carpenteri*** Quatrefages 1866 (Antarctica), and ***T. nisseni*** Rosa 1908 (North Pacific)

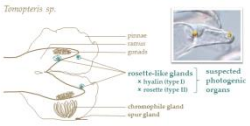
photophores → photocytes → microsources

structure ⟸ optical microscopy ⟹
⟸ transmission electron microscopy ⟹

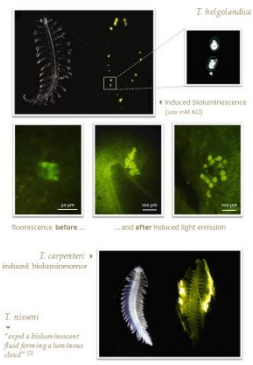
light signals
× bioluminescence ⟸ intensified camera ⟹
⟸ confocal laser scanning microscopy ⟹
× fluorescence ⟸ epifluorescence microscopy ⟹

Results

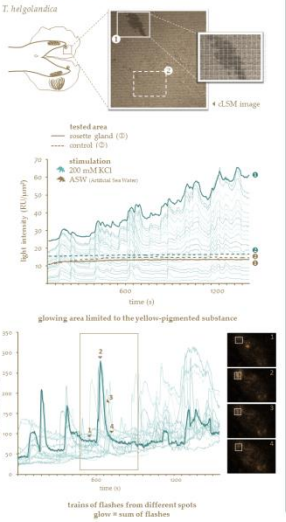
Parapodial glandular structures



Bioluminescence and fluorescence patterns

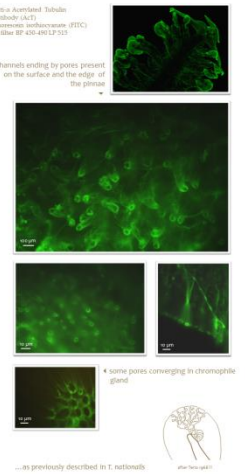


Live monitoring of light emission



Branched channel system

Revealed *in T. helgolandica*, *T. carpenteri* and *T. nisseni* by immunohistochemistry



Conclusions

→ ***localization and monitoring of bioluminescent activity*** *at cellular level, associated to the yellow- pigmented substance of the rosette-glands*

→ ***colocalization of fluorescence reactivity*** *and* ***modulation of the signal pattern*** *during the light emission*

→ *observational* ***evidence for a channel system*** *in the three studied species despite intra- and extracellular emission patterns:* ***what involvement in the light emission process?***

Acknowledgements

We sincerely thank all our collaborators, the Espegrend Marine Biological Station of the **University of Bergen**, the Department of Zoology of the **University of Gothenburg**, the **Monterey Bay Aquarium Research Institute** and the Institute of Life Sciences of the **Catholic University of Louvain**, for their contributions.

This research is supported by a Research Foundation for Industry and Agriculture (FRIA) research grant (to A.G.). J.M. is an FNRS research associate. This is a contribution to the Biodiversity Research Centre of the Earth and Life Institute (UCL) and to the Interuniversity Centre of Marine Biology (CIBIM).

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Characterization of photogenic sites in deep-sea planktonic worms: a comparative approach within Tomopteridae

Gouveneaux A. and Mallefet J.

Luminescence **2014**; 29(S1): 20
Special Issue: Abstracts of the 18th International Symposium on Bioluminescence and Chemiluminescence, June 23-28 2014, Uppsala, Sweden

From the intracellular microsources to the most sophisticated photophores, the characterization of photogenic structures may, in some cases, require the development and combination of original approaches and criteria.
While the majority of bioluminescent pelagic organisms are blue emitters, some tomopterid polychaetes are able to produce yellow light emissions.[1] After Greeff described in 1880 the parapodial rosette glands containing fluorescent yellow-pigmented cells as the specific photogenic organs,[2] some species have been reported as bioluminescent referring on this morphological criterion only.[3] However, although it seems consistent hence being commonly admitted, the structure-function coupling has never been clearly demonstrated. Moreover, how to explain that both intracellular and secreted bioluminescence have been reported?[1,2] Here we compare the bioluminescent signals and structures from *T. helgolandica* Greeff, 1879, *T. nisseni* Rosa, 1908 and *T. carpenteri* Quatrefages, 1866 which are East Atlantic, Pacific and Antarctic species respectively. Through combined optic, fluorescent, confocal microscopy techniques, we provide the first experimental data for the clarification of the ambiguous status of the rosette glands in Tomopteridae. Thus, the bioluminescent activity and fluorescence reactivity have been co-localised in the yellow pigmented cells of T. helgolandica, confirming the close link between these cells and the photogenic events. Moreover, we observed that the fluorescence tissue regions extend during the light emission. Finally, we have recently demonstrated by immunohistochemical staining a dense network of tubules largely developed in the pinnae. Some similar structures have been described in 1964 by Terio in *T. nationalis* as the excretion pathway of the yellow fluorescent substance.[4]
However, these channels partially converge to form the chromophile glands – another glandular type of unknown function – whereas the morphoanatomical continuity with the rosette glands remains uncertain. We are currently performing additional analysis using confocal and transmission electronic microscopy in order to improve the dynamic morphological description of these structures.

