

RESEARCH ARTICLE

Pupil light reflex in the Atlantic brief squid, *Lolliguncula brevis*

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SUMMARY

Coleoid behavioral ecology is highly visual and requires an eye capable of forming images in a variety of photic conditions. A variable pupil aperture is one feature that contributes to this visual flexibility in most coleoids, although pupil responses have yet to be quantitatively documented for squid. The pupil light reflex (PLR) of the Atlantic brief squid, *Lolliguncula brevis*, was analyzed by directly exposing one eye of individual squid to light stimuli of varying irradiance and imaging the reflex, while simultaneously recording from the opposite, indirectly stimulated eye to determine whether the constriction was consensual between eyes. A PLR was measured in *L. brevis*, with an asymmetrical constriction observed under increasing irradiance levels that was not consensual between eyes, although a response of some level was observed in both eyes. Response thresholds ranged between 12.56 and 12.66 log photons cm⁻² s⁻¹. The PLR was rapid and dependent upon the stimulus irradiance, achieving half-maximum constriction within 0.49–1.2 s. The spectral responsivity of the PLR was analyzed by measuring the magnitude of the reflex in the eye directly stimulated by light of equal quantal intensity at wavelengths from 410 to 632 nm. The responsivity curve showed a maximum at 500 nm, indicating the eye is especially well suited for vision at twilight. These results, when considered in the context of the ambient light characteristics, show that the PLR of *L. brevis* contributes to a dynamic visual system capable of adjusting to the highly variable composition of light in its estuarine habitat.

Key words: lateral asymmetry, visual ecology, cephalopod, Coleoidea.

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INTRODUCTION

In order to meet the requirements for acute visual capabilities, the members of the subclass of cephalopods containing squid, cuttlefish and octopus (Coleoidea) possess advanced camera-type eyes. To enter the eye, light passes through a mobile pupil aperture, which typically constricts and centralizes incoming light reaching the graded refractive index lens. This minimizes spherical aberration of the light, which is focused on the retina by accommodative changes of the lens (Sivak, 2004). The pupil light reflex (PLR) enhances dynamic range and enables rapid focus under varying light conditions (Land and Nilsson, 2002). When the eye is exposed to high light intensity, the size of the pupil aperture decreases, which protects the retinal photopigments and limits the incoming light to a particular area of the retina. At low light levels, dilation of the pupil allows greater photon flux to the photopigments (Land and Nilsson, 2002; Talbot and Marshall, 2011). Cephalopod pupils are highly variable in shape, and may serve as a focusing mechanism, behavioral adaptation or means of camouflage (Froesch, 1973; Muntz, 1977; Hanlon and Messenger, 1996; Talbot and Marshall, 2011). Teuthids show a circular or crescent-shaped pupil surrounded by a highly reflective iris (Holt et al., 2011). An extension of the iris, termed the ‘iris flap’ or ‘lid’, descends from the dorsal margin in the Myopsina, a sub-order of near-shore squid with corneal eye covers (Arnold, 1967; Boyle and Rodhouse, 2005; Bozzano et al., 2009).

A PLR has been quantitatively documented in cephalopods: cuttlefish *Sepia officinalis*, octopus *Eledone cirrhosa*, and nautilus *Nautilus pompilius* (Muntz, 1977; Hurley et al., 1978; Douglas et

al., 2005). While there have been several studies of the teuthid visual system including eye development, lens shape and composition, and visual acuity (e.g. Sivak, 2004; Sweeney et al., 2007; Makino and Miyazaki, 2010; Bozzano et al., 2009), a complete description of the pupil reflex, and its ecological relevance to the animal, is lacking. A light-induced pupil reflex in teuthids was suggested in whole eyes of developing larvae, at the retinal level with respect to migrating pigments, and in regards to the shape and design of the pupil (Suzuki and Takahashi, 1988; Bozzano et al., 2009; Talbot and Marshall, 2011).

Many organisms such as humans, some amphibians and most rays show a consensual visual reflex, which is commonly observed in the PLR (e.g. Bateson, 1890; Henning et al., 1991; Kankipati et al., 2010). In these organisms, the two eyes respond in the same manner when one is exposed to a stimulus, while in the majority of sharks, reptiles and birds each eye functions independently (Denton, 1956; Kuchnow, 1971) (reviewed in Bisazza et al., 1998). Lateral asymmetry is the difference of morphology or function between the two sides of the brain leading to hemispheric specializations, observed in visual systems, appendage use and musculature (Bisazza et al., 1998). Perhaps the most striking example of lateral asymmetry in coleoids is that observed in the genus *Histioteuthis*, which shows morphological differences in the size of its eyes and optic lobes, as well as in lens color between the eyes, which are presumably used for separate tasks (Young, 1975; Wentworth and Muntz, 1989). Lateral asymmetry has been observed in the eyes of *Octopus vulgaris*, which relies primarily on monocular vision and also shows more active skin pigmentation on the arms

of the side of the preferred eye (Byrne et al., 2002). In regards to the PLR, Douglas and colleagues quantitatively described the lack of a consensual response (i.e. lateral asymmetry) in the pupils of *E. cirrhosa* and *S. officinalis* (Douglas et al., 2005). The presence or absence of lateral asymmetry in an organism could be an indication of certain visual capabilities (e.g. monocular *versus* binocular vision) related to its behavioral ecology.

The Atlantic brief squid, *Lolliguncula brevis* (Blainville), is a small Loliginid with a mantle length ≤ 100 cm (Hixon, 1980). It inhabits near-shore and estuarine environments from Delaware Bay (DE, USA) south to the Gulf of Mexico and Argentina/Uruguay, and relies heavily on visual hunting (Voss, 1956; Laughlin and Livingston, 1982; Boyle and Rodhouse, 2005). Various aspects of *L. brevis* physiology such as its anaerobic metabolism (Finke et al., 1996) and euryhaline tolerance (Hendrix et al., 1981) give it the ability to survive the rapidly changing physical conditions of estuarine environments. Because of the variable nature of light in near-shore habitats, *L. brevis* likely also possesses sensory adaptations to enable consistent visual function.

The primary goals of this study were to definitively test the PLR in a member of the squid order Teuthidae, and, if present, to observe whether the response was consensual between eyes or whether the eyes demonstrated lateral asymmetry. It was hypothesized that *L. brevis* would show a light-induced pupillary reflex at ecologically relevant irradiance levels and wavelengths with a non-consensual response between directly and indirectly stimulated eyes, and therefore demonstrate lateral asymmetry as observed in other coleoids.

MATERIALS AND METHODS

Specimen collection

Lolliguncula brevis were collected by otter trawl from shallow areas (1.5–6 m depth) in Boca Ciega Bay (FL, USA) and lower Tampa Bay (FL, USA) between December 2010 and February 2011. Specimens were transported and treated to reduce stress following a previously published protocol (Hanlon et al., 1983). After collection, *L. brevis* were brought back to the laboratory where they were transferred to individual covered, opaque, aerated buckets. The mantle length of each individual was measured; squid were then used in a single experimental trial and subsequently released ~2 km from the collection site.

Twenty-six individuals were collected in total. Experimental trials were selected for subsequent analysis based on the following criteria: (1) individuals showed good health (able to swim, actively resisting capture, showing significant siphon movement once immobilized for the experiment indicating normal physiological respiration), (2) squid were of acceptable physical condition (no damage around eyes, little damage to the skin, arms or body), and (3) video quality and experimental procedure were adequate (in focus, correct zoom, constant recording conditions, little to no rotation of animal during experiment). Out of the 26 individual trials, 15 trials met the above criteria. Seven individuals with mantle length 5.6 ± 0.18 cm (mean \pm s.e.m.) were used in the PLR experiment. Eight individuals with mantle length 6.6 ± 0.26 cm (mean \pm s.e.m.) were used in the spectral responsivity experiment.

Experimental set-up

Individual squid were immobilized during experiments in a holder (see Preuss and Budelmann, 1995): a 10 cm long PVC pipe was fitted into a stand-hole of an acrylic tank (50 \times 50 \times 50 cm, filled with 5 μ m-filtered seawater) (Fig. 1A), and a 5 \times 5 cm PVC elbow extended from the top of the pipe, terminating in a section of flexible

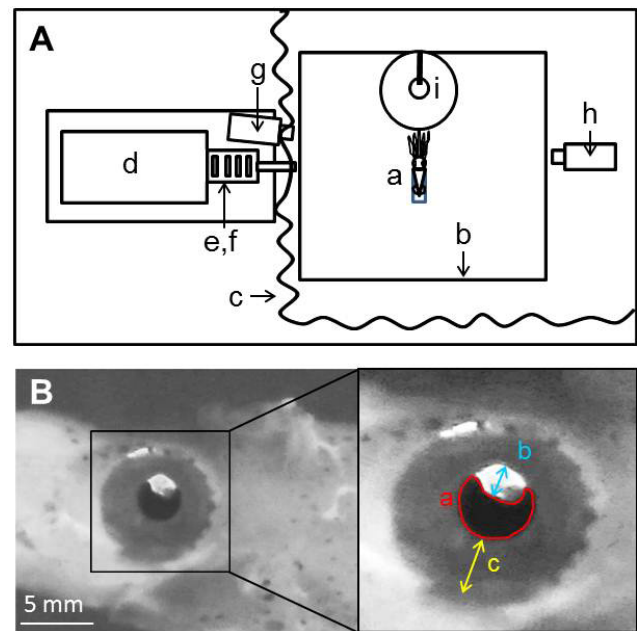


Fig. 1. Experimental set-up and eye parameter measurements. (A) Laboratory design viewed from above. Individual *Lolliguncula brevis* were placed in a holder (a) in an acrylic experimental tank (b) surrounded by black cloth on the walls and a curtain to limit stray light (c). Experimental light was provided by a voltage-regulated lamp (d), the quality of which was changed by inserting neutral density and interference filters into slots (e) and controlled using an electronic shutter (f). Responses were captured by camcorders recording the eye under direct stimulation (g) and indirect stimulation (h). Constant background illumination was provided by fluorescent light (740 nm half-maximum emission) through a red sleeve, and a red compact fluorescent bulb above the tank (i). (B) Eye parameters analyzed. Measurements included pupil area (a, enclosed by the red line), lid length (b, blue arrow) and ventral iris length (c, yellow arrow), captured using ImageJ software.

Tygon tubing. An individual specimen was secured by inserting the mantle into the tubing, which was then tightened with a hose clamp, allowing enough movement to facilitate breathing. Experiments were conducted in a windowless room, with a light-tight flat black curtain surrounding the aquarium containing the test subject. The aquarium was constantly illuminated from above throughout all experiments by dim red fluorescent lighting (T12 bulb with red safety sleeve, Creative Lighting Solutions, Cleveland, OH, USA; and 13 W red compact fluorescent bulb; 740 nm half-maximum spectral emission) to provide sufficient light for video recording. Based on previous reports of Loliginid visual pigments (e.g. Daw and Pearlman, 1974; Hanlon and Messenger, 1996), *L. brevis* was not expected to have long-wavelength visual sensitivity. Accordingly, while low levels of red background light may have been perceived by test subjects, these same background conditions were maintained throughout the experiment and represented a baseline sufficient for testing subject pupil dilation.

Experimental light stimuli were provided by a voltage-regulated 150 W quartz halogen lamp (Fiber-Lite DC-950, Dolan Jenner, Boxborough, MA, USA). Light from the lamp passed through a hot mirror to reduce heat, and then through a combination of neutral density and interference filters according to each light stimulus (25 mm diameter, Edmund Optics, Barrington, NJ, USA), with stimulus duration controlled by an electromagnetic shutter and driver (models VS25 and 310, Uniblitz, Rochester, NY, USA). The stimulus light passed through a small hole in the curtain *via* an

extension tube extending from the shutter towards the aquarium and onto the test subject – a distance of ~22 cm. This optical arrangement resulted in a beam of light centered on the eye receiving the direct stimulus, extending only slightly wider than the entire eye at high irradiance levels, and creating very little stray light in the aquarium at the level of the test subject's opposite, indirectly stimulated eye. Absolute irradiance was measured under red light and for all experimental intensities integrated from 400 to 700 nm using a spectrometer radiometrically calibrated with a 1 m long (600 μm core) cosine-corrected fiber optic cable (Ocean Optics USB 2000, LS-1-CAL calibration source, Dunedin, FL, USA). The diffuser of the cosine corrector, approximately the diameter of the squid's eye, was pointed both towards the light source and away from the light source to measure irradiance at the level of the directly stimulated eye and indirectly stimulated eye, respectively.

Two digital camcorders (Sony DCR-SR88) were used to simultaneously record pupil constriction in each eye. One camera was positioned with its lens projecting through the curtain viewing the eye of the test subject along the axis of the light from the stimulus lamp (i.e. directly stimulated eye). The other camera was positioned on the opposite side of the tank recording the eye that did not receive direct stimulation from the light source (i.e. indirectly stimulated eye).

PLR

Experimental video recordings began after a specimen was placed in the holder and allowed to acclimate to the experimental setting for 5 min, illuminated only by red background light. The specimen was then exposed to 532 nm light (10 nm full width at half maximum, FWHM) for 1 min at the lowest stimulus intensity, followed by a return to background red light for 1 min to allow for pupil dilation to its initial size, then a higher stimulus intensity, etc., until nine stimuli of different intensity had been presented. Light exposure for the eye under direct stimulation began with the lowest intensity (12.54 log photons $\text{cm}^{-2} \text{s}^{-1}$) and increased through to full intensity light (14.24 log photons $\text{cm}^{-2} \text{s}^{-1}$), while intensity remained constant for the eye under indirect stimulation during all light stimuli (~12.77 log photons $\text{cm}^{-2} \text{s}^{-1}$). Each stimulus event was labeled with an audio time stamp and video frames were captured at select times, autocorrected for saturation and contrast (Sony PMB software), and analyzed using ImageJ software (NIH Image, <http://rsb.info.nih.gov/nih-image/>). Data from this experiment were used to quantify the PLR as a function of time and irradiance.

The time course of pupil constriction at low, intermediate and high irradiance levels was determined for the above experiment by measuring the pupil area in red light immediately before the light exposure, then over a 2 s period at 0.2 s intervals following the onset of the light stimulus. Additional measurements were made 3, 4, 5, 20, 35 and 50 s after stimulus initiation. The data at each irradiance were normalized to the dilated pupil area immediately before stimulus exposure and fitted with a 3-parameter exponential decay function (SigmaPlot 12.2, SPSS, Chicago, IL, USA). The resulting equation was used to calculate the time elapsed before the pupil reached 50% of the maximum constriction for that light level (t_{50}) (Douglas et al., 1998; Douglas et al., 2005).

The PLR was measured in both the directly stimulated eye and the indirectly stimulated eye during the above experiment to determine the magnitude of the constriction as a function of irradiance and the presence or absence of a consensus between eyes. For the directly stimulated eye, measurements were made 50 s after the onset of a stimulus and after 50 s of dilation under background red illumination. Similarly, frames of the indirectly stimulated eye

were also captured after 50 s of dilation under red light. However, the required positioning of the camera slightly ventral to the optical axis included a small portion of the light source in the lens wide field of view, resulting in an oversaturated image during delivery of intermediate to high light stimuli to the direct eye. Because the pupil outline was difficult to observe in frames of the indirect eye 50 s after stimulus initiation, frames were captured as soon as the camera focused following shutter closure (~1 s). Data from this time point at the low to intermediate light stimuli experienced by the indirect eye were representative of the constricted pupil; a comparison of the pupil areas 50 s after stimulus initiation and 1 s after stimulus removal showed no significant difference (data not shown). To fully describe the PLR, measurements of the pupil included: (1) pupil area, defined as the area of the open pupil, (2) lid length, measured vertically from the top to the bottom of the lid, and (3) ventral iris length, from the ventral edge of the pupil aperture to the ventral edge of the iris (Fig. 1B). Pupil area is routinely used to quantify the pupil reflex (e.g. Hurley et al., 1978; Douglas et al., 1998; Douglas et al., 2005) and both lid length and ventral iris length are of interest for near-shore squid such as *L. brevis* with a mobile extension of the iris (i.e. lid) and the possibility of asymmetrical pupil constriction. The pupil area, lid length and ventral iris length were each analyzed for significance using a 2-way repeated measures analysis of variance (2-way RM ANOVA) with Tukey *post hoc* testing (SigmaPlot 12.2, SPSS). The PLR threshold was defined as the lowest light level that showed a significant ($P < 0.05$) difference in the parameter during a light stimulus and after dilation under red light before that stimulus was given. Additionally, the magnitude of the PLR between the eyes under direct and indirect stimulation was compared at the maximum irradiance stimulus during both light exposure and the preceding dilation under red light using a rank sum test.

Spectral responsivity

A response spectrum for the *L. brevis* PLR was determined in a second experiment by measuring the pupil constriction in the directly stimulated eye after exposure to a range of wavelengths at equal quantal intensity. Irradiance at each wavelength was measured at the position of the directly stimulated eye as described above and quantally matched using neutral density filters to the lowest value measured (14.05 log photons $\text{cm}^{-2} \text{s}^{-1}$). The response spectrum experiment was conducted as described above for the PLR in the direct eye, except light stimuli were only presented for 30 s, as the constriction appeared to be fully established before that point, and the dilation period under red light was extended to 2 min to allow the pupil to completely return to its original state. Eight spectral stimuli were given in decreasing order from 632 to 410 nm (10 nm FWHM). Frames were captured and pupil area was measured 20 s after stimulus initiation and after 1 min 50 s dilation in red light, and analyzed for significance using a 2-way RM ANOVA with a Tukey test, as above.

Environmental light measurements

Environmental light data were collected in *L. brevis* habitat during March 2011 to compare with the PLR threshold and spectral responsivity data collected in the laboratory. All measurements were initiated 30 min before the official time of solar noon (sun center at highest elevation) and civil twilight (sun center 6 deg below horizon), and concluded ~30 min after noon and 60 min after twilight. All measurements were made under clear skies, with those at solar noon made during flood tide, and civil twilight measurements made during ebb tides. Data were collected at a depth of 0.5 m facing

south from a north/south-oriented dock in Boca Ciega Bay (FL, USA; 27°42.640'N, 82°41.464'W), which is a documented *L. brevis* habitat (Dragovich and Kelly, 1967) (L.R.M., unpublished observations). Spectral irradiance and photosynthetically active radiation (PAR 400–700 nm) were sampled every 5 min using a spectrometer radiometrically calibrated with a 10 m long (600 μm core) cosine-corrected fiber optic cable (Ocean Optics USB 2000, LS-1-CAL calibration source) oriented to measure sidewelling light along a squid's predicted line of sight. Cosine-corrected sidewelling PAR at 0.5 m was also measured at 5 min intervals (LI-192 sensor, LI-1400 logger, LI-COR, Lincoln, NE, USA). Depth profiles measuring the sidewelling spectral irradiance as PAR were taken at twilight and noon to calculate the attenuation coefficient (k_{PAR}).

RESULTS

PLR

A distinct PLR was observed in both directly and indirectly stimulated eyes, where the pupil constricted during periods of light exposure, and dilated under red light after removal of the stimulus (Fig. 2). The PLR constriction time course was measured at three stimulus irradiances: 12.66, 13.35 and 14.24 log photons $\text{cm}^{-2} \text{s}^{-1}$. The full PLR constriction occurred within 1–3 s, and the speed (t_{50} , time to 50% maximum constriction) was dependent on the stimulus irradiance (Fig. 3). At the lowest irradiance at which the PLR was observed (12.66 log photons $\text{cm}^{-2} \text{s}^{-1}$; see below) the t_{50} was 1.2 s, whereas at higher irradiances of 13.35 and 14.24 log photons $\text{cm}^{-2} \text{s}^{-1}$ the t_{50} was 0.90 s and 0.49 s, respectively. After the initial constriction, the pupil area changed very little throughout the duration of the stimulus, with the 20–50 s time points representing the most developed constriction (Fig. 3).

In the directly stimulated eye, the lowest irradiance to evoke a significantly smaller pupil area during a light stimulus relative to the dilated pupil area under red light immediately before the stimulus (i.e. threshold) was 12.66 log photons $\text{cm}^{-2} \text{s}^{-1}$ ($P < 0.001$, 2-way RM ANOVA; $P = 0.005$, Tukey test) (Fig. 4A). The threshold of the PLR for the eye under indirect stimulation was greater than that of the eye under direct stimulation, with the pupil area threshold shifted to a higher irradiance of 12.98 log photons $\text{cm}^{-2} \text{s}^{-1}$ in the indirect eye ($P = 0.003$, Tukey test) (Fig. 4B). The lid length parameter showed the same threshold (12.98 log photons $\text{cm}^{-2} \text{s}^{-1}$) in the eye under direct stimulation and the eye under indirect stimulation ($P < 0.001$ and $P = 0.032$, respectively, 2-way RM ANOVA; $P < 0.001$ and $P = 0.006$, respectively, Tukey test) (Fig. 5). The measurement of ventral iris length showed no significant differences across light stimuli in either the directly or indirectly stimulated eye ($P = 0.922$

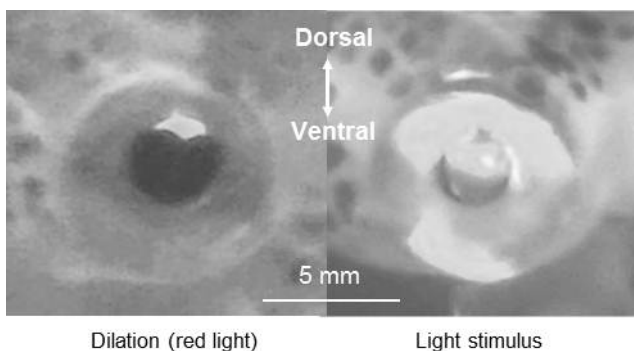


Fig. 2. Pupil light reflex (PLR) in *L. brevis*. The eye of *L. brevis* showing dilation under red light (12.50 photons $\text{cm}^{-2} \text{s}^{-1}$, left) and a PLR during the highest experimental irradiance (14.24 photons $\text{cm}^{-2} \text{s}^{-1}$, right).

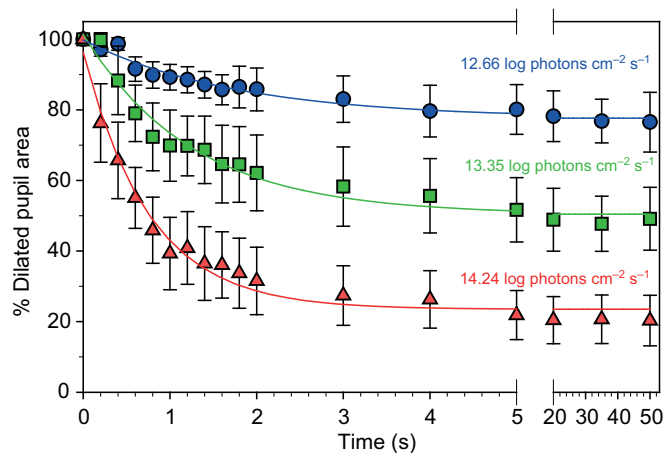


Fig. 3. Time course of the PLR in *L. brevis*. Pupil constriction measured 0–50 s after exposure to stimuli at three irradiances plotted as a percentage of the fully dilated pupil in red light immediately before initiation of the stimulus. Data are means \pm s.e.m. for 7 replicate individuals. Lines represent significant fits ($P < 0.05$) of 3-parameter exponential decay functions for each light level used to calculate time to 50% maximum constriction (t_{50}) as described in Materials and methods: 12.66 photons $\text{cm}^{-2} \text{s}^{-1}$, % dilated area = $77.6 + 22.1e^{-0.567t}$; 13.35 photons $\text{cm}^{-2} \text{s}^{-1}$, % dilated area = $50.4 + 50.9e^{-0.798t}$; 14.24 photons $\text{cm}^{-2} \text{s}^{-1}$, % dilated area = $23.5 + 72.8e^{-1.32t}$.

and $P = 0.121$, respectively, 2-way RM ANOVA) (Fig. 5). During the maximum irradiance light stimulus (i.e. 14.24 log photons $\text{cm}^{-2} \text{s}^{-1}$), the magnitude of the PLR in the directly stimulated eye was greater than that in the indirectly stimulated eye in terms of the pupil area ($P < 0.001$) but not the lid length ($P = 0.073$). There was no difference in the PLR magnitude between the eyes during dilation immediately prior to the maximum light stimulus exposure in any parameter ($P > 0.05$).

Spectral responsivity

While pupil area remained constant during dilation periods under red light, exposure of the directly stimulated eye to different wavelengths of light at equal quantal intensities resulted in varying degrees of constriction. The PLR response spectrum showed a significant difference in pupil area between light stimuli and dilation under red light for wavelengths between 436 and 550 nm ($P < 0.001$, 2-way RM ANOVA; $P \leq 0.003$, Tukey test), with a peak at 500 nm (Fig. 6).

Environmental light

Sidewelling light levels at 0.5 m in *L. brevis* habitat ranged from the highest irradiance of 16.54 log photons $\text{cm}^{-2} \text{s}^{-1}$ at solar noon to 11.17 log photons $\text{cm}^{-2} \text{s}^{-1}$ at twilight, when the greatest rate of change occurred (Fig. 7). At twilight the spectral composition peaked at ~ 500 nm with a smaller peak at ~ 650 nm (Fig. 7). At midday, dominant wavelengths were ~ 565 nm with an absolute irradiance ~ 5 orders of magnitude greater than at twilight (Fig. 7). The calculated k_{PAR} for *L. brevis* habitat in Boca Ciega Bay was 1.26 m^{-1} .

DISCUSSION

The present results clearly demonstrate a PLR in *L. brevis* with sensitivity well suited to the irradiance and spectral composition of light in its habitat. The response was fast, taking between 0.49 and 1.2 s to reach half the maximum pupil constriction (t_{50}). The time

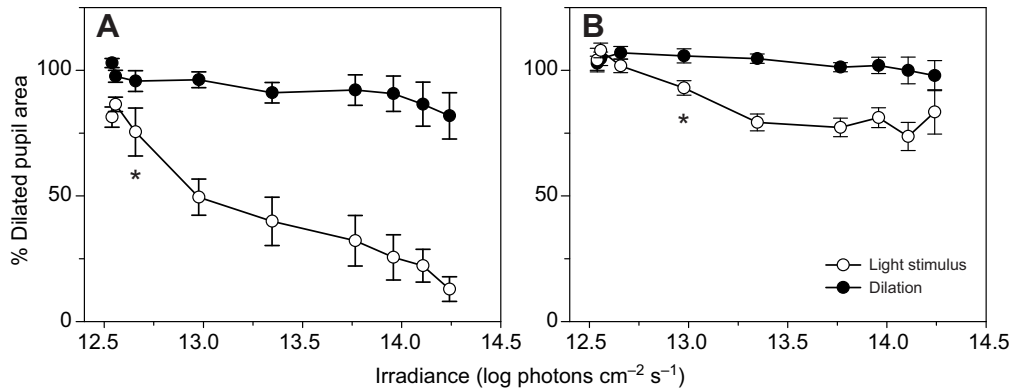


Fig. 4. PLR of *L. brevis* defined by the mean pupil area. Pupil area is plotted for the directly stimulated eye (A) and indirectly stimulated eye (B). All values are shown as a percentage of the fully dilated pupil area directly before any experimental light exposure. Data points indicate the eye during a light stimulus and during dilation under red light immediately before the stimulus, and are means \pm s.e.m. for 7 replicate individuals. Asterisks indicate the response threshold (2-way RM ANOVA with Tukey test; $P < 0.05$).

course of the PLR was dependent upon the stimulus irradiance, with a faster response and greater overall constriction at higher irradiances. This rapid, irradiance-dependent response is similar to that reported for the PLR in cuttlefish *S. officinalis* and octopus *E. cirrhosa* ($t_{50} = 0.32$ and 0.65 s, respectively) (Douglas et al., 2005), and much faster than the ~ 39 s to half-maximum constriction we calculated for *N. pompilius* based on published data (Hurley et al., 1978). Comparisons can be difficult because of experimental differences in exposure time, irradiance and spectral quality of the stimuli, but the constriction time of the cephalopod pupil is similar to that of mammals and birds, which show some of the fastest responses in the animal kingdom (Barbur et al., 2002).

Threshold irradiance values for the PLR differed when the pupil response was calculated as pupil area, lid length and ventral iris length, which gives insight into the mechanism of the response. During exposure to light stimuli, the lowest threshold observed among the parameters measured was that of the pupil area, with a threshold of $12.66 \log \text{ photons cm}^{-2} \text{ s}^{-1}$. The next parameter to show sensitivity as the intensity increased was the lid length, which had a threshold at $12.98 \log \text{ photons cm}^{-2} \text{ s}^{-1}$. Accordingly, the PLR in *L. brevis* is controlled by adjusting pupil area by the extent of the lateral iris in lower light levels or subtle irradiance changes, while extension of the dorsal iris lid occurs at higher irradiances or upon large light changes. Additionally, there was no experimental difference in the ventral iris length at any irradiance, indicating that the pupil constriction is not uniform and must come from lateral and dorsal extension of the iris, rather than from the ventral margin

of the pupil or eye. The neuro-muscular control of this extension remains an open question.

While the primary function of the pupil aperture is to regulate and direct the light reaching the lens and retina, the asymmetrical anatomy of this constriction could interfere with the visual gaze of the organism. In other species of cephalopods, variations in the retinal topography and photoreceptor cell density create specialized visual axes, and are dependent on their photic environment and habitat (Muntz, 1977; Makino and Miyazaki, 2010; Talbot and Marshall, 2011). Based on the centralized areas of retinal cell density and crescent-shaped pupils found in coastal and inshore coleoids, the visual axis is directed downward and forward in these animals to enable detection of changes both on the sea floor and in the water column (Makino and Miyazaki, 2010; Talbot and Marshall, 2011). If the same were true for *L. brevis*, the asymmetrical pupil constriction would enable it to maintain the expected downward visual axis and still effectively shield the eye from downwelling and sidewelling light.

Light observed at solar noon in shallow *L. brevis* habitat was over three orders of magnitude greater than the irradiance at which the lid length saturated in the directly stimulated eye ($13.95 \log \text{ photons cm}^{-2} \text{ s}^{-1}$). To compensate for the limits of the PLR, *L. brevis* likely employ synergistic methods such as pigment migration (Daw and Pearlman, 1974) or behavioral preferences for deeper daytime habitats (Inada, 1996; Gilly et al., 2006). Using light data, the threshold for pupil area ($12.66 \log \text{ photons cm}^{-2} \text{ s}^{-1}$) and the irradiance at which the lid length appeared to saturate ($13.95 \log$

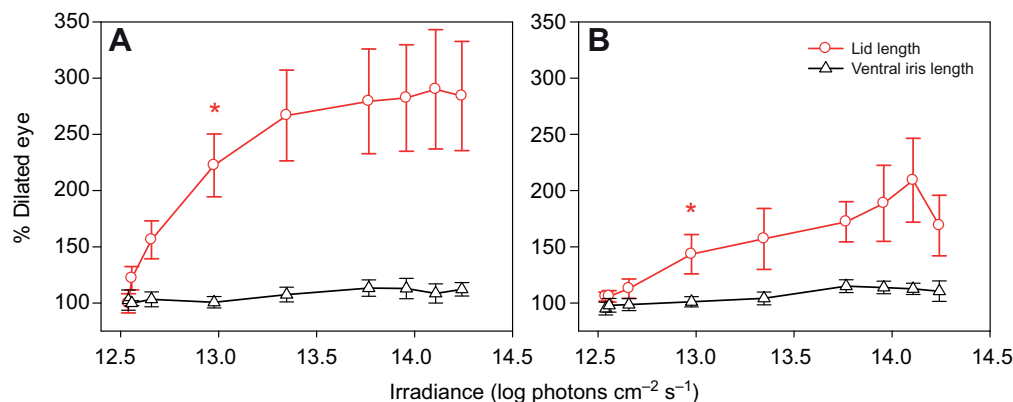


Fig. 5. PLR of *L. brevis* defined by the mean lid length and ventral iris length. Each parameter is plotted for the directly stimulated eye (A) and indirectly stimulated eye (B). All values are shown as a percentage of the fully dilated pupil directly before any experimental light exposure. Each panel shows lid length and ventral iris length measurements during light stimuli. Data are means \pm s.e.m. for 7 replicate individuals. Asterisks indicate the response threshold for the lid length parameter (2-way RM ANOVA with Tukey test; $P < 0.05$); ventral iris length did not change across light stimuli.

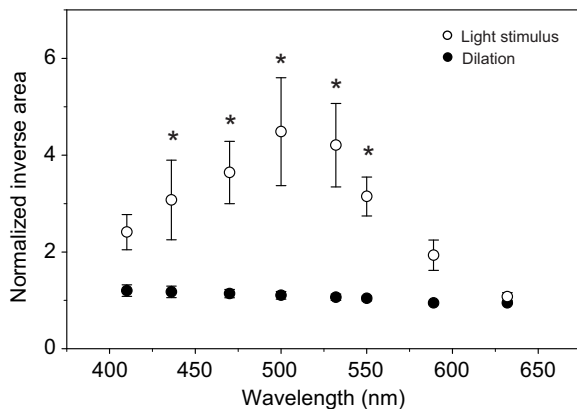


Fig. 6. Spectral responsivity of *L. brevis* PLR. The inverse pupil area is plotted as a function of wavelength during the light stimulus and the dilation under red light immediately before the stimulus. Asterisks indicate a significant response between the PLR during periods of light stimulus and dilation (2-way RM ANOVA with Tukey test; $P < 0.05$). Data are means \pm s.e.m. for 8 replicate individuals normalized to the fully dilated pupil directly before any experimental light exposure.

photons $\text{cm}^{-2} \text{s}^{-1}$), the optimal depth range for maximum PLR function during daytime hours is calculated to be 4.3–6.7 m, which is consistent with our collections and previous habitat studies of *L. brevis*. During daylight sampling, Bartol and colleagues determined that *L. brevis* were more commonly found at depth ranges of 5–15 m, as opposed to depths shallower than 5 m in the Chesapeake Bay (VA, USA) (Bartol et al., 2002), and Laughlin and Livingston described a preference for channels or areas with a current and depths greater than 4 m in Apalachicola Bay (FL, USA) (Laughlin and Livingston, 1982). Similarly, in the present study, individuals were captured during the day at a depth of ~ 2 –3 m, and we had no success at daytime capture in shallower water.

The PLR varied not only with light intensity but also with the spectral composition of light. While the PLR clearly occurred in response to light stimuli with maximum sensitivity peaking at ~ 500 nm, the photopigments responsible for activating the response are unknown. Ambient light can be detected by both visual and non-visual photopigments, with various non-visual photopigments controlling circadian rhythms, hormone secretions and/or pupillary constriction in both vertebrates and invertebrates (reviewed in Foster and Helfrich-Förster, 2001; Fu et al., 2005). It is possible that the photopigment responsible for light detection controlling the PLR is a rhodopsin (visual), commonly found in cephalopods and partially sequenced in *L. brevis* (Strugnell et al., 2005), or a melanopsin (non-visual), which shows biophysical similarities to rhodopsin (e.g. Koyanagi et al., 2005). Regardless of the type of photopigment, a λ_{max} at 500 nm is beneficial to an estuarine organism by providing a good spectral match to dominant wavelengths both during the day (~ 565 nm) and at twilight when the peak ambient light shifts to shorter wavelengths (495–545 nm) and when *L. brevis* shows increased feeding activity (Coelho et al., 2010).

When comparing the PLR between eyes, lateral asymmetry was found. Similarly, a previous study of other cephalopods demonstrated lateral asymmetry in the PLR of both the cuttlefish *S. officinalis* and octopus *E. cirrhosa*, showing a significant reflex in the stimulated eye and no constriction in the opposite eye (Douglas et al., 2005). In the present study, the light threshold at which the reflex became apparent in pupil area measurements was at a higher irradiance in the indirectly stimulated eye than in the eye under direct stimulation.

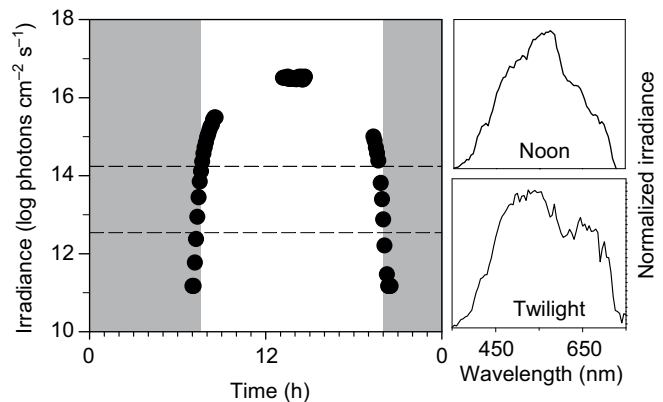


Fig. 7. Environmental irradiance and spectral composition measured at 0.5 m depth on 20–24 March 2011. Irradiance values are plotted as photosynthetically active radiation (PAR, log photons $\text{cm}^{-2} \text{s}^{-1}$) as a function of time over 24 h (shading indicates night bounded by exact times of civil twilight), with the highest and lowest experimental light stimuli presented in PLR experiments indicated by the dashed lines. The normalized spectral irradiance at solar noon (13:38 h) and civil twilight (07:35 h) are plotted as a function of wavelength.

Furthermore, the pupil areas showed a difference in reflex magnitude, with the directly stimulated eye having a greater constriction during exposure to maximum irradiance. Accordingly, the *L. brevis* PLR showed lateral asymmetry. However, there were differences in *L. brevis* pupil area and lid length in the eye under indirect stimulation during light exposure of the opposite eye. This occurred despite a constant irradiance measured at the position of the indirectly stimulated eye (i.e. 12.77 log photons $\text{cm}^{-2} \text{s}^{-1}$). It is possible that the reduced PLR in the indirectly stimulated eye was caused by stray light scattered in the experimental chamber or transmitting through the test subject's body. If this were the case, stray light should have increased with stimulus intensity, and it would be expected that the response magnitude would continue to increase at higher irradiances. In contrast, the response in the indirectly stimulated eye appeared to saturate in both the pupil area and lid length well below the maximum constriction observed in the directly stimulated eye. This suggests the response may be due to some level of consensus between the eyes rather than to stray light.

The weak consensual response of *L. brevis*, an estuarine species involved in active predation, could be a function of the habitat, physiology and behavior associated with this species when compared with others more associated with the seafloor (see Douglas et al., 2005). For example, the variety and number of extraocular muscles in squid (14) and cuttlefish (13) in comparison to those in octopus (7) show adaptation in the former groups for the increased eye maneuverability necessary for convergent eye movements (Budelmann, 1994). While octopus, such as *O. vulgaris*, use primarily monocular vision for locating and capturing prey in caves and crevices, where the independent use of eyes would be advantageous, some cuttlefish and all squid have the ability of convergent eye movements and binocular vision where the visual fields of the two eyes overlap (Budelmann and Young, 1993; Hanlon and Messenger, 1996; Byrne et al., 2002; Makino and Miyazaki, 2010). A consensual or binocular visual system would benefit such visual predators by providing higher accuracy of location and attack distance estimation for objects in the frontal field (Messenger, 1968; Messenger, 1977), and is especially useful given their open-water environment and body design (Budelmann and Young, 1993; Makino and Miyazaki, 2010). For *L. brevis*, the lid length showed

the same PLR threshold in both the directly and indirectly stimulated eyes and no difference in PLR magnitude between the eyes at maximum irradiance. This indicates the extension of the dorsal iris lid was primarily consensual, which is advantageous given the downwelling light the organism is exposed to in its habitat.

The estuarine habitat shows high variability in light intensity and spectral composition over both diel and tidal cycles. The observed intensity and wavelength sensitivity of the PLR, the rapid reflex to stimuli and the lack of complete lateral asymmetry to allow for binocular vision indicate the visual system of *L. brevis* is capable of high performance in a demanding habitat where few other cephalopods are found.

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