

**Have the eyes of bioluminescent scale worms adapted to see their own light? A comparative study of eyes and vision in *Harmothoe imbricata* and *Lepidonotus squamatus***

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**Abstract**

Annelids constitute a diverse phylum with more than 19000 species, exhibiting greatly varying morphologies and lifestyles ranging from sessile detritivores to fast swimming active predators. The lifestyle of an animal is closely linked to its sensory systems not least the visual equipment.

Interestingly, many errant annelid species from different families such as the scale worms, Polynoidae, share the same two pairs of eyes on their prostomium. These eyes are typically 100-200 µm in diameter

and structurally similar judged from the gross morphology. The polynoids, *Harmothoe imbricata* and *Lepidonotus squamatus* from the North Atlantic are both benthic predators preying on small invertebrates but only *H. imbricata* can produce bioluminescence in their scales. Here we have examined their eye morphology, photoreceptor physiology, and light guided behaviour in order to assess their visual capacity and visual ecology. Whereas the structure and physiology of the two pairs of eyes are remarkably similar within species, the only difference being the gaze direction, the photoreceptor physiology differs between the two species. Both species express a single opsin in their eyes but in *H. imbricata* the peak sensitivity is green shifted and the temporal resolution is lower, suggesting that the eyes of *H. imbricata* are adapted to detect their own bioluminescence. The behavioural experiments showed that both species are strictly night active but yielded no support to the hypothesis that *H. imbricata* are repelled by their own bioluminescence.

## Introduction

Light reception takes place in almost all animals and ocelli and eyes are found in about half of the animal phyla (Land and Nilsson, 2012). Within annelids ocelli and eyes are present in a large number of species and the majority of larger clades. Still, in the derived lineage of clitellates (earth worms and leeches) only a few species have small pigment cup eyes and light guided behaviours seem to be limited to simple phototaxis in most of them (Verger-Bocquet, 1992). The tube dwelling or burrowing species of the large clade Sedentaria, are also often eyeless, but some species have highly sophisticated compound eyes like the Christmas tree worm, *Spirobranchus giganteus*, and the fan worm *Acromegalomma vesiculosum* (Bok et al., 2017; Bok et al., 2019). These eyes can have more than 1000 ommatidia each, putatively having spatial resolution rivaling many insect eyes,

but it remains unknown if and how this image information is used and thus what functional significance the eyes have.

In the large sister group of Sedentaria, the Errantia, eyes are common. They vary in size and complexity from pigment cups with only a few receptors to highly advanced camera type eyes with large spherical lenses and 1000s of photoreceptors (Hermans and Eakin, 1974; Wald and Rayport, 1977). An often-found arrangement of the visual system in Errantia is having two pairs of eyes on the prostomium such as seen in many nereids and syllids, e.g., *Alitta virens* (as *Neries virens*) and *Odontosyllis enopla* (Dorsett and Hyde, 1968; Wolken and Florida, 1984). These eyes often appear similar in structure though there might be a size difference between the two pairs. They are typically made of two cell types only, pigmented cells forming the pigment screen and rhabdomeric photoreceptors (Suschenko and Purschke, 2009). In some species the pigment cells send in processes between the photoreceptors, which expand distally and form a lens-like structure. Whether this structure does have any optical functions is questionable since it is sometimes irregularly shaped and often lies directly next to the photoreceptor outer segments without a vitreous space in between (Verger-Bocquet, 1992; Purschke et al., 2006).

Eye structure has been examined in a large number of errantian species (Purschke et al., 2010) but little is known about the functional significance of the eyes. Perhaps the most extreme case is the pelagic alciopids having a single pair of huge camera type eyes, tripling the width of the prostomium (Hermans and Eakin, 1974). They are assumedly visual predators but it has so far not been possible to test this experimentally. The limited available electrophysiological data indicates that the complex morphology is backed by complex physiology, possibly supporting colour vision (Wald and Rayport, 1977). In the bioluminescent syllid, *O. enopla*, there is a striking match between the spectral sensitivity

of one of their photoreceptor populations and the peak wavelength of the emitted light suggesting visual communication during mating (Nicol, 1978). Based on the general eye structure in Errantia other possible light guided behaviours could be phototaxis, diurnal activity patterns, depth gauge and predator detection (Nilsson, 2009; Purschke et al., 2010), but as a general rule experimental evidence is lacking.

A major group within Errantia are the scale worms, Aphroditiformia. A few deep sea and cave living species are continuous swimmers in the water column, but most scale worms are benthic crawlers found in most marine habitats (Rouse and Pleijel, 2001; Gonzalez et al., 2017). They are characterized by two rows of dorsal scales, elytra, and many of them have the mentioned two pairs of prostomial eyes (Suschenko and Purschke, 2009). They occupy several different niches and trophic levels but a larger number of them are predators feeding on a broad variety of benthic invertebrates (Fauchald and Jumars, 1979; Jumars et al., 2015). In the shallow waters of the North-East Atlantic two species of polynoid scale worms are commonly found, *Lepidonotus squamatus* and *Harmothoe imbricata*. They typically inhabit rocky shores and stony reefs (with *H. imbricata* preferring blue mussel beds) where they feed on small benthic invertebrates (Fauchald and Jumars, 1979). Their two pairs of eyes appear similar and from examinations of other species of *Harmothoe* it is seen that they consist of pigmented photoreceptors and pigmented supporting cells also forming a lens-like structure (Suschenko and Purschke, 2009). An interesting difference between the two species is that in *H. imbricata* the scales emit light when disturbed and become strongly bioluminescent when autotomized, which is not the case for *L. squamatus* (Nicol, 1953). This is a defense mechanism allowing *H. imbricata* to escape, while an attacking predator chases the autotomized scales (Livermore et al., 2018). The two species belong to each their clade or subfamily within Polynoidae. *Lepidonotus squamatus* belongs to Lepidonotinae, whereas *Harmothoe imbricate* belongs to Polynoinae (Zhang et al., 2018). It

is especially within the larger clade Polynoinae that several examples of bioluminescent species have been reported (Moraes *et al.*, 2021). Although these subfamilies both occupy derived positions within Aphroditiformia (scale worms), scale worms have been proposed to date back to Devonian times and the split of the Polynoidae subfamilies may also date back >100 million years (Rouse and Pleijel, 2001).

Here we use *H. imbricata* and *L. squamatus* to obtain experimental data on the visual capacity of the typical eyes of Errantia. Through behavioural, morphological, and physiological examinations, we seek to test the following two hypotheses. 1) Each pair of eyes serve different purposes as seen in some other multi-eyed visual systems (Garm and Mori, 2009; Menda *et al.*, 2014), which is reflected in differences in physiology and/or morphology. 2) The eyes of the bioluminescent *H. imbricata* will be optimized to detect the light emitted by their autotomized scales. Our results provide some support the second hypothesis but only to a lesser degree the first hypothesis.

## **Materials and Methods**

### Animals

Fifteen specimens of *Lepidonotus squamatus* (Linnaeus, 1758), 2 - 5 cm long, were collected at approx. 35 m in the Northern part of Øresund, Denmark, using a standard triangular dredge. The specimens were brought back directly to the Marine Biological Section in Copenhagen. About 50 specimens of *Harmothoe imbricata* (Linnaeus, 1767), 1 - 3 cm long, were hand collected from mussel beds attached to ropes at a pier in the Kaldbak Fjord, Faroe Islands. They were transported back alive to Copenhagen in pairs in 50 ml falcon tubes with seawater and a small piece of rope. In Copenhagen the two species were kept in a 35 l tank and a 150 l tank, respectively, with sea water of 10 °C and 32-33 psu. About

half of the water was exchanged every two-three weeks. Large stones were scattered on the bottom of the tanks and served as hiding places. They were fed a variety of local amphipods and isopods along with small benthic annelids from aquarium cultures (*Ophryotrocha* spp.), but only the annelids were documented to be consumed.

### Eye morphology and visual fields

Three specimens of each species were anesthetized in sea water mixed 1:1 with 7 % MgCl<sub>2</sub> for 10 min. Afterwards they were decapitated and had the prostomia with their two sets of eyes photographed under a standard dissection microscope equipped with a c-mount camera (Evolution MP 5.0). In order to visualize the gaze direction of the pupils, pictures were taken dorsally, laterally and in the case of *H. imbricata* also anteriorly.

The cut off prostomia were fixed in seawater mixed 1:1 with 3 % glutaraldehyde and 3.7 % paraformaldehyde in 0.1 M sodium cacodylate buffer for 3 days at 4 °C and post fixed in 2% osmium in 0.1 M cacodylate buffer for 2 hours at room temperature. Afterwards they were dehydrated in a standard series of ethanol, transferred to acetone, and embedded in Epon 812 resin. All prostomia of *H. imbricata* and one from *L. squamatus* were serial sectioned in approximately 1 µm sections on a Leica EM UC7 microtome equipped with a Diatome HistoJumbo diamond blade. The direction of sectioning was aligned to obtain sections through the optical axis of the eyes. The sections were stained with toluidine blue and selected sections photographed with a VS110-S5 Olympus slide scanner equipped with a XM10 Olympus camera.

Two prostomia of *L. squamatus* were scanned using synchrotron X-ray microtomography at the TOMCAT beamline, Swiss Light Source (Paul Scherrer Institute, Switzerland), at 40 x resulting in effective voxel sizes of 163 nm. The samples were scanned with a monochromatic 12 keV beam, using a 20  $\mu\text{m}$  thick LuAG:Ce scintillator with an exposure time of 380 ms and a propagation distance of 12 mm. Approximately two thousand projections were recorded during the 180° rotation of the sample. The radiographic projections were reconstructed into aligned tiff image stacks and Paganin filtered ( $\delta = 1^{-7}$ ,  $\beta = 1^{-9}$ ; Paganin, Mayo, Gureyev, Miller, & Wilkins, **2002**) using custom in-house software (Marone & Stampanoni, **2012**).

The morphological examinations showed that all pupils were close to circular and that the vitreous bodies in the center of the eyes were unlikely to have a lens function (see results section for details). We estimated the visual field of the eyes, therefore, from a section through the optical axis of the eyes assuming that the only influencing factor was shading by the screening pigment of the eyes.

## Behaviour

The diurnal activity patterns of the scale worms were examined in their holding tanks, which had a light scheme of 18 hours room light and 8 hours darkness mimicking local summertime. They were filmed using a standard handycam in nightshot mode for 15 hours, starting at 4 pm with three hours of room light followed by eight hours in darkness and ending with another four hours of room light. During the eight hours of darkness the tank was illuminated by an array of infrared (IR, 940nm) LEDs. This was repeated three times for both species. To check for an endogenous diurnal rhythm, two trials were done where the light scheme in the holding tanks were changed such that the 8 hours of darkness occurred between 11 am and 7 pm.

The response to flashes of light mimicking the bioluminescent flashes of *H. imbricata* were examined with the animals placed one at a time in a small container (1.5 l) filled with water from the holding tank and a single stone to hide under. They were transferred to the experimental tank under room light conditions but once in the tank light was turned off and they were left to dark adapt for 15 - 20 min. Afterwards the setup was illuminated by IR light only and filmed by a handycam in night shot mode. At the end of the tank opposite the stone a 1 mm light guide was placed 0.5 cm above the bottom pointing at the stone. The light guide was illuminated by a computer-controlled optical bench, which supplied flashes of dim green light (520 nm, half width = 12 nm) of  $8 \times 10^{-1} \text{ W/sr/m}^2$ . Two different 30 min long experiments were conducted: 1) flashes were given at random intervals (1 – 8 min between flashes) and with varying duration between 5 and 60 s (average of 30 s), 2) flashes of 30 s were given every time the animals were with 10 cm of the light guide and faced it. This resulted in 4 - 10 and 2 - 8 stimulations in each trial, respectively. The intensity, peak wavelength and duration of the flashes were chosen to mimic the bioluminescence of a single scale of *H. imbricata* (Plyuscheva and Martin, 2009). The light emitting surface of the light guide was also matching the scale size of a mid-sized *H. imbricata*. In total seven *H. imbricata* and five *L. squamatus* were used in the behavioural tests.

Due to the observation that *L. squamatus* would occasionally walk around with the anteriormost two scales elevated (or laterally retracted) while the rest were laying down flat across the dorsal body an additional experiment was conducted for this species. When the two anterior most scales were lifted the animal would be approached slowly with a histology needle to test if they would lower the scale to protect the prostomium sacrificing vision. This was repeated twice with two specimen.



## Electrophysiology

The prostomium was dissected of each animal and had the tentacles and palps removed. Afterwards it was transferred to a petri dish in the electrophysiological setup containing about 5 ml of sea water from the holding tank. A custom-made glass suction electrode (outer diameter ~50  $\mu\text{m}$ , pore size 5-10  $\mu\text{m}$ ) was placed at the edge of either one of the posterior or anterior eyes and a 1 mm light guide was placed immediately in front of the pupil. The light guide was several times larger than the diameter of the pupil ensuring a close to even illumination of the retina. The light stimuli were produced by an optical bench with an ultra-bright white LED (Luminus CBT 90), a series of neutral density filters and a spectral filter wheel. The LED was controlled by a custom made program for LabView (National Instruments, TX).

Initially 25 ms white flashes were presented to the eye and the electrode was moved around until a decent looking impulse response was obtained and then the preparation was dark adapted for 30 min. The duration of the dark adaptation was determined by applying the same stimuli every 5 min and wait for two consecutive stimulations to give the same response magnitude. In the two preparations tested in this way, it happened after 20 min and 25 min respectively, thus ensuring a safety margin. In a similar way the longevity of the preparation was tested, and it was found that no significant change in responsiveness happened within the first 2.5 h after dark adaptation.

Each protocol started with an intensity series covering 5 log units of white light,  $2.5 \times 10^{-3} \text{ W/sr/m}^2 - 2.5 \times 10^2 \text{ W/sr/m}^2$  in steps of 0.3 or 0.7 log units using neutral density filter (Linos, Goettingen, Germany), starting at the low intensity end. This was followed by an equal quanta stimulation,  $1.7 \times 10^{19} \text{ photons/s/sr/m}^2$ , with spectral filters (half width = 12 nm, CVI Laser, Bendheim, Germany) covering 420 – 680 nm in steps of 10 or 20 nm. The spectral data were transformed by the v-Log I curves (Coates et al., 2006) and compared to the absorption spectrum of a theoretical opsin

(Govardovskii et al., 2000) using the least squares method. Following the spectral series a second intensity series was conducted in steps of 1 log unit to ensure that the sensitivity of the preparation had not changed during the protocol. All stimuli were 25 ms flashes with an interstimulus interval of 1.5 min. and the entire protocol lasted approximately 65 min excl. dark adaptation. For each of the prostomia used tests were done on either one eye only, on both an anterior and a posterior eye or on one eye only but followed by second series also testing the temporal resolution of that eye.

The temporal resolution was tested by flicker fusion frequency (fff) experiments. Due to differences in the dynamic range between the two species, *H. imbricata* being more sensitive, the protocols differed. For *H. imbricata* the eyes were initially light adapted for 5 min to  $5.1 \times 10^{-1}$  W/sr/m<sup>2</sup>. This was followed by a series of sinusoidal stimuli with an amplitude of  $\pm 5 \times 10^{-1}$  W/sr/m<sup>2</sup> starting at the intensity of the adaptation light. The frequency span tested was 0.5 – 8 Hz in steps of 0.5 Hz. For *L. squamatus* the eyes were light adapted for 5 min to  $5.1 \times 10^0$  W/sr/m<sup>2</sup>, which was followed by a series of sinusoidal stimuli with an amplitude of  $\pm 5 \times 10^0$  W/sr/m<sup>2</sup>. The frequency span tested for this species was 0.2 – 3.8 Hz in steps of 0.4 Hz. The stimulations for *H. imbricata* lasted 10 s with interstimulus intervals of 30 s and stimulations for *L. squamatus* lasted 60 s and had interstimulus intervals of 30 s. Fff curves were constructed through Fourier transformations of the recordings using the normalized power of the principal frequency (stimulus frequency) of each recording. In all cases a time-period matching 10 stimulation cycles was analyzed.

For *H. imbricata* the intensity and spectral series were obtained for seven anterior and seven posterior eyes and fff series was obtained from five anterior and five posterior eyes. For *L. squamatus* the intensity and spectral series were obtained for eight anterior and eight posterior eyes and fff series was obtained from six anterior and six posterior eyes.

The signals from the electrode were amplified 1000 times using a differential AC Amplifier (1700, A-MSystems, USA) and filtered through a 50 Hz notch filter, and 0.1 and 1000 Hz high and low pass filters respectively. The recordings were stored and processed on a laptop using a custom made program for LabView (National Instruments, TX).

## Results

### Eye arrangement and gaze direction

The posterior eyes are similar in the two species and situated on the dorso-lateral part of the prostomium close to the peristomium. They are oval and 100 – 150  $\mu\text{m}$  along the largest diameter in adult specimens (Fig. 1). Their pupils are round and approx. 50  $\mu\text{m}$  in diameter and they gaze dorso-laterally in both species (Fig. 1, 2). The anterior eyes are also oval but slightly larger in both species, spanning 150-200  $\mu\text{m}$  along the largest diameter. In *H. imbricata* they are situated on the anterior-most part of the prostomium under the cephalic peaks, whereas in *L. squamatus* they are placed midway on the prostomium on the dorso-lateral side (Fig. 1). Similar to the posterior eyes, the pupils are approx. 50  $\mu\text{m}$  in diameter and in *H. imbricata* they gaze anteriorly (Fig. 2) whereas in *L. squamatus* they gaze antero-laterally.

### Eye morphology and visual fields

The overall morphology is similar for the two species and for both pairs of eye. The outer layer is formed by the nuclei for the two involved cell types: pigment cells forming most of the screening pigment and the pigmented photoreceptors. The pigment is packed in vacuoles, the layer is 10 – 15  $\mu\text{m}$  thick, and from sub-illuminated stereo microscopy, it seems to be highly light absorbant. The

rhabdomeric photoreceptors are less densely pigmented (Fig. 2) but have dense microvilli on the outer segments. The outer segments of *H. imbricata* are longer than those of *L. squamatus* (20 - 30  $\mu\text{m}$  versus 10-15  $\mu\text{m}$ ) (Fig. 2b-c). In both species, the pigment cells send slim processes in between the outer segments, but they expand distally where are filled with a uniform material forming a homogeneous lens-like body (Fig. 2a). The pupil is in all eyes covered by a layer of epithelial cells and the cuticle, which does not appear to have any optical specializations above the eyes.

From the morphology it is seen that there is no vitreous space between the lens-like body and the photosensitive part of the photoreceptors (Fig. 2). Further, the material filling the lens-like body is homogeneous and does not appear densely packed from the staining, and we therefore assume that any optical function it might have is minor. The visual fields of the eyes can thus be estimated from the arrangement of the screening pigment and outer segments alone (Fig. 2d-e). For both eye pairs in both species this arrangement reveals rather broad visual fields spanning 130-140 degrees.

#### Diurnal activity patterns

Both species have a distinct diurnal activity pattern and are both strictly night active. During light hours they would sit motionless in a dark hiding place, typically under one of the rocks in the tank. When light was turned off they would start exploring the tank typically after 5 – 10 min (Fig. 3). There was no sign of an internal diurnal rhythm since their activity patterns also followed the light in the experiments where the light scheme was shifted. Still, if the worms were stressed in room light with no place to hide, e.g., in a small petri dish, they would start crawling around. Here *L. squamatus* displayed a to our knowledge novel behaviour. They would crawl around with only the distalmost pair of elytra lifted exposing the prostomium and the two pairs of eyes (Fig. 4a). A mechanical threat (needle) made them lower the scales again along with withdrawal of their tentacles (Fig. 4b, Supplementary video).

After making this discovery we looked through the recordings of their natural nocturnal activity under IR light but interestingly they were never seen to lift the scales here. This behaviour was not observed for *H. imbricata* in either situation.

#### Response to the mimic of bioluminescence

After approx. 15 min of dark adaptation in the experimental tank with only IR light most specimens of both species would start crawling around but some moved very little. Overall the active specimens behaved similarly. In almost all trials they had a strong tendency to walk along the edge of the tank and only rarely crossed the central part (Fig. 5). No matter whether the bioluminescence mimic was turned on randomly or only when the animal was facing the light guide there was no detectable response: they did not pause or speed up nor did they change their direction of walking during periods of light on.

#### Eye physiology

Both species respond normally to short flashes of light with a two-phased potential (Fig. 6). The amplitude of the response is graded following the intensity of the flashes. From the V-Log I curves it is seen that the anterior and posterior eyes of *H. imbricata* respond very similar and have a dynamic range of approx. 2.5 log units from  $5 \times 10^{-1}$  W/Sr/m<sup>2</sup> to  $1 \times 10^2$  W/Sr/m<sup>2</sup> (Fig. 6c). At maximum intensity, the curves flatten indicating the beginning of photoinhibition. The two pairs of eyes of *L. squamatus* also respond in the same way but the dynamic range is broader than for *H. imbricata* covering at least 4 log units and with no signs of photoinhibition at maximum intensity (Fig. 6F).

All four spectral sensitivity curves have a good match with the theoretical absorption spectrum of opsins (Fig. 7). For *H. imbricata* the two pairs of eyes again show similar peak sensitivity of the matched opsins, being 506 +/- 3 and 499 +/- 10 nm for the anterior and posterior eyes, respectively. In *L. squamatus* the peak sensitivities are shifted towards the short wavelength part of the spectrum and the matched opsins show maximum sensitivity at 494 +/- 6 and 487 +/- 9 nm for the anterior and posterior eyes, respectively. The peak sensitivity in the anterior eyes of *H. imbricata* was significantly different from both the anterior and posterior eyes of *L. squamatus* (One way ANOVA,  $F_{2,24} = 7.62$ ,  $P < 0.001$ , followed by Tukey HSD posthoc test,  $P = 0.036$  and  $0.0006$  respectively). Further, the peak sensitivities of the posterior eyes were also significantly different in the two species (One way ANOVA,  $F_{2,24} = 7.62$ ,  $P < 0.001$ , followed by Tukey HSD posthoc test,  $P = 0.036$ ).

The temporal resolution of the two species appears rather different but again, within each species the anterior and posterior eyes are similar (Fig. 8). Both eyes of *H. imbricata* have a fff of 4-5 Hz and interestingly the power of the Fourier transformation declines rapidly with increasing frequency of stimulation (Fig 8b). In *L. squamatus* the stimulations did not reach fff and at the highest frequency, 3.8 Hz, a response is still visible (Fig. 8c). Even though unfortunate, we do not consider this a major problem, since the most important for the intra and interspecific comparison is not the absolute fff but rather the shape of the initial part of the curve. Still, if the curves for *L. squamatus* are extended following the shape of the decline between 1.8 and 3.8 Hz the 0.05 cut off level is reached between 5 and 7 Hz for both the anterior and posterior eyes (not shown). The frequency curves are again similar for the two pairs of eyes even though the large standard errors indicate larger variation between preparations in this species. The power of the response in *L. squamatus* remains high until about 2 hz where it starts to decline (Fig. 8d).

## Discussion

In the presented work we have examined and compared the eye morphology, eye physiology and light guided behaviours, between two scale worm species both having two pairs of prostomial eyes. *H. imbricata* is found to have more sensitive photoreceptors with a green shift in the spectral sensitivity matching their bioluminescence, which supports our hypothesis that they may detect their own and conspecifics' bioluminescence. Further, their low temporal resolution strongly favors detection of slowly changing low intensity signals, which again matches their light emission lasting several seconds. However, it was not possible to detect a behavioral response to a mimic of their bioluminescence. Despite both species being strictly night active, the results suggest that the less sensitive eyes in *L. squamatus* could function during daytime possibly detecting hiding places or threats like predators. Except for a difference in gaze directions, the two eyes pairs within each species are morphologically and physiologically close to identical, hereby opposing the hypothesis of a functional difference between the eye pairs.

### Seeing your own bioluminescence

When *H. imbricata* autotomize their scales they become bioluminescent, which works as a defense against, at least, crustacean predators (Livermore et al., 2018). While the initial escape is important, it is equally important that the worm will keep following an escape route away from the predator. The obvious way to do this is to see your own glowing scales and crawl/swim in the opposite direction. Whereas our *in vitro* behavioural experiments with a mimic of the glowing scale could not provoke such a behavioural response, the physiological data does support this theory. The intensity of their bioluminescent signal is low (Nicol, 1953; Widder, 2010) and declines with the square of the distance, so the eyes should have enhanced sensitivity in order to detect this signal. We do find that when

compared with the closely related non-bioluminescent polynoid species, *L. squamatus*, the outer segments of the photoreceptors are longer in *H. imbricata* and so is the integration time as seen by the steep decline on the flicker fusion frequency graphs. This will result in enhanced sensitivity as also seen on the V-Log I curves, which is shifted 1-1.5 log units to the low intensity side for *H. imbricata*. Further, it is equally important that the spectral sensitivity of the eye matches the spectral peak of the emitted light (Haddock et al., 2010; Garm et al., 2016). The light emitted from the scales is estimated to peak between 510 and 520 nm (Nicol, 1953; Lecuyer and Arrio, 1975), which has a rather good match with the green shift in sensitivity of especially the anterior eyes in *H. imbricata*, peaking at around 506 nm. A green shift in the spectral sensitivity is also often seen as an adaptation to the colour of the water in the habitat with open water species having opsins typically peaking in the deep blue part of the spectrum around 470 nm and opsins of coastal living animals peaking closer to 500 nm (Cheroske and Cronin, 2005). Still, despite the difference in depth distribution *H. imbricata* and *L. squamatus* live in the same coastal greenish water and should not differ in the spectral sensitivity if the ambient light was the determining factor.

In addition to detecting your own light emission, it will also be beneficial to detect light emitted from conspecifics, which warns you about the whereabouts of predators. It could in fact also be advantageous for *L. squamatus* to get this predator warning since the two species co-occur in some habitats, but judging by the physiology of their eyes and their behavioural response to the mimic this is not supported.

Placing our trust in the physiological data, we see two possible explanations for why *H. imbricata* did not respond to a mimic of their own bioluminescence and both might have been at play in our experiments. First of all, the animals were possibly too stressed to respond after being moved from the holding tank to the experimental set-up. This is indicated by the trajectories showing that they



spend most of the time crawling along the sides, which is a typical escape/stress response. Second, the flashes were designed to mimic the bioluminescence of a single scale, which might not be enough to elicit a behavioural response. Under an attack *H. imbricata* will typically shed four - eight scales (Livermore et al., 2018, own observations), and even though the shed scales do not stick together they stay relative close to each other and the combined light emission could be needed to trigger an escape response. Additional behavioural experiments are needed to explore these possibilities further.

Why have two pairs of prostomial eyes?

Eyes are some of the most expensive organs to build and maintain (Moran et al., 2015), but still several animals groups have a high number of eyes. This is seen in box jellyfish (Nilsson et al., 2005), spiders (Harland et al., 2012), chitons (Li et al., 2015), scallops (Speiser and Johnsen, 2008), fan worms (Bok et al., 2017), starfish (Garm and Nilsson, 2014) and many others. One reason for this common pattern in animals with sparse nervous systems is to allow for special purpose eyes, where the visual tasks are divided between eyes minimizing the need for post-processing (Land and Nilsson, 2006). However, our results do not support such a functional differentiation among the anterior and posterior prostomial eyes, often found in scale worms and other members of Errantia. In both examined species the anterior and posterior eyes are close to identical, only with the anterior eyes being slightly larger. The most conspicuous difference lies in the gaze direction of the two pairs of eyes, suggesting that the benefit of having four prostomial eyes is an extended visual field, as also seen in other animals, e.g. starfish (Garm and Nilsson, 2014).

Interestingly, the anterior eyes of *H. imbricata* are situated underneath the anterior-most edge of the prostomium looking forwards. This means that they gaze out under the scales with the anterior eyes, whereas the scales at least partly obscure the vision of the posterior eyes. As a notion the scales are somewhat transparent in this species and will allow some light to penetrate, especially since it is mainly found in shallow water blue mussel beds where the light intensity is high during daytime hours. In the deeper living *L. squamatus* the scales are dark pigmented and normally cover all four eyes, but here we observed a to our knowledge novel behaviour. This species occasionally lifts the first pair of scales only and hereby expose all four eyes, which might be the only times where it is actively using vision since the thick and pigmented scales are not see-through. Even though we did not observe this behaviour for *H. imbricata* we suggest that it probably is present in a number of polynoid scale worms with eyes and light-obscuring scales, as obtaining visual input seems to depend on it. The stalk (elytraphore) on which the scales are attached is muscular in all scale worms and the scales have been observed to be lifted in the sigalionid scale worm *Pholoe minuta*, but this is normally done on larger parts of the body and suggested to serve ventilation purposes (Heffernan, 1990).

#### Visual ecology of *H. imbricata* and *L. squamatus*

As described above one of the visual tasks of *H. imbricata* seems to be detection of their own and possibly conspecifics' bioluminescence to escape from predators. But there are likely several other behaviours associated with the eyes. Both species are strictly night active and our results also show that this is not controlled by an endogenous rhythm since turning the light off during the day activates them and turning it on at night makes them hide and become quiescent. Such diurnal activity pattern can be achieved by any type of photoreceptor and does not require any spatial information (Nilsson, 2009). It could be controlled by the prostomial eyes even when covered by the scales as the relative intensity

change throughout the day is not affected by the shading. Their ability to seek out a dark hiding place, e.g., a rock or mussel shell, at dawn could be guided by low spatial resolution vision, which is supported by the morphology. We do not have data that allow us to elaborate further on this, though.

Other behaviours where vision is putatively involved in other species of errantian annelids are prey detection and mating (Hermans and Eakin, 1974; Nicol, 1978). We only observed our animals to be active and feeding in pitch darkness under IR-light and under these conditions it is highly unlikely that their small eyes are sensitive enough to visually detect their invertebrate prey (Plyuscheva et al., 2010). We did not test their behaviours at full moon intensities (approx.  $10^{-2}$  W/m<sup>2</sup> at the surface on clear nights) though, but judging by the V-Log I curves of dark adapted eyes this could allow for visually guided behaviours especially for the shallow water species *H. imbricata*. Still, most night will have intensities several orders of magnitude lower and in general prey detection is probably done through chemical and tactile cues picked up by their palps, tentacles and nuchal organs (Rouse and Pleijel, 2001; Lindsay, 2009; Jumars et al., 2015). During the mating behaviour of *H. imbricata*, they hook up in pairs with the male resting on the dorsum of the female in order to fertilize the spawned egg mass released by the female (eggs thereafter brooded under the scales) (Daly, 1972). Although we found them to be strictly night active, this might not be true for their reproductive behaviour. Daly (1972) observed them in daylight pairing up, though seemingly guided by chemical and tactile cues rather than vision.

It is noteworthy, that the photoreceptors in *L. squamatus* are less sensitive and somewhat faster than those of *H. imbricata*, which indicate that they are used under conditions with higher light intensities. One possibility is that they visually detect predators sneaking up on them in their hiding places during the day, which is supported by the differential scale lifting only happening in bright light.

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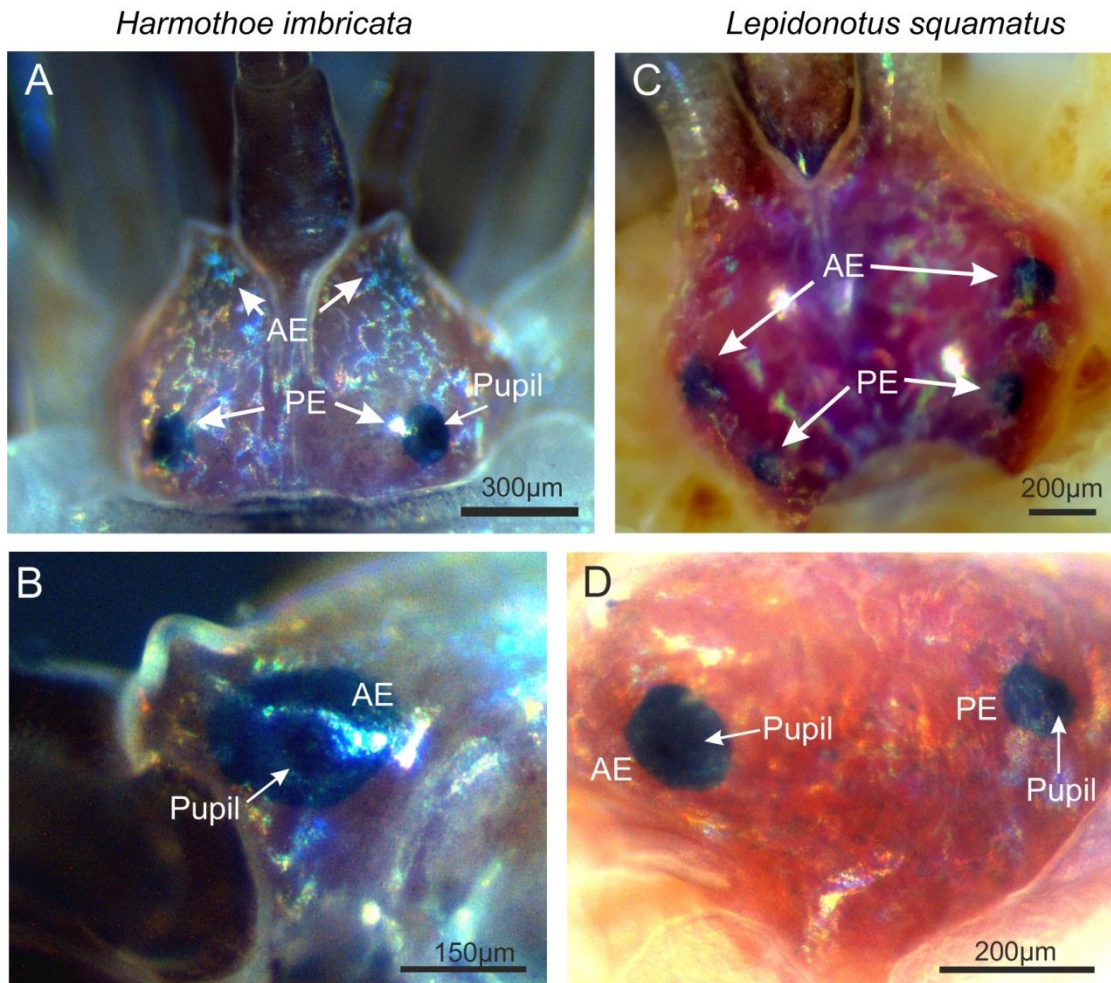
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## Figures

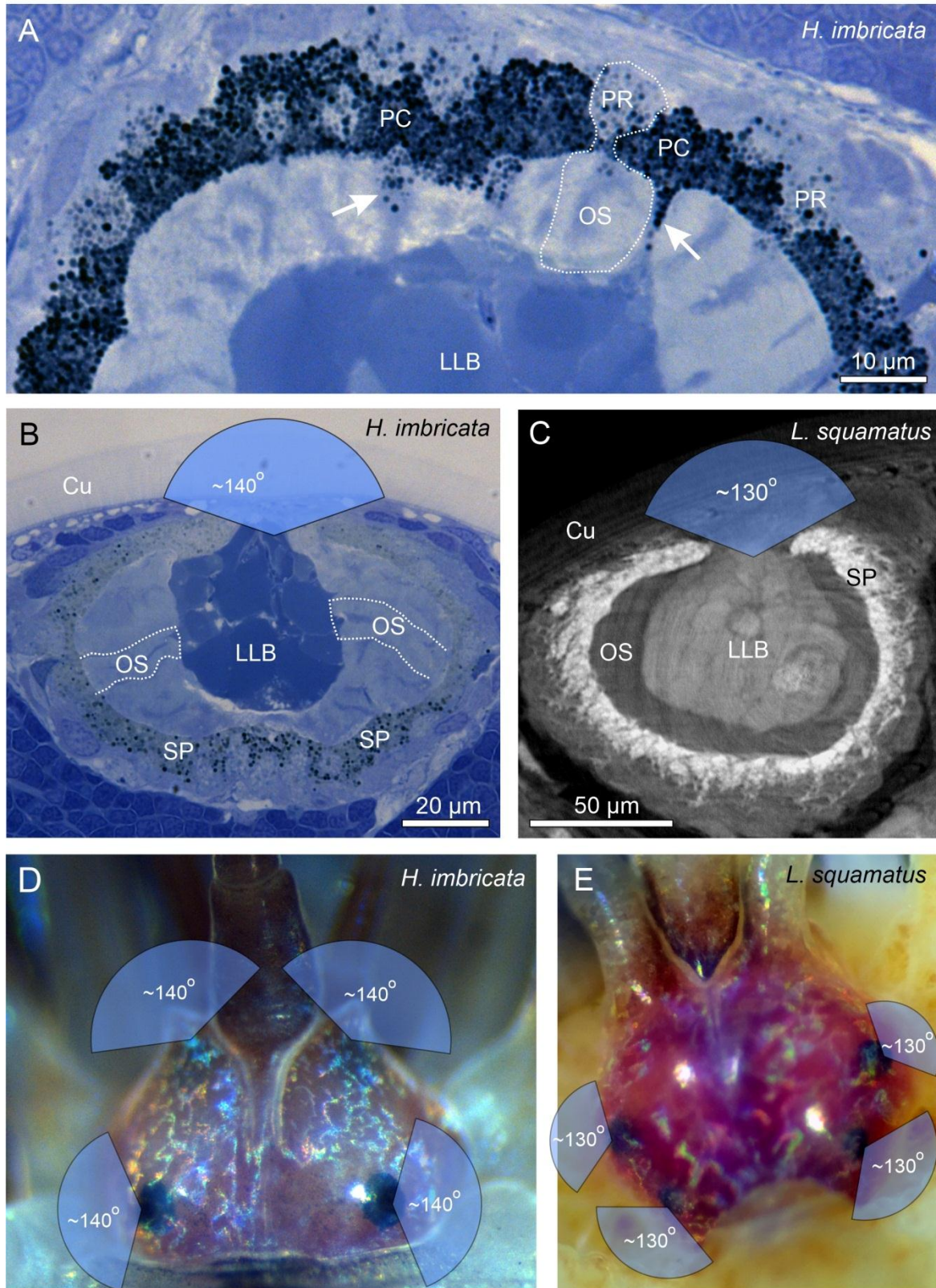
Figure 1



**Figure 1.** Arrangement of the eyes. Both species of scale worms have the classical set of two eyes on their prostomium as seen on many species of errant marine annelids. **A)** In *H. imbricata* the posterior pair of eyes (PE) are situated on the back of the prostomium on the dorso-lateral side. The anterior eyes (AE) are situated on the ventral side just next to the lateral tentacles on the frontal part of the prostomium. **B)** Anterio-lateral view of the prostomium of *H. imbricata* showing the left anterior eye.

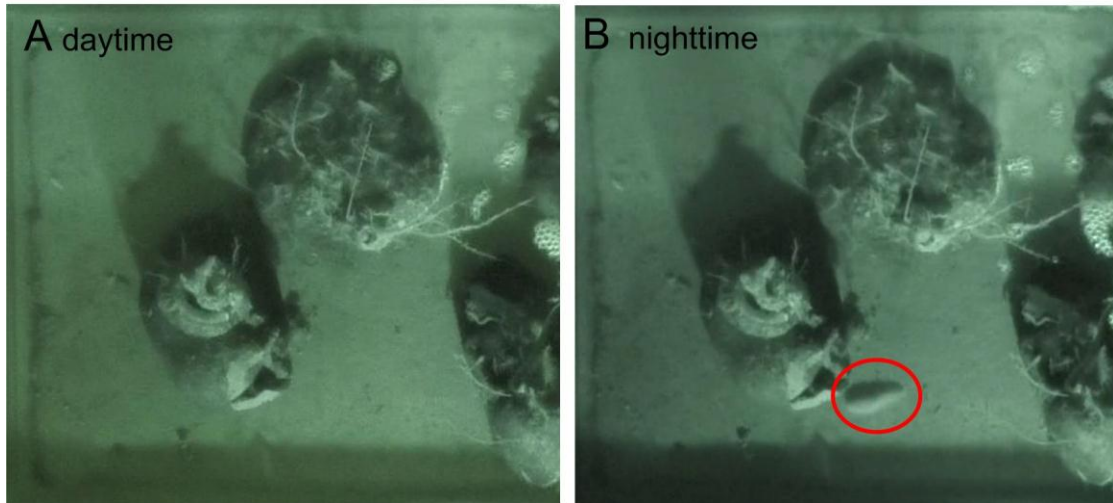
Note the pupil pointing antero-laterally. **C)** In *L. squamatus* both pairs of eyes are clearly visible on the dorsal side of the prostomium. Compared to *H. imbricata* the AE are moved backwards and are situated midway on the prostomium. **D)** In lateral view it is seen that the pupils in *L. squamatus* are pointing dorso-laterally for the PE and antero-laterally for the AE.

Figure 2



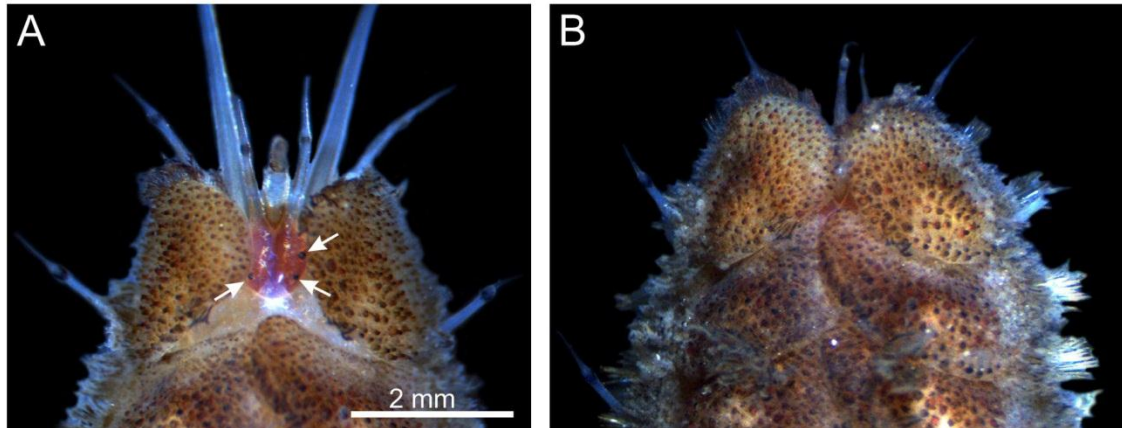
**Figure 2.** Eye morphology and gaze direction. **A)** Close up of the retina in a posterior eye of *H. imbricata*. Broken white line outlines a pigmented photoreceptor (PR) including the out segment (OS). Arrows indicate extensions of the pigment cells (PC) forming the lens-like body (LLB). **B)** LM section close to the optical axis of the anterior eye of *H. imbricata*. Broken white lines indicates the outer segments (OS) of the photoreceptors. Note that the LLB lies directly adjacent to the OS. The visual field of the eye as estimated by the screening pigment alone is a cone of approx.  $140^\circ$ . **C)** X-ray microtomographic scan through the optical axis of a posterior eye of *L. squamatus*. The morphology is very similar to that of *H. imbricata* (A) but note the shorter outer segments. Here the estimated visual field is a cone of approx.  $130^\circ$ . **D)** When the estimated visual fields are added to the direction of the pupil (see Fig. 1), it is seen that the anterior eyes of *H. imbricata* are gazing forwards and slightly to the side whereas the posterior eyes are gazing to the side and upwards. **E)** In *L. squamatus*, the anterior eyes gaze to the side and slightly forwards whereas the posterior eyes gaze to the sides and backwards. Cu = cuticle, SP = screening pigment.

## Figure 3



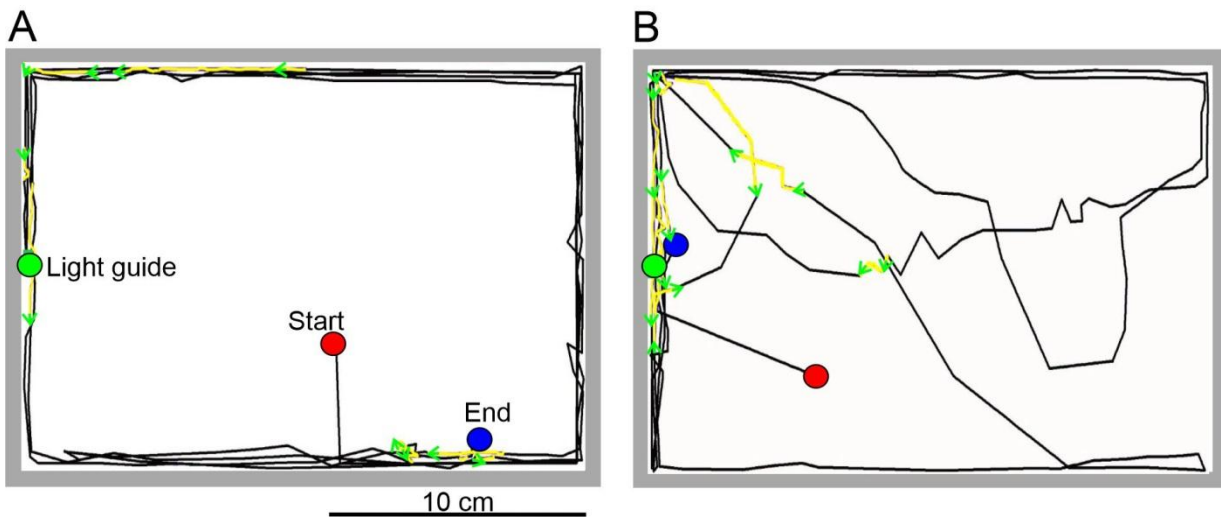
**Figure 3.** Diurnal rhythm. Both species are strictly night active. **A)** During light hours the scale worms would typically sit still in the shade under the provided stones in the tank. They did occasionally move a little but were never seen crawling around. **B)** After 5-10 min in darkness the worms would start moving around exploring the tank. Red circle marks *L. squamatus* crawling over the floor of the tank. Nighttime was filmed under infra-red light.

Figure 4



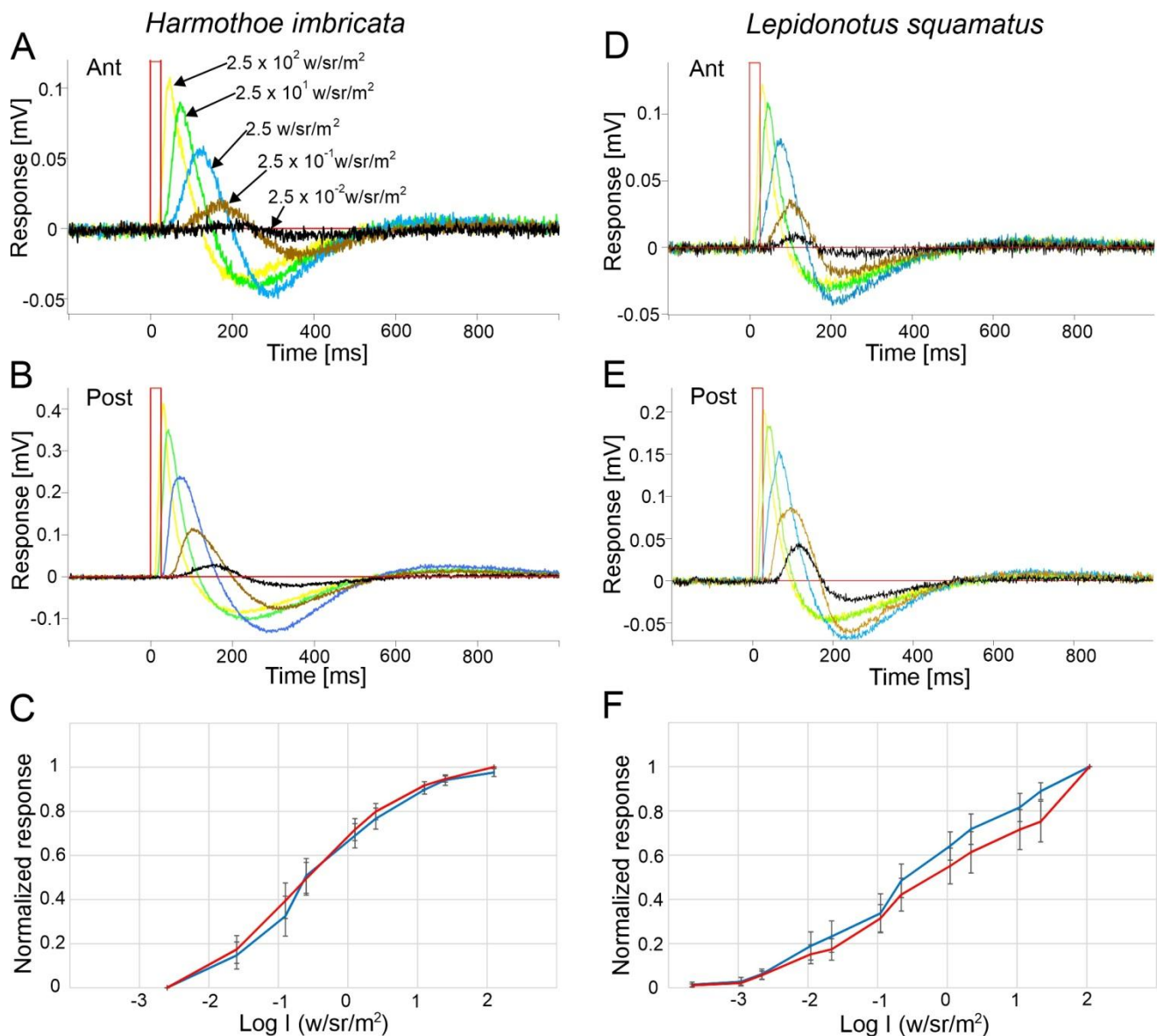
**Figure 4.** Scale lift. **A)** When crawling around in daytime light intensities undisturbed *L. squamatus* often lifts up the anteriormost pair of scales, which would reveal the prostomium and the eyes (arrows). **B)** If disturbed the anteriormost scales are lowered to protect/hide the prostomium. Note that the tentacles are also withdrawn. See also supplementary video.

Figure 5



**Figure 5.** Behavioural response of *H. imbricata* to light flashes. In otherwise darkness the scaleworms would be presented with short and weak flashes of green light mimicking the bioluminescence from the scales. **A)** Example of trajectories with light turned on at random times for 5 – 60 s. **B)** Examples of trajectory from experiments with light turned on for 30 s when the worm was with 10 cm of the light guide and faced it. Yellow part of trajectories indicate when light was on and green arrowheads the heading at light-on and light-off. Note that the worms had a strong tendency to crawl along the edges of the chamber in both types of experiments.

Figure 6

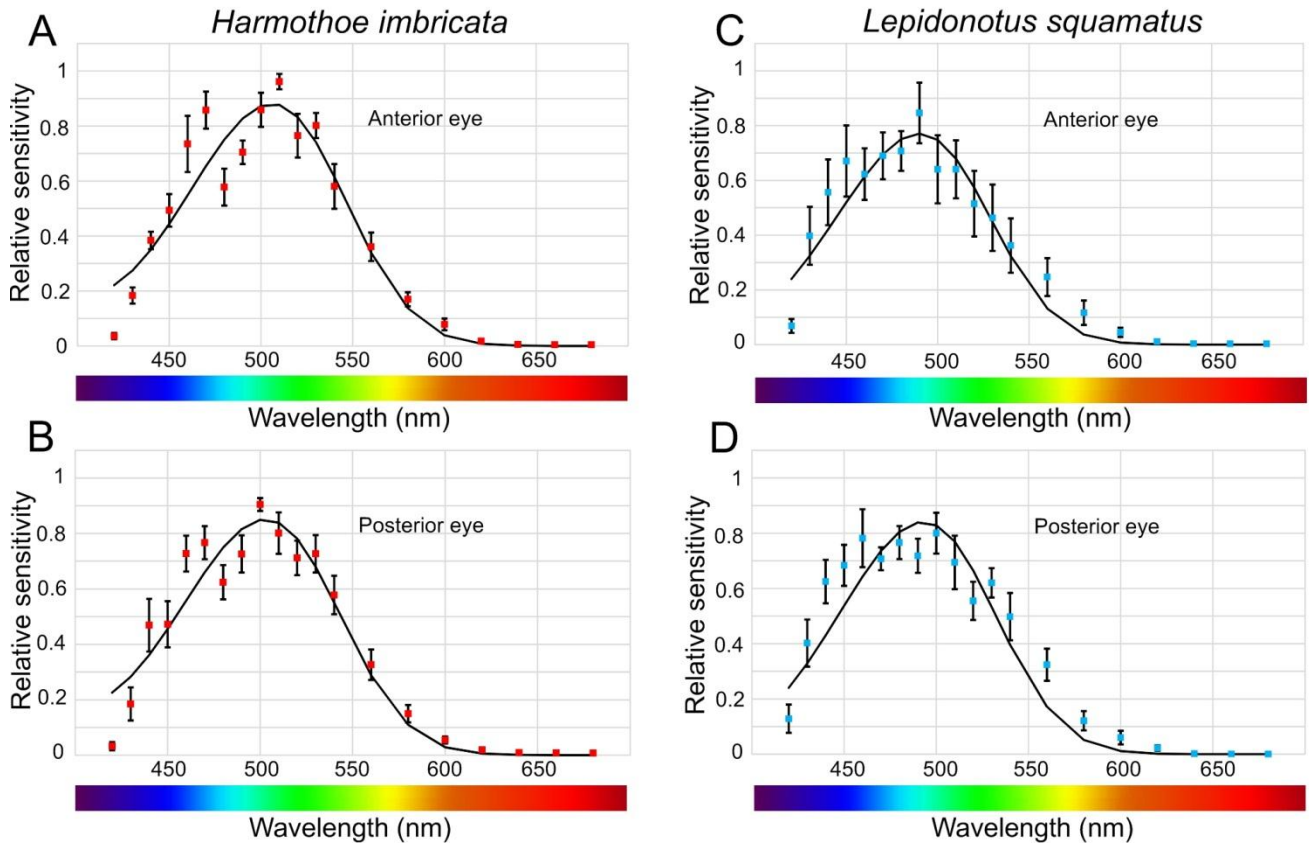


**Figure 6.** Absolute sensitivity of the posterior and anterior eyes. **A, B)** Examples of impulse responses from the anterior eyes (Ant) and posterior eyes (Post) of *H. imbricata* covering five log units of light intensity. The red curve indicates the 25 ms light stimulation. **C)** V-log I curves from Ant (blue line) Post (red line) eyes of *H. imbricata*. Both curves are close to sigmoidal and have a dynamic range of



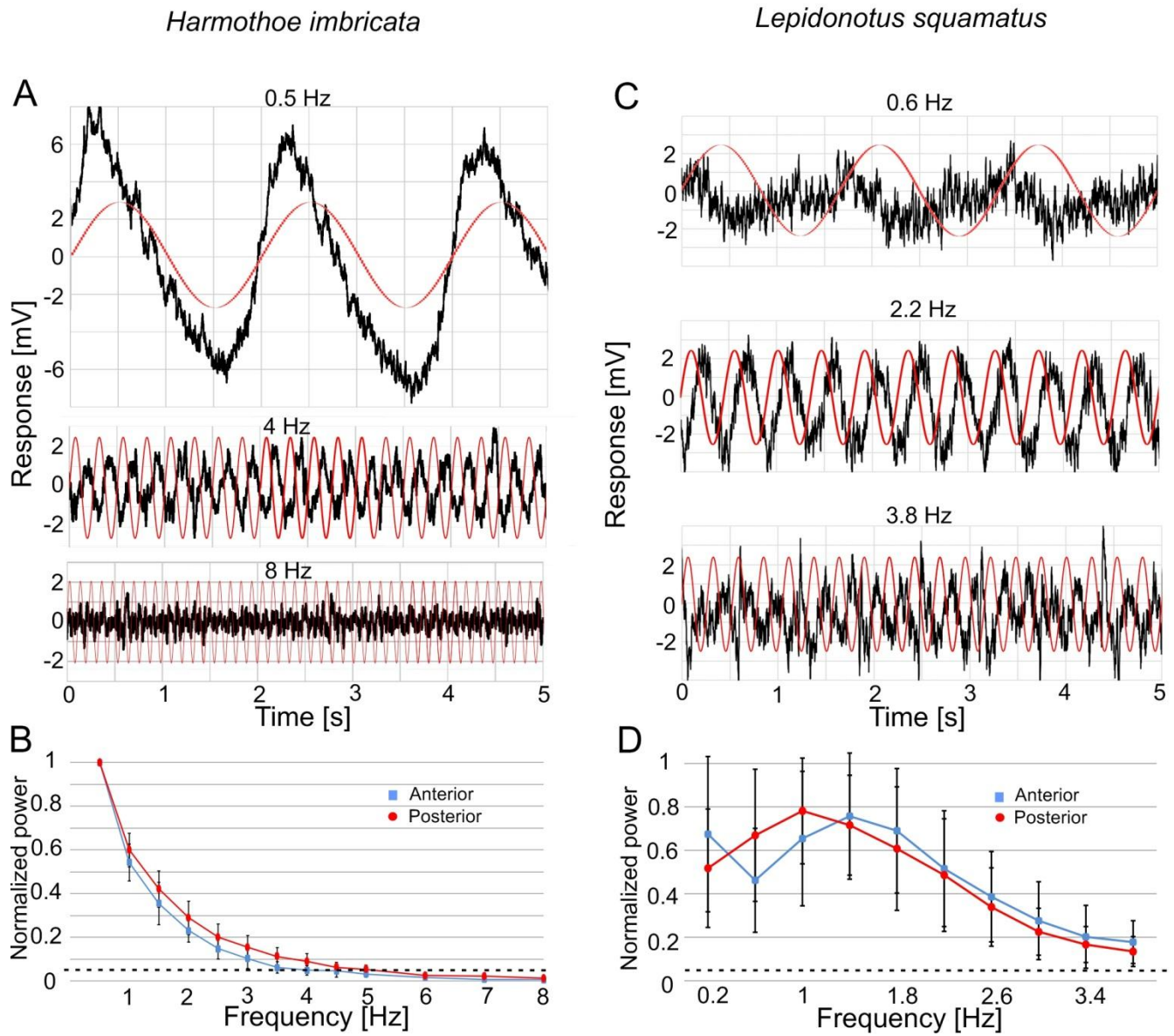
approx. 2.5 log units. **D, E**) Examples of impulse responses from the anterior eyes (Ant) and posterior eyes (Post) of *L. squamatus* covering five log units of light intensity. The color-coded intensities follow A and the red curve indicates the 25 ms light stimulation. **F**) V-log I curves from Ant (blue line) Post (red line) eyes of *L. squamatus*. No saturation is seen in the high intensity end and both eyes have a dynamic range of at least four log units. Error bars indicate standard error of mean, n=7 in C, and 8 in F.

Figure 7



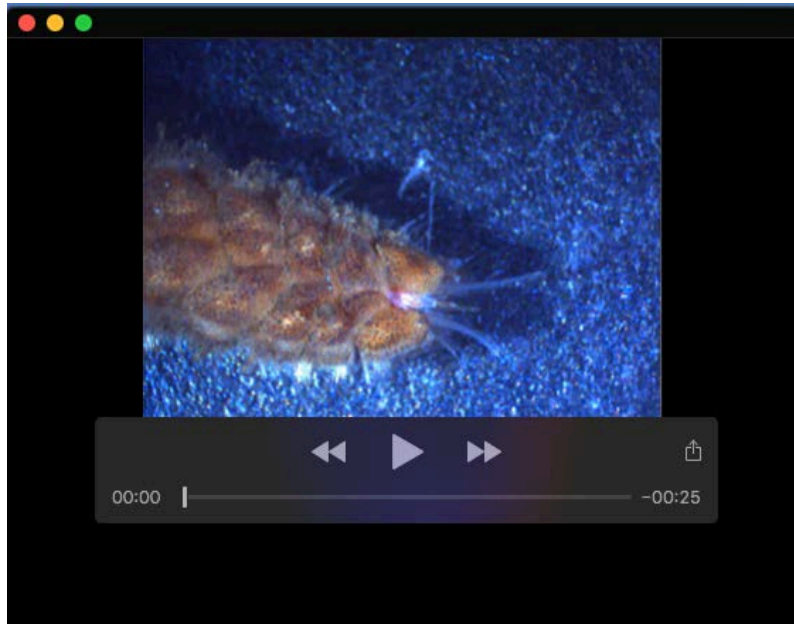
**Figure 7.** Spectral sensitivity curves. **A, B)** Spectral sensitivity curves from the two eyes of *H. imbricata* (red squares). The two curves are close to identical and the templates for a 506 and 499 nm opsin (black lines) (Govardovskii et al., 2000) are the best matches for the anterior and posterior eye respectively using the least squares method. **C, D)** Spectral sensitivity curves from the two eyes of *L. squamatus* (blue squares). The two curves are also close to identical and a 494 and 487 nm opsin (black lines) are the best matches for the anterior and posterior eye respectively. Error bars indicate the standard error of mean,  $n=7$  in A and B,  $n=8$  in C and D.

Figure 8



**Figure 8.** Temporal resolution measured as flicker fusion frequency. **A)** Examples of responses to 5 s sinusoidal stimulations (red lines) of 0.5, 4, and 8 Hz, respectively, from an anterior eye of *H. imbricata*. Note the great change in amplitude from 0.5 to 4 Hz (Responses are to scale) and the lack of response at 8 Hz. **B)** *H. imbricata*, when the relative response measured as the normalized power of the

Fourier transformation is mapped against frequency of stimulation it is seen that the response reaches 0.05 around 5 Hz for both eyes (broken black line). Note the steep decline with increased frequency of stimulation. **C)** Examples of responses to 5 s sinusoidal stimulations (red lines) of 1, 2.2, and 3.8 Hz, respectively, from a posterior eye of *L. squamatus*. Responses are to scale. **D)** For *L. squamatus* the normalized power does not reach 0.05 with the 3.8 Hz tested for either of the eyes. From the shape of the decline it is estimated that both would do this at 5 – 6 Hz. Blue lines are anterior eyes, red lines are posterior eyes, error bars indicate SD and n = 5 in B and 6 in D.



**Movie 1.** A large specimen of the scale worm *Lepidonotus squamatus* crawling in a petri dish under bright light. When undisturbed the anterior pair of scales are lifted exposing the two pairs of prostomial eyes but when disturbed mechanically the scales are lowered and the tentacles and palps are retracted for protection.