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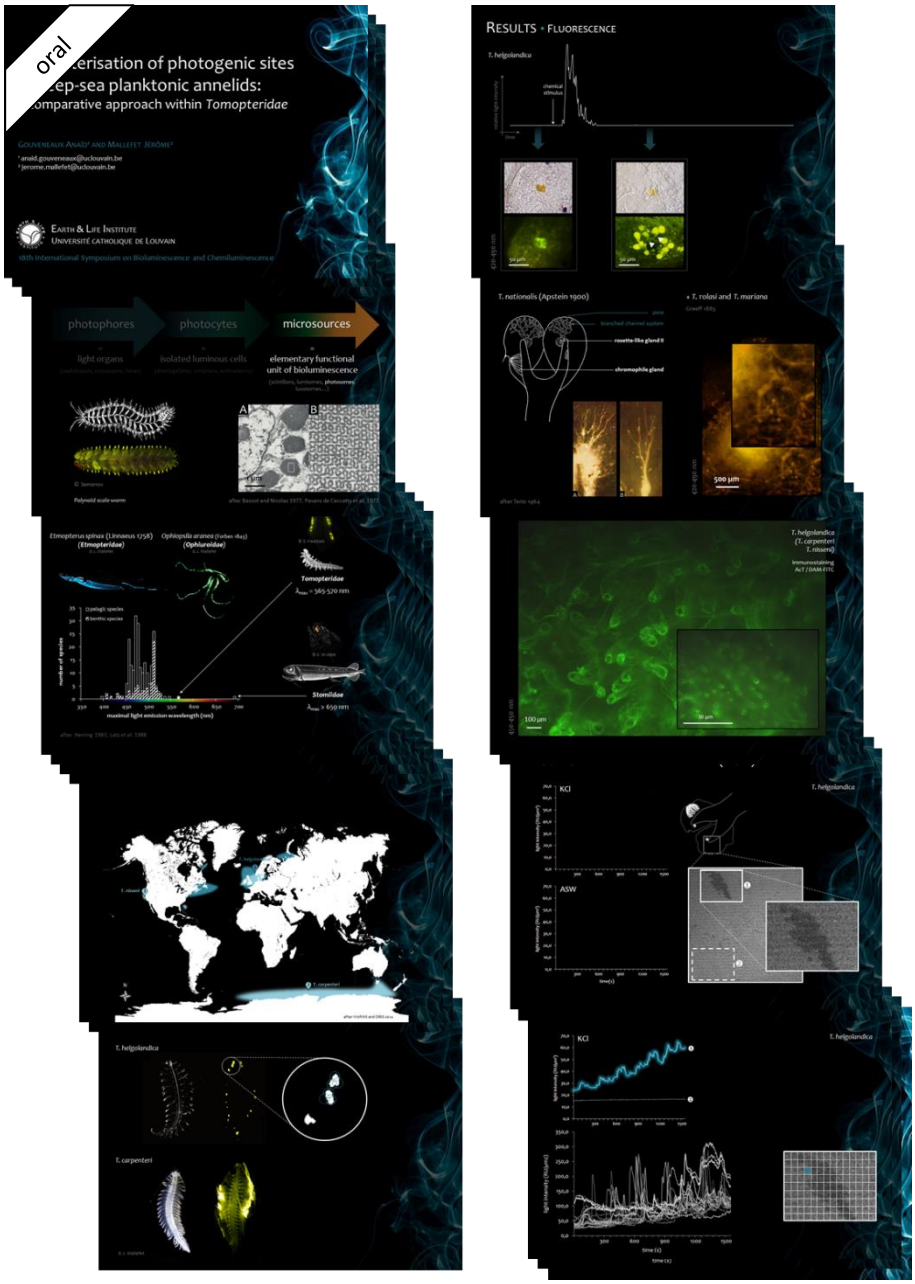
oral
erisation of photogenic sites
ep-sea planktonic annelids:
omparative approach within Tomopteridae
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18th International Symposium on Bioluminescence and Chemiluminescence
photophores
photocytes
microsources
light organs
isolated luminous cells
elementary functional unit of bioluminescence
Polynoid scale worm
Etmopterus spinax (Linnaeus 1758)
(Etmopteridae)
Ophiopsila aranea (Forbes 1843)
(Ophiuroidae)
Tomopteridae
Stomiidae
number of species
maximal light emission wavelength (nm)
T. helgolandica
T. carpenteri
RESULTS • Fluorescence
T. helgolandica
chemical stimulus
T. nationalis (Apstein 1900)
+ T. rolasi and T. mariana
branched channel system
rosette-like gland II
chromophile gland
500 µm
T. helgolandica
(T. carpenteri
T. nisseni)
Immunostaining
AcT / DAM-FITC
100 µm
KCl
ASW
T. helgolandica
KCl
T. helgolandica

