Hiding opaque eyes in transparent organisms: a potential role for larval eyeshine in stomatopod crustaceans

K. D. Feller* and T. W. Cronin

ABSTRACT
Opaque screening pigments are a fundamental requisite for preserving resolution in image-forming eyes. Possession of any type of image-forming eye in a transparent, pelagic animal will thus undermine the ability of that animal to be invisible in the water column. Transparent, pelagic animals must therefore deal with the trade-off between the ability to see and the ability of other animals to see them. Stomatopod larvae, like many transparent crustaceans, possess specialized optics in their compound eyes that minimize the volume of the opaque retