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Visual physiology underlying orientation and diel behavior in the sand beach amphipod *Talorchestia longicornis*

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SUMMARY

Talitrid amphipods employ vision for zonal recovery behaviors on sand beaches and for entraining circadian activity rhythms. Using a hierarchy of methods, we examined visual spectral and response–intensity functions in *Talorchestia longicornis*, a species in which orientation and rhythm entrainment are wavelength-specific behaviors. Microspectrophotometry, electroretinogram recording and behavioral assays were used to determine visual pigments, retinal spectral sensitivity and whole-animal spectral responsivity, respectively. Diel changes in absolute sensitivity were also investigated at retinal and whole-animal levels. Two receptor spectral classes were identified, with values for visual pigment λ_{max} of 427 and 518 nm. Retinal spectral sensitivity varied with electrode position along the distal–proximal axis. Chromatic adaptation of distal and proximal photoreceptors resulted in sensitivity peaks at 430 and 522 nm, respectively. In accordance with identified visual pigments and spectral sensitivity, *T. longicornis* photobehavioral responsivity covered a broad range (420–580 nm). Collectively, a dual-pigment visual system underlies wavelength-specific behavior in *T. longicornis*, with the short-wavelength pigment likely to be localized in the distal R5 retinular cell. While response–intensity functions did not change over the diel cycle at the retinal level, behavioral photoresponsiveness varied between day and night. At a wavelength used by *T. longicornis* for celestial orientation (420 nm), photobehavior was heightened at night, potentially aiding in nocturnal orientation. By contrast, at a wavelength used to entrain its circadian rhythm (520 nm) and for routine visual tasks, photobehavior was heightened during the day, and spectral sensitivity matched to the twilight spectrum, facilitating crepuscular vision and entrainment by irradiance at sunrise and sunset.

Key words: vision, wavelength, talitrid, microspectrophotometry, electroretinogram, phototaxis.

INTRODUCTION

Talitrid amphipods routinely use vision to orient on sand beaches. During *y*-axis orientation or zonal recovery, amphipods use celestial and landmark cues for orientation from the intertidal and supratidal zones back to their burrow zone in sand dunes (for reviews, see Ugolini et al., 2002; Scapini, 2006). They also have a circadian rhythm in which they are active during the night and inactive during the day that is entrained by the light:dark and diel (24 h) temperature cycles (Ugolini, 2003; Forward et al., 2009a). In conjunction with overall activity, visual orientation in response to local cues (e.g. phototaxis and scototaxis) has been shown to vary over the diel cycle, although species, populations and life-history stages differ in the way these responses change over the diel cycle (Mezzetti et al., 1997; Nardi et al., 2000). Although extraretinal photoreception has been reported in talitrids, photoreceptors in the eyes are required for entrainment of activity rhythms and, presumably, for local and celestial orientation as well (Frelon-Raimond et al., 2002; Forward et al., 2009a; Forward et al., 2009b).

Despite the central role of vision and the light:dark cycle in talitrid orientation and entrainment of the circadian rhythm in activity, little is known about the visual physiology of these animals. Mezzetti and colleagues (Mezzetti et al., 2010) recently reported differences in eye morphology between *Talorchestia spinifera* and *Talitrus saltator* that might be related to differences in habitat and how their eyes are used in orientation. *Talorchestia spinifera*, which lives lower intertidally and is less nocturnal than *Talitrus saltator*, has more extensive interommatidial cells and, probably, a less sensitive eye. Furthermore, both species have vesicles between each set of photoreceptors and the overlying lens of currently unknown function, but it has been suggested that the distinct horizontal alignment of vesicles across the eye in *Talorchestia spinifera* plays a role in seaward orientation (Mezzetti et al., 2010). Preliminary electrophysiological studies (Ugolini et al., 1996) and several behavioral studies (e.g. Mezzetti et al., 1994; Mezzetti and Scapini, 1995; Ugolini et al., 2006) suggest that talitrids use chromatic choice to maintain their supratidal position on beaches. Of particular importance are wavelengths in the blue and green spectral regions, considered to represent positions of ‘sea’ and ‘land’, respectively (Ugolini et al., 2006).

Forward and colleagues (Forward et al., 2009a; Forward et al., 2009b) recently reported that the talitrid *Talorchestia longicornis* exhibits wavelength-specific behavior. During sun compass orientation, they move perpendicularly to shorelines using light in the violet/blue region of the spectrum, whereas longer wavelengths are required for entrainment of the circadian rhythm to the light:dark cycle. It was argued that using short-wavelength violet/blue light (~420 nm) to localize the position of the sun aids in improving the contrast of the sun against background skylight or might facilitate

detection of the skylight radiance distribution (Forward et al., 2009b). By using longer green-wavelength light (~520 nm) for entrainment of the endogenous rhythm, *T. longicornis* might be better able to identify the time of twilight because green wavelengths remain at that time, whereas there is a loss of atmospheric wavelengths <450 nm and in the range 540–640 nm [i.e. the Chappuis effect (Forward et al., 2009b)].

Here, we employ a hierarchy of methodologies to examine visual physiology in the sand beach amphipod *T. longicornis* at the level of the visual pigments by means of microspectrophotometry, retinal spectral sensitivity through extracellular ERG recording and the whole-animal by means of behavior. Collectively, these approaches were used to test whether a dual-visual-pigment system is present in *T. longicornis* that could underlie the wavelength-specific behavior described above and whether absolute sensitivity increases during nighttime to facilitate vision during the active phase of the circadian rhythm.

MATERIALS AND METHODS

Specimen collection

Talorchestia longicornis Say were collected on the Neuse River (North Carolina, USA) from a beach lacking tides and having a salinity of approximately 16 practical salinity scale units (PSS). Amphipods were collected using pitfall traps buried in the sand overnight. After collection, amphipods were maintained in the laboratory in aquaria of volume 4 liters filled with ~12 cm of moistened sand. They were fed cellulose tissues and fish food flakes (TetraMin, Melle, Germany) and exposed to the ambient light:dark cycle. Amphipods retain their normal activity rhythms and visual orientation behaviors when kept under these conditions (Forward et al., 2009a; Forward et al., 2009b).

Microspectrophotometry

Specimens were sent to University of Maryland Baltimore County, where they were kept in the dark, at least overnight, and for as long as several days before experiments. While being dark adapted, they were housed in small plastic containers and fed as described earlier. All further procedures were performed under dim red-illumination and have been described elsewhere in more detail (Cronin, 1985; Cronin et al., 1996). Animals were sacrificed by decapitation, and eyes were prepared for microspectrophotometry (MSP) by first quick-freezing them with a cryogenic spray, mounting them on cryostat stubs and placing them in a cryostat maintained at approximately –25°C. Sections 14 µm in thickness were cut, mounted on coverslips within a drop of marine crustacean Ringer solution containing 1.25% glutaraldehyde and placed in the microspectrophotometer for scanning.

Procedures for MSP generally followed the sequence described by Cronin and colleagues (Cronin et al., 1996). Briefly, photoreceptors were selected under far-red illumination, and a spot of 600-nm monochromatic light either 1 µm or 5 µm in diameter was placed in the center of the receptor, illuminating it parallel to the long axis of the receptor. Receptors were scanned at 1-nm intervals from 400 to 700 nm when fully dark adapted and again after a saturating 2-min exposure to white light, which bleached most of the visual pigment originally present. Absorbance spectra of the visual pigments in the dark-adapted receptor were taken as the difference between the first and second spectrum. Difference spectra for photobleaching were averaged and fitted mathematically with standard Stavenga et al. (Stavenga et al., 1993) rhodopsin templates, using a least-squares procedure as described by Cronin and colleagues (Cronin et al., 1996). During the course of the study, it became obvious that more than one class of rhodopsin was present, and so individual analyzed photobleaches were grouped by wavelength of maximum absorption, averaged and reanalyzed to provide the best possible spectral fit.

Electrophysiology

For recording of extracellular electroretinograms (ERGs), *T. longicornis* were attached to the plastic head of a pin by their dorsal carapace with cyanoacrylate gel adhesive (Loctite, Rocky Hill, CT, USA) and mounted on an acrylic support. The specimen was suspended above a static, room-temperature (23°C) seawater bath of 15 PSS, providing sufficient humidity to prevent desiccation. One metal microelectrode (125-µm shank, <1-µm tip; FHC, Bowdoinham, ME, USA) was placed subcorneally and served as the recording electrode. Another electrode was placed subcuticularly along the dorsal midline of the head, a region between the eyes lacking ommatidia, and served as the differential reference. Differential AC signals were amplified (Xcell3, FHC), then digitized and stored in LabView (Version 6.1, National Instruments, Austin, TX, USA) for later analysis of peak-to-peak response heights. In this configuration, *T. longicornis* remained alive and healthy during experiments lasting 1 to 2 days.

Monochromatic light stimuli were provided by a 100 W quartz halogen lamp (LSH-T100, Horiba Jobin Yvon, Edison, NJ, USA), coupled to a monochromator (Spectral Products CM110, Putnam, CT, USA) under computer control. Blocking filters were used to enhance spectral purity, and the spectral quality at test wavelengths was verified by a spectroradiometer (~9 nm FWHM, 350–700 nm; USB4000, Ocean Optics, Dunedin, FL, USA). An electromagnetic shutter (VS25, Uniblitz/Vincent Associates, Rochester, NY, USA) under computer control provided a stimulus flash duration of 100 ms, and stimulus irradiance was adjusted using a neutral-density wheel driven by a computer-controlled stepper motor. Light was directed onto the eye of *T. longicornis* through one branch of a bifurcated, randomized fiber optic light guide (EXFO, Richardson, TX, USA). Irradiance was measured at 10-nm intervals with an optometer and calibrated radiometric probe (model S471 optometer, model 260 sensor head, UDT Instruments, Baltimore, MD, USA). A fiber optic illuminator (DC-950, Dolan Jenner, Boxborough, MA, USA) was connected to the other branch of the light guide to provide accessory illumination for specimen preparation and chromatic adaptation experiments. White light from the 150 W lamp was filtered for specimen preparation using a red longpass filter (RG630, Schott, Elmsford, NY, USA) and for chromatic adaptation using either a 380-nm interference filter (10 nm FWHM) or an orange longpass filter (OG590, Schott). Irradiance was controlled by neutral-density filters.

Dark-adapted spectral sensitivity

An individual was considered dark adapted when the response to a dim test flash had remained constant for 1 h. Spectral sensitivity was determined using the criterion-response method described by Cohen and Frank (Cohen and Frank, 2006). Briefly, the dark-adapted eye was stimulated with test flashes of monochromatic light, and irradiance was adjusted to reach a defined criterion response (50 µV) at each wavelength tested (350–650 nm, 20 nm intervals). A test flash of standard wavelength and irradiance was periodically given throughout the experiment to confirm that the eye remained dark adapted. In preliminary experiments, it was determined that the polarity of the ERG waveform reversed predictably in accord with the depth of the recording electrode. Waveforms were corneal positive when the electrode was recording from a population of cells

more distal in the retina, then reversed to corneal negative if the electrode was moved deeper into the retina to record from a more proximal population of cells. Spectral sensitivity experiments with dark-adapted *T. longicornis* were therefore conducted with the recording electrode at both distal (N=4) and proximal (N=5) locations within the retina. The polarity of the ERG waveform served as the indicator of the cell populations (distal or proximal) being recorded. Dark-adapted spectral sensitivity data were plotted as the reciprocal of irradiance required to evoke the criterion response at each wavelength and normalized to the wavelength of maximum sensitivity. These data were fit with standard Stavenga et al. (Stavenga et al., 1993) rhodopsin templates, using a least-squares procedure as described by Cohen and Frank (Cohen and Frank, 2006).

Chromatic-adapted spectral sensitivity

The presence of multiple photoreceptor spectral classes was assessed by measuring spectral sensitivity under chromatic adaptation after the determination of dark-adapted spectral sensitivity (e.g. Frank and Case, 1988). Chromatic adaptation consisted of light-adapting the eye, as described earlier, until a constant response to a dim test flash was observed for 1 h. Spectral sensitivity was then re-tested using the criterion-response method at all wavelengths, presenting monochromatic test flashes over the adaptation light. With the electrode in the distal position, five replicate (380 nm light adapted) and four replicate (>590 nm light adapted) chromatic adaptation experiments were conducted. With the electrode in the proximal position, three replicate chromatic adaptation experiments were conducted under both 380 nm and >590 nm light adaptation and analyzed using the ratio of the ERG responses at blue (410–450) and green (510–550) wavelengths (e.g. Cohen and Frank, 2007). Ratios were calculated using the log-transformed inverse irradiance required to generate a criterion response at test wavelengths, where wavelengths with greater sensitivity are less negative. Accordingly, the blue:green ratio is expected to decrease if green sensitivity is selectively affected, whereas blue sensitivity remains unchanged. Blue:green response ratios were compared by one-factor ANOVA, with Tukey's *post hoc* testing (SigmaStat 3.10, Systat Software, Chicago, IL, USA).

Response–intensity functions

Talorchestia longicornis is nocturnally active (Forward et al., 2007; Forward et al., 2009a), and therefore absolute sensitivity of the visual system might differ between night and day. To test for wavelength-specific diel changes in photoreceptor sensitivity, V log$I$ curves were constructed from ERGs of dark-adapted *T. longicornis* measured during the day (09.00–15.00 h) and night (22.00–00.30 h) at two wavelengths (420 nm with distal electrode placement, N=4; and 520 nm with proximal electrode placement, N=3). The same individuals were tested at a given wavelength during the day, then again at night and were not used in subsequent experiments. Light flashes lasting 100 ms were presented in order of increasing irradiance. An experimental flash was not given until the ERG response to a dim test flash indicated that the eye was dark adapted. Peak-to-peak response heights (V) were calculated and modeled as a function of log irradiance (log I) using the Zettler modification of the Naka–Rushton equation in order to determine: (1) slope; (2) log K, the log irradiance evoking 50% of the maximum response amplitude; and (3) dynamic range, the log irradiance range from 5 to 95% of V_{max} (Frank, 2003). Differences in response–intensity functions for the *T. longicornis* eye were assessed through signed rank (day *vs* night) and rank sum (420 *vs* 520 nm) tests on these variables (SigmaStat 3.10, Systat Software).

Temporal resolution

To determine whether temporal summation varies in the *T. longicornis* retina between day and night, ERGs evoking 50% of the maximum response from V log$I$ experiments for 420 and 520 nm light described above were also analyzed for: (1) response latency, the amount of time elapsed from the onset of the light flash until the onset of the photoreceptor response; and (2) time-to-peak, the period of time elapsed from the onset of the light flash until the photoreceptor response reaches an initial peak (Cohen and Frank, 2007). Differences in response latency and time-to-peak of the *T. longicornis* eye between day and night for 420 and 520 nm were assessed through signed rank tests (SigmaStat 3.10, Systat Software).

Behavior

Phototactic behavior was used to complement the MSP and electrophysiological data by providing an integrative assessment of visual sensitivity in *T. longicornis*. Phototaxis was measured in the horizontal plane in a Plexiglas trough (length 41 cm, width 6.5 cm, height 8 cm), which was divided into five equal sections along the longitudinal axis. The sections were separated by thin slides constructed so that all could be moved vertically in unison. Light was delivered along the long axis of the trough from a slide projector with either a 300 W tungsten or 150 W quartz halogen lamp (420 nm response–intensity experiment only), interference filtered to wavelengths from 420 to 620 nm (Ditric Optics, Marlboro, MA, USA; half band-pass for filters ranged from 7.1 to 11.2 nm). Irradiance was controlled by neutral-density filters and measured by a radiometer (model QSP-170BD, Biospherical Instruments, San Diego, CA, USA).

Animals were dark adapted for at least 1 h before testing. A single trial consisted of eight animals that were tested in a volume of 11 of 16 PSS seawater. The amphipods were placed in the center compartment of the test trough for 30 s in complete darkness, after which the dividers were lifted and the light source was turned on. After 45 s, the dividers were replaced, and the amphipods in the different chambers counted. The control experiment consisted of repeating the procedure but not turning on the stimulus light. Amphipods that moved to the section closest to the light source were considered to show positive phototaxis, whereas those in the section furthest from the light were considered to show negative phototaxis. Animals were not used more than twice and never more than once in a given night or day.

Response spectrum

A response spectrum was determined by testing responses to wavelengths from 420 to 620 nm at 20 nm intervals using the tungsten lamp. Light intensity at each wavelength was adjusted to be approximately equal at an average irradiance of 3.8×10^{7} photons cm^{-2} s^{-1}. This intensity was used because it induced a significant positive phototactic response at 520 nm but not a maximal response. Responses to each wavelength were replicated five times, and all experiments were conducted at night (22.00–03.00 h). Wavelengths that evoked significant phototactic responses were determined by a one-factor ANOVA, using Dunnett's test versus the control (SigmaStat 3.10, Systat Software).

Response–intensity

Phototactic responses were measured at different irradiances of either 420 nm (quartz halogen lamp) or 520 nm (tungsten lamp) during the day (14.00–16.00 h) and night (22.00–03.00 h). Irradiance values ranged from the highest available irradiance to the level where the light no longer induced a phototactic response. These experiments

were designed to determine the pattern of phototaxis (positive or negative) at different irradiances and the lowest irradiance value (threshold) to induce a significant response. Phototactic responses for each condition were replicated three times. The threshold was defined as the lowest irradiance value that induced a mean phototactic response that was significantly greater than the mean control value as determined by a one-factor ANOVA, using Dunnett's test versus the control (SigmaStat 3.10, Systat Software).

RESULTS

Spectral sensitivity

Analyzed MSP scans of *T. longicornis* rhabdoms in retinal sections fell into two distinct spectral classes corresponding to two distinct Stavenga et al. (Stavenga et al., 1993) rhodopsin templates. A short-wavelength spectral class displayed maximal absorbance at 427 nm, and a middle-wavelength spectral class peaked at 518 nm (Fig. 1). Other spectra (not shown) were observed, but these did not fall into clear spectral classes, suggesting they were either mixtures of rhodopsins or combinations of rhodopsin and metarhodopsin or of other unknown pigments.

The ERG waveform and the shape of the electrophysiological spectral sensitivity curve were both dependent on electrode position (Fig. 2A). Placement of the recording electrode in the shallow, distal portion of the *T. longicornis* eye resulted in a corneal-positive waveform, with a spectral sensitivity peak in the dark-adapted eye at short wavelengths and a shoulder at middle wavelengths (Fig. 2B). With the recording electrode positioned in the deeper, proximal portion of the eye, the ERG waveform became corneal negative (Fig. 2A), and the spectral sensitivity curve displayed a peak in the dark-adapted eye at middle wavelengths and a shoulder at shorter wavelengths (Fig. 2B).

The short-wavelength peak present in the dark-adapted distal cell population shifted to middle wavelengths under chromatic adaptation with 380 nm light, with some residual sensitivity at shorter wavelengths (Fig. 3A). Chromatic adaptation upon exposure to >590 nm light resulted in a loss of the middle-wavelength shoulder, leaving only the short-wavelength peak that was best fit by a 430 nm Stavenga et al. (Stavenga et al., 1993) rhodopsin template (Fig. 3A). Blue:green response ratios differed significantly among dark-adapted, 380 nm chromatic-adapted and >590 nm chromatic-adapted

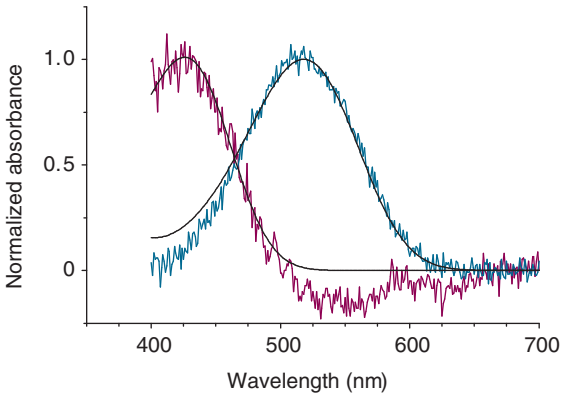

Fig. 1. Visual pigments in *T. longicornis* rhabdoms. Average-difference spectra for photobleached rhodopsins in short-spectral-class photoreceptors (violet line, *N*=10) and of the middle-spectral-class photoreceptors (blue line, *N*=5). Best-fit rhodopsin templates are plotted as black lines for each difference spectrum (short spectral class λ_{max}=427 nm; middle spectral class λ_{max}=518 nm).

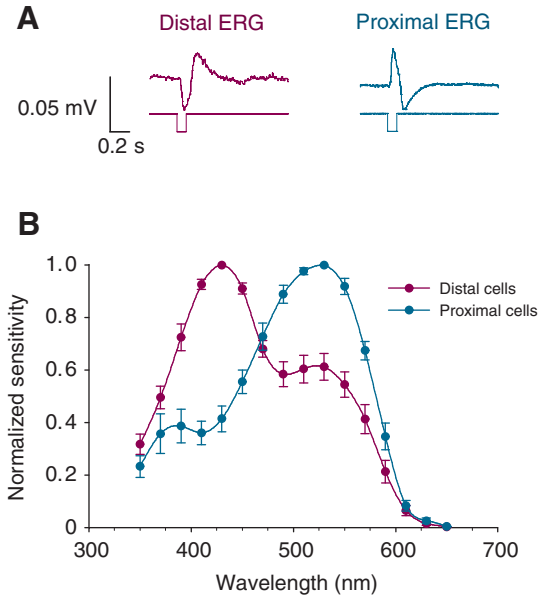

Fig. 2. Electrophysiological spectral sensitivity of the dark-adapted *T. longicornis* eye. (A) Upper traces show representative ERG responses to a 100 ms flash of 530 nm light at an irradiance sufficient to evoke a 0.050 mV response with the electrode placed distally (violet) and proximally (blue) in the eye. The lower traces show the shutter position, which is open during the negative square wave. Note the change in ERG polarity from corneal positive in the distal ERG (violet), to corneal negative in the proximal ERG (blue). (B) Spectral sensitivity curves of dark-adapted *T. longicornis*, constructed using the criterion-response method, with normalized sensitivity plotted as a function of wavelength. Separate curves are shown for specimens with the recording electrode placed within distal cells (*N*=4; violet symbols/line) and within proximal cells (*N*=5; blue symbols/line). The data are means ± s.e.m.

distal cell populations ($F_{2,9}$=168.7, P<0.001; P<0.05 for all Tukey's pairwise multiple comparisons) (Fig. 4).

The middle-wavelength peak in the dark-adapted proximal cell population narrowed under 380 nm chromatic adaptation, with loss of sensitivity at shorter wavelengths. A 522 nm Stavenga et al. (Stavenga et al., 1993) rhodopsin template best fit these data (Fig. 3B). Chromatic adaptation of the proximate cell population with >590 nm light resulted in a loss of sensitivity at middle wavelengths and a peak at shorter wavelengths (Fig. 3B). Chromatic adaptation to >590 nm light in proximate cell populations resulted in significantly lower blue:green response ratios than in either dark-adapted or 380 nm chromatic-adapted treatments, which did not differ statistically ($F_{2,7}$=32.9, P<0.001; P<0.05 for Tukey's pairwise multiple comparisons dark *vs* >590 nm and 380 nm *vs* >590 nm) (Fig. 4).

Compared with the controls, the behavioral response spectrum for positive phototaxis in *T. longicornis* displayed significant behavioral responsivity over a broad wavelength range (420–580 nm) (Fig. 5). The levels of positive phototaxis significantly exceeded control levels in two spectral ranges, from 440 to 520 nm and from 560 to 580 nm ($F_{12,51}$=4.3, P<0.001; P<0.05 for Dunnett's test versus the control) (Fig. 5).

Response–intensity functions and temporal resolution

Parameters generated from Naka–Rushton models fit to electrophysiological Vlog$I$ curves for light flashes at either 420

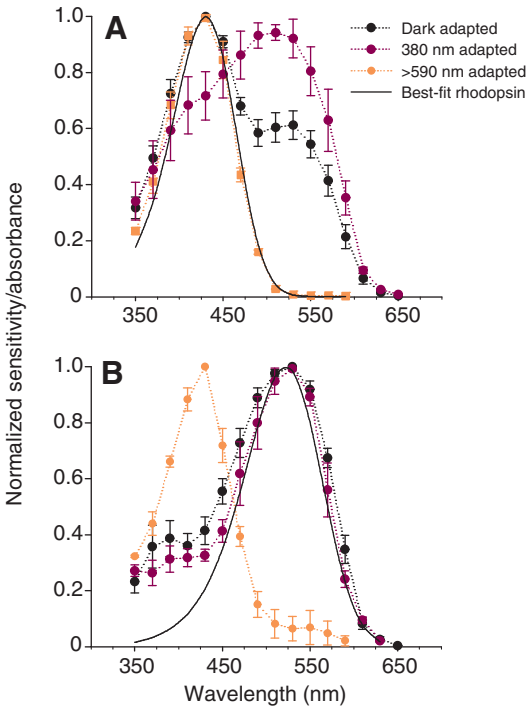

Fig. 3. Electrophysiological spectral sensitivity of the chromatic-adapted *T. longicornis* eye. (A) Distal cells. Spectral sensitivity curves are shown for dark-adapted (N=4; black circles), 380 nm-adapted (N=5; violet circles) and >590 nm-adapted (N=4; orange circles) specimens. The data are means ± s.e.m. The rhodopsin λ_{max} best-fit to the >590 nm-adapted data is at 430 nm (black unbroken line). (B) Proximal cells. The data are plotted as in (A), except three replicates were conducted for both 380 nm- and >590 nm-adapted specimens. The rhodopsin λ_{max} best-fit to the 380 nm-adapted data is at 522 nm (black unbroken line).

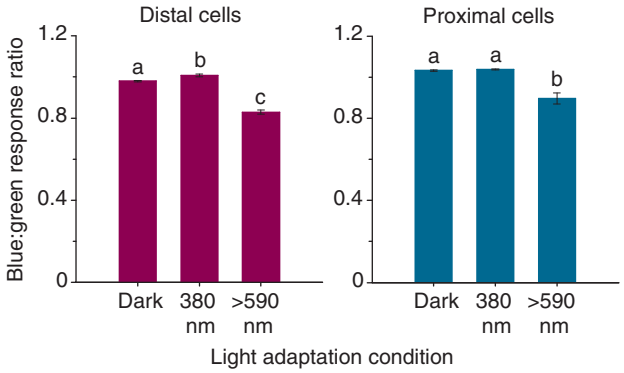

Fig. 4. Blue:green response ratios for chromatic-adapted *T. longicornis* eyes. Average log irradiance required to generate a 50 μV response at blue (410–450 nm) wavelengths was divided by those at green (510–550 nm) wavelengths to construct the ratio. The plotted data are means ± s.e.m. for three to five replicates per treatment (see Figs 2, 3). Significant differences among the treatments (dark-adapted, 380 nm light-adapted, and >590 nm light-adapted) for recordings at distal (violet) and proximal (blue) cells were determined by one-way ANOVAs (P<0.05). Letters above each bar indicate significant differences (P<0.05) within each cell location determined using Tukey's *post hoc* tests.

or 520 nm (Fig. 6) showed no day–night difference in slope, log K or dynamic range (P>0.05, signed rank test) (Table 1). When paired day and night data were pooled and compared between 420 and 520 nm, the slope was higher and the dynamic range was lower at 420 nm than at 520 nm (rank sum test, P=0.003 for both). V log$I$ curves at these test wavelengths did not differ in log K (rank sum test, P>0.05). Both response latency and time-to-peak (parameters representing temporal resolution of the photoreceptors) showed slight increases between night and day for 420 and 520 nm, but these differences were not significant (P>0.05, signed rank test) (Table 1).

Behavioral response–intensity curves determined for *T. longicornis* during both day and night at 420 and 520 nm showed a positive phototactic response, increasing with increasing irradiance (P<0.05, one-factor ANOVA for each time/wavelength treatment), then decreasing at high irradiance levels (Fig. 7). Treatments differed in the lowest irradiance required to evoke a significantly greater positive phototactic response than was observed in controls (response threshold). At 420 nm, the nighttime response threshold (5.5×10^7 photons cm^{-2} s^{-1}; F_{10}=11.099, P<0.001, Dunnett's test *vs* control) was lower than the threshold during the day (5.5×10^8 photons cm^{-2} s^{-1}; F_9=14.125, P<0.001) (Fig. 7A). The opposite was true at 520 nm, where the response threshold during the day (4.2×10^8 photons cm^{-2} s^{-1}; F_9=7.343, P<0.001) was lower than the threshold during the night (1.1×10^9 photons cm^{-2} s^{-1}; F_9=7.462, P<0.001) (Fig. 7B).

DISCUSSION

It was hypothesized that *T. longicornis* would possess multiple visual pigments, encompassing both violet/blue (~420 nm) and green (~520 nm) wavelengths. The presence of at least two distinct spectral classes in the *T. longicornis* eye is indicated collectively by the three measures of wavelength sensitivity employed in the present study (MSP, ERG and behavior). These experimental approaches are increasingly integrative in their assessment of visual sensitivity (Marshall et al., 1999; Marshall et al., 2003). MSP best represents visual pigment λ_{max}, whereas ERG recording is representative of retina-level spectral sensitivity influenced by factors including pigment density, lateral screening by photostable pigments such as ommochromes, serial screening within a given photoreceptor by rhodopsin and its photoproducts, and photoreceptor

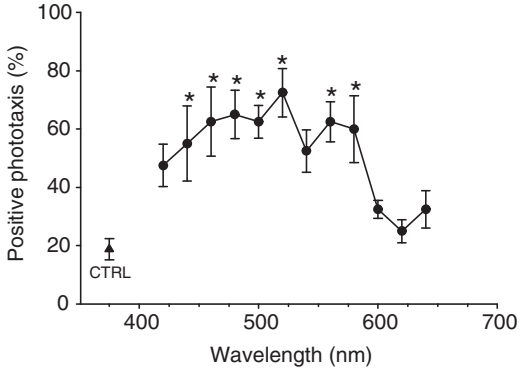

Fig. 5. Behavioral response spectrum for dark-adapted *T. longicornis*. The mean percentage (±s.e.m.) of positive phototaxis in five trials is plotted as a function of wavelength. Positive phototaxis was defined by an individual swimming into the chamber of a horizontal trough nearest to the light source during a 45 s light stimulus at 3.8×10^7 photons cm^{-2} s^{-1}. Significant increases in positive phototaxis relative to control conditions in darkness ('CTRL') at each wavelength were determined by a one-way ANOVA, with Dunnett's test versus the control (*P<0.05).

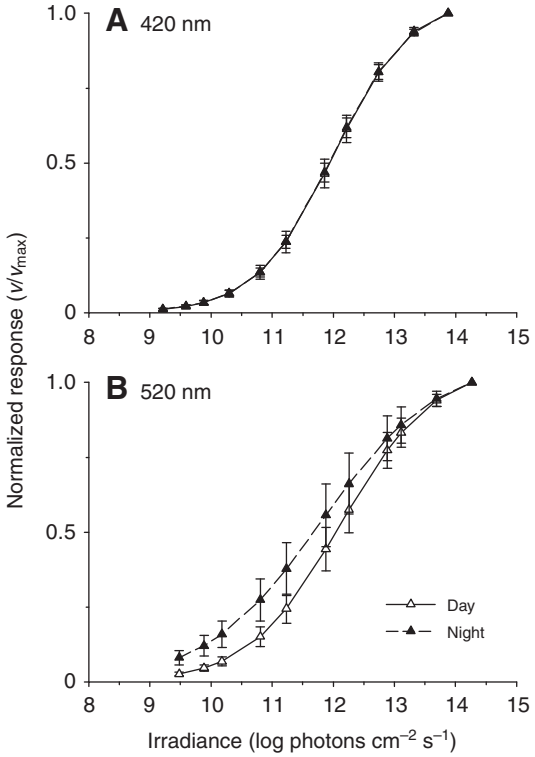

Fig. 6. Electrophysiological response–intensity (*V*/log*I*) relationship for the *T. longicornis* eye. ERG responses at a given irradiance were normalized to the maximum response for that amphipod preparation, and means (±s.e.m.) plotted as a function of log irradiance (log photons cm^{-2} s^{-1}). Daytime experiments (open triangles) and nighttime experiments (filled triangles) conducted with (A) 420 nm light (*N*=4) and (B) 520 nm light (*N*=3) are shown. Naka–Rushton model fits are provided for experiments conducted during the day (unbroken lines) and night (broken lines) at both wavelengths.

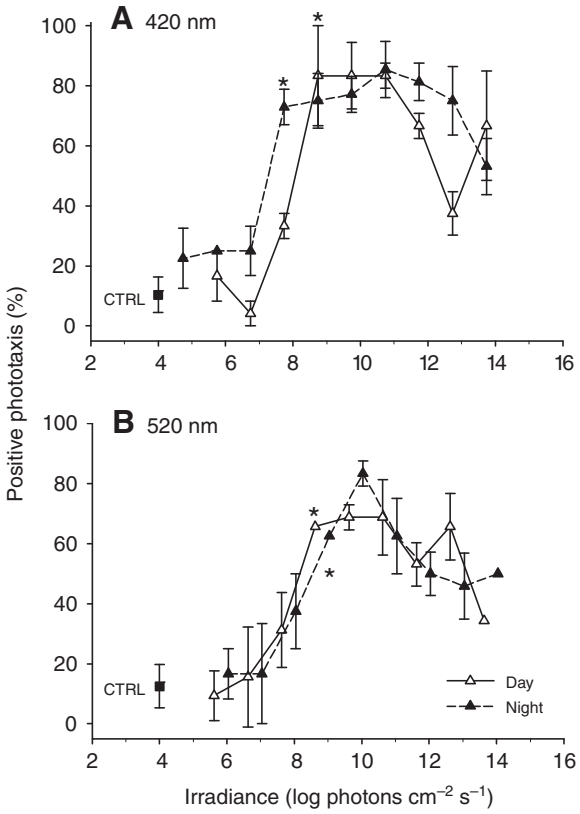

Fig. 7. Behavioral response–intensity relationship for the *T. longicornis* eye. The mean percentage (±s.e.m.) of positive phototaxis in three trials is plotted as a function of log irradiance (log photons cm^{-2} s^{-1}). Daytime experiments (open triangles, unbroken lines) and nighttime experiments (filled triangles, broken lines) conducted with (A) 420 nm light and (B) 520 nm light are shown. The response threshold (*), defined as the lowest irradiance needed to evoke increased positive phototaxis relative to control conditions in darkness ('CTRL', filled square), was determined by a one-way ANOVA, with Dunnett's test versus the control (P<0.05).

size. Behavior is the most integrative in that neural processing influences the wavelength response, in addition to any filtering occurring at the retina level.

MSP and ERG recording indicate the presence of a short-wavelength spectral class located distally in the rhabdom, containing a rhodopsin with MSP λ_{max} at 427 nm and with the ERG peak sensitivity of the receptor as a whole shifted to 430 nm. A middle-wavelength spectral class is located proximally, with a rhodopsin MSP λ_{max} at 518 nm and ERG peak sensitivity at 522 nm. Lateral filtering by ommochromes and serial filtering by distal rhodopsins and metarhodopsins probably explain the slight shift in λ_{max} between MSP and ERG measurements, as these pigments are known to shift spectral sensitivity to longer wavelengths (Goldsmith, 1978; Jordão et al., 2007). Ommochromes in *T. longicornis* retinular cells have an absorption maximum at 500 to 550 nm and a smaller peak at ~450 nm (data not shown). This is similar to the absorption spectrum of screening pigments in the amphipod *Pontoporeia affinis*, in which a similar long-wavelength shift was reported between MSP and ERG data (Donner et al., 1994). Shifting of spectral sensitivity could also result from vesicles overlying the ommatidia of talitrids (e.g. Mezzetti et al., 2010), although such vesicles have yet to be reported for *T. longicornis*, and, if they are present, their spectral properties are unknown. Two peaks are also evident in the behavioral response spectrum for phototaxis, yet the behavioral peaks (440 to 520 nm and 560 to 580 nm) are shifted to longer wavelengths than the electrophysiological measure of sensitivity. Lateral and serial filtering could result in such a shift in the behavioral response spectrum, particularly in the dark-adapted state, if the pigment shield has retracted proximally within the eye, increasing light passing laterally through non-mobile screening pigments (Goldsmith, 1978; Hallberg and Elofsson, 1989).

The existence of a dual-visual-pigment system fits with the known eye structure of gammarid amphipods. The typical ommatidial structure of shallow-water and semi-terrestrial gammarids includes rhabdomeres from five retinular cells (R1–R5), with the fifth retinular cell (R5) positioned distally and not extending the full length of the rhabdom (Hallberg et al., 1980). This situation is analogous to the 8-retinular-cell arrangement common among crustaceans, with R1–R7 cells containing blue/green sensitive visual pigments and an additional, typically smaller, R8 cell containing either an ultraviolet (UV)-sensitive or a violet-sensitive visual pigment (for a review, see Marshall et al., 1999). It has been speculated that the R5 cell of amphipods is UV sensitive and might play a role in the detection of polarized light, as is commonly observed for short-wavelength polarization-sensitive cells in insects (Hallberg et al., 1980). In *T. longicornis*, only the ERG recordings from distal cell populations contained a substantial contribution from

Table 1. Variables describing response–intensity functions and temporal resolution in dark-adapted *Talorchestia longicornis*

		N	Slope	log *K* (log photons cm^{-2} s^{-1})	Dynamic range (log photons cm^{-2} s^{-1})	Response latency (ms)	Time-to-peak (ms)
Day	420 nm	4	0.71±0.02	12.01±0.13	3.63±0.09	30.0±2.9	72.5±2.3
	520 nm	3	0.66±0.05	11.97±0.23	3.95±0.27	27.7±4.3	60.7±2.2
Night	420 nm	4	0.71±0.04	12.00±0.09	3.67±0.24	33.8±1.4	76.3±5.5
	520 nm	3	0.57±0.08	11.89±0.41	4.65±0.59	30.3±5.2	64.0±6.4

Slope, log *K* and dynamic range values are derived from a Naka–Rushton model fit to ERG-derived *V*log*I* curves at two wavelengths (420 and 520 nm). Response latency and time-to-peak values are calculated from individual ERG traces evoking 50% of the maximum *V*log*I* response. For each wavelength, the same individuals were tested during both the day and night. Means ± s.e.m. are provided.

both short- and middle-wavelength-absorbing visual pigments, resulting in significant changes in blue:green response ratios upon adaptation to both 380 and >590 nm light. This suggests a distal location for the 427 nm spectral class in *T. longicornis* and fits with the hypothesis for the R5 cell containing the short-wavelength-sensitive pigment in amphipods. Further MSP and immunohistochemical studies could provide additional evidence to confirm this.

In general, shallow-water crustacean visual systems have R1–R7 spectral sensitivity maxima at 460–525 nm, presumably to enhance photon capture during the day or at twilight (for a review, see Marshall et al., 2003). The semi-terrestrial fiddler crab (*Uca* spp.) represents a more direct comparison to *T. longicornis*, given their similar adult habitats. *Uca* possesses a two-pigment visual system much like that of *T. longicornis*, with peaks at violet (~430 nm) and green (500–530 nm) wavelengths (Hyatt, 1975; Horch et al., 2002; Jordão et al., 2007). The green-sensitive receptors in both species are well positioned to capture both daytime/nighttime and twilight spectra (e.g. Johnsen et al., 2006).

Compared with other crustacean groups, visual spectral sensitivity in amphipods is poorly studied (for a review, see Marshall et al., 2003). Spectral sensitivity in the Antarctic amphipod *Abyssorchomene plebs* peaked at 487 nm, with heightened sensitivity between 350 and 400 nm, but chromatic adaptation did not suggest a second, UV-sensitive visual pigment (Cohen and Frank, 2006). Single-pigment visual systems have been reported for several other aquatic amphipod species that live in light-limited habitats. The coastal species *Pontoporeia affinis* and *P. femorata*, which inhabit low-light waters of the Baltic Sea, have sensitivity maxima near 550 nm (Donner, 1971). In accordance with the 'sensitivity hypothesis' (Munz, 1958) (for a review, see Marshall et al., 2003), spectral sensitivity maxima are considerably shorter (~470 nm) in the open-ocean mesopelagic species *Phronima sedentaria* and *Scina crassicornis*, as spectral sensitivity is tuned to ambient daytime downwelling light in their habitat (Frank and Widder, 1999; Cohen and Frank, 2007). Among talitrids, previous studies have suggested the existence of multiple visual pigments in *Talitrus saltator*. Initial electrophysiological and behavioral evidence presented by Mezzetti and Scapini (Mezzetti and Scapini, 1995) found a single visual spectral sensitivity peak at ~450 nm, and, upon chromatic adaptation with blue light, a second spectral class at longer wavelengths was suggested. Subsequent ERGs and short-wavelength chromatic adaptation in *T. saltator* conducted by Ugolini and colleagues (Ugolini et al., 1996) found spectral sensitivity peaks at ~450 nm and ~525 nm. Additional behavioral experiments by Galanti and colleagues (Galanti et al., 2007) provide some support for the presence of distinct UV and blue sensitivity in *T. saltator*. Thus, a multiple-pigment visual system might be common in talitrids, and further comparative studies of proximate and ultimate aspects of visual spectral sensitivity are warranted, given differences in talitrid ecology among species and known morphological diversity in eye morphology (e.g. Mezzetti et al., 2010).

The dual-visual-pigment system we describe for *T. longicornis* provides a physiological mechanism for a spectral component to several previous observations on talitrid orientation behavior and activity patterns. An extensive range of studies has established that talitrids, including *T. longicornis*, can use both local landscape and sky features to orient on sand beaches, with considerable phenotypic plasticity among populations in terms of which cue(s) are preferentially used (for a review, see Scapini, 2006) (Walsh et al., 2010). Common among these local orientation cues is a role for the wavelength of light. Regarding landscape features, silhouetted dunes and vegetation provide reliable orientation cues along the sea–land axis that are enhanced by colored features of the sea (blue) and land (green) for *Talitrus saltator* (Ugolini et al., 2006). Local sky features are also prominent orientation cues for talitrids. The skylight intensity pattern is used for orientation and might involve a spectral component (Ugolini et al., 2009). Additionally, Forward and colleagues (Forward et al., 2009b) reported that short-wavelength light is required for sun compass orientation behavior in *T. longicornis*; animals lost their sun compass orientation capability when tested under a natural sky filtered to peak wavelengths of 510–580 nm yet retained the behavior when the sky was filtered to 420–480 nm. In a related study, Forward and colleagues (Forward et al., 2009a) found that middle-wavelength light (~520 nm) is required to entrain the *T. longicornis* circadian activity rhythm to the light:dark cycle. The present results with the *T. longicornis* eye show that the spectral positions of its visual pigments (427, 518 nm) and similar spectral sensitivity peaks (430, 522 nm) are consistent with behavioral evidence for the role of wavelength in the discrimination of landward and seaward landscape features, directing the sun compass orientation and entraining the endogenous circadian rhythm.

While the presence of at least two spectral channels provides some capacity for differentiating the spectral quality of light, thereby providing a visual cue to control specific behaviors, it is not clear at this point whether these wavelength–behavior associations are associated with true color vision or, instead, a wavelength-specific behavior system. True color vision would provide a far more flexible system with color discrimination based solely on hue, whereas wavelength-specific behavior is a more rigid system whereby behaviors are controlled by spectrally distinct receptor classes (for reviews, see Menzel, 1979; Pichaud et al., 1999). While the two spectral classes in *T. longicornis* clearly align with the two wavelengths required for celestial orientation and entrainment of circadian rhythms, it is likely that these spectral channels contribute to other aspects of vision beyond these behaviors. For example, the middle wavelength (green) spectral class makes up most of the volume of the rhabdom and likely plays a major role in typical visual tasks.

It has been hypothesized that visual sensitivity would be heightened during the night for improved visual function during nighttime foraging. This was based on the nighttime active phase of the locomotor rhythm in *T. longicornis* (Forward et al., 2009a) and on evidence that phototactic behavior in talitrids increases in magnitude during the night phase (Nardi et al., 2000). At the photoreceptor level, visual sensitivity, as indicated by log *K* measurements from electrophysiological *V*log*I* curves, and temporal resolution did not change between day and night for either photoreceptor spectral class, suggesting that visual sensitivity at the retinal level is the same during day and night. However, the thresholds for phototaxis changed between day and night for both 420 and 520 nm light, which perhaps results from neural pooling (for a review, see Schiff, 1987) or receptor-level changes not detected in the present study. The threshold was lower for 420 nm during the night (increased sensitivity) and lower for 520 nm during the day. Talitrids use lunar cues in *y*-axis orientation behavior (for a review, see Ugolini et al., 2002). Accordingly, the short-wavelength receptor class, which has heightened sensitivity at night, might be involved in localizing the position of the moon, given its role in localizing the position of the sun during the day and the similar spectral composition of sun and moon light (Johnsen et al., 2006). While a similar spectral channel might be involved in the sun and moon compass orientation, there is some evidence that two independent clocks regulate solar and lunar orientation behavior (Ugolini et al., 1999).

Middle (green) wavelengths are involved in entraining the circadian activity rhythm to the light:dark cycle (Forward et al., 2009a), and *T. longicornis* remain in burrows during the day (Forward et al., 2007). The transitions of lighting at dawn and/or dusk are used to adjust the timing of the circadian rhythm in activity (e.g. Williams, 1980a; Williams, 1980b; Williams, 1982). At twilight, the light spectrum in air changes owing to the Chappuis effect, in which there are photon peaks in the blue/green (from about 450–540 nm) and in the red region with a suppression of photons at wavelengths below 450 and from 540–640 nm (e.g. Forward et al., 1988). Thus, the beginning and ending of the day for an amphipod will depend upon its spectral sensitivity and threshold intensity for light sensitivity. The results indicate that the threshold light intensity for phototaxis to 520 nm light is lowest during the day. In addition, the 520 nm pigment class is well positioned for photon capture at twilight. Thus, the combination of increased sensitivity in the 520 nm region and the match to the twilight spectrum would enable *T. longicornis* to most accurately phase its circadian activity rhythm and burrow emergence/entry to the light:dark cycle.

LIST OF ABBREVIATIONS

ERG	electroretinogram
I	irradiance
MSP	microspectrophotometry
PSS	practical salinity scale
UV	ultraviolet
V	electroretinogram response amplitude

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