Conversation with a bioluminescent planktonic worm

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Tomopteris helgolandica is a transparent planktonic annelid (Polychaeta: Tomopteridae) able to produce yellow flashes (λ_{max} = 572 ± 2 nm).[1] This cryptic species lives in the mesopelagic zone where the majority of organisms are visually adapted to the blue colour dominating this environment.[2] Thus, it has been suggested that such an original bioluminescent signal could be used for intraspecific private communication.[3] But, is *T. helgolandica* able to perceive its own light? We know that 3-days larvae develop a pair of pigmented ocelli consisting of seven large rhabdomeric sensory cells topped by a lens.[4] The early stages are positively phototactic [5] but behavioral observations of adult specimens of the yellow emitter *T. septentrionalis* have revealed photophobic responses to blue light flashes simulating dinoflagellate bioluminescence.[6] Through a similar approach, we have tested the behavioral effect of simulated bioluminescent signals on our model species. Isolated specimen were placed under infra-red lighting in a round aquarium and filmed by a coupled CCD video recording system. One camera was sensitive to the IR so we can track the animal moving. Simultaneously, an intensified camera only recorded the bioluminescent events. Manually controlled light signals were applied through a fake worm – provided with optic fibres reproducing the distribution pattern of *T. helgolandica*'s photogenic organs – immersed in the seawater. We tested 0,2 s^{-1} flashes and continuous signals as well as five different light colours (blue, green, yellow, orange and red). The 50h of video collected were analysed using the video tracking software Ethovision XT (Noldus Information Technology). Spontaneous light emissions have been observed during physical contacts with the fake worm and during stressfull situation like emersion but they did not seem to appear in response to the simulated light signals. They did not demonstrate specific interest in the yellow light emission but seemed attracted by the continuous blue light signals. These results lead us to reassess the virtually admitted hypothesis of intraspecific communication. We are now currently testing the hypothesis that *T. helgolandica* might use light signals against its predators as suggested by its responses to mechanical stimuli. Finally, independently to its own bioluminescence capabilities, it might take advantage of the bioluminescence emitted by its preys.

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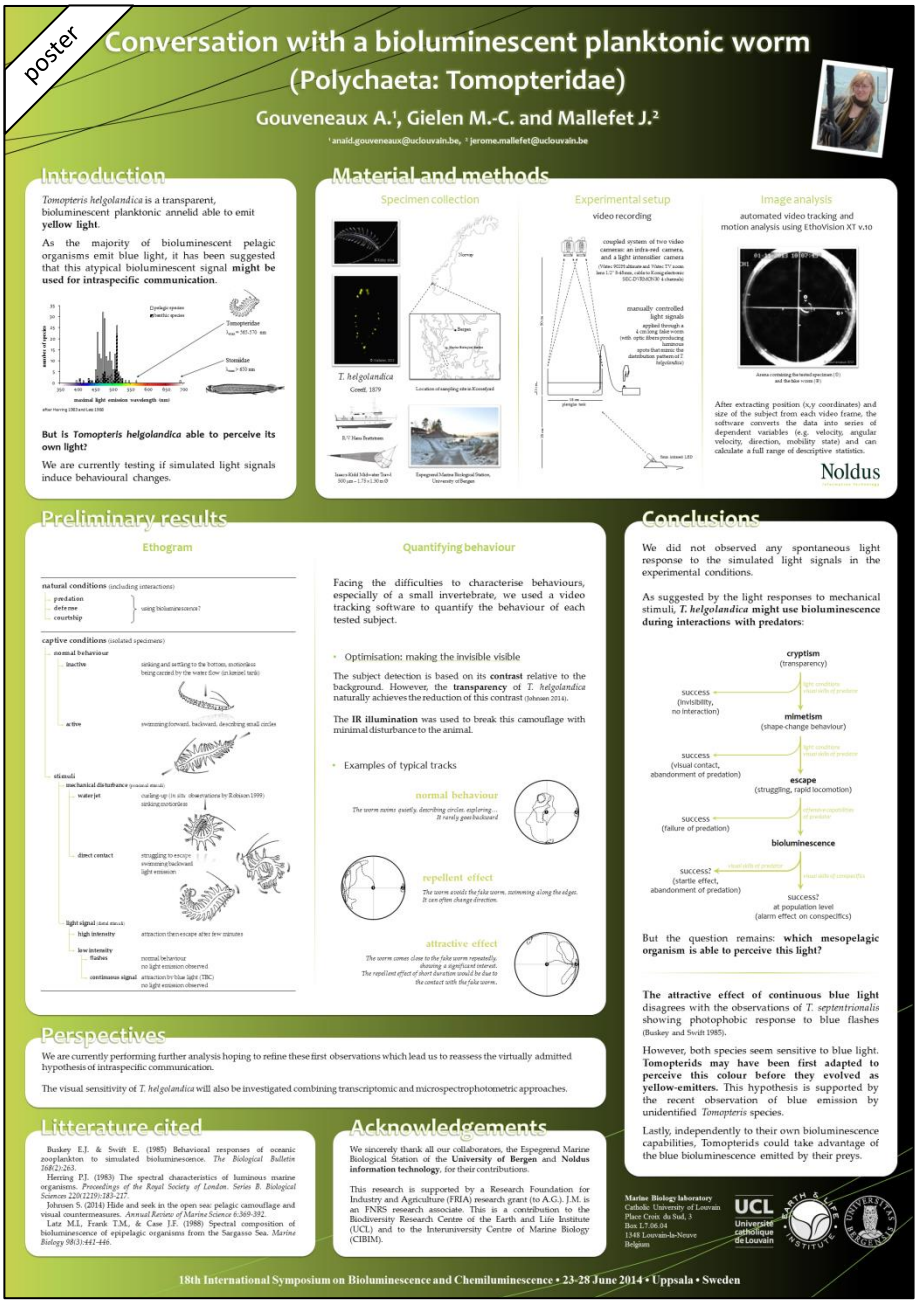

Opsin diversity in a luminous marine worm

Delroisse J., Duchatelet L., Gouveneaux A., Flammang P. and Mallefet J.

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Tomopteris helgolandica Greeff 1879 (Annelida, Tomopteridae) is a transparent holoplanktonic polychaete able to emit a bright light under various circumstances [1]. In contrast to most bioluminescent marine organisms, this worm emits a yellow light. Biological functions of the worm bioluminescence remain enigmatic even though several hypotheses have been suggested in the literature. A private communication channel between conspecifics (*i.e.* intraspecific communication) was proposed by previous authors despite a lack of evidence. In this context, the investigation of ocular photoreception could reveal crucial information for the understanding of the bioluminescence function in *T. helgolandica*. Additionally, extraocular photoreception was revealed at the level of bioluminescent organs of different luminous metazoans such as ctenophores, cephalopods and brittle-stars [2-4]. This potentially points to a paradigm in the light-mediated control of the bioluminescence and make the study of extraocular opsins of great interest in the bioluminescence context. This study presents the first data on the visual and non-visual light sensitive pigment diversity of the luminous worm *T. helgolandica*. Transcriptome analyses revealed both ocular and extraocular opsins. Opsin mRNA expression was investigated *in silico* in cephalic and body regions. Nine potential opsins were shown to be expressed in head and body regions (including 6 full-length *bona fide* opsin sequences) and belong to various metazoan opsin classes (4 full-length rhabdomeric opsin sequence, 1 full-length peropsin sequence, 1 full-length neuropsin sequence and 3 partial neuropsin sequences belonging to up to 3 neuropsins). Th r-opsin 1 to 3, Th peropsin, and Th neuropsin 3 and 4 were shown to be mainly expressed in the cephalic region (most probably the eye region for r-opsins and peropsins). Only Th r-opsin 4 showed a higher expression within the body region. Whole mount immunohistological study is in progress in order to document immunoreactive cells in the body using commercial anti-r-opsin antibodies. This study opens a window on the study of interaction between light emission and light perception in luminous marine invertebrates.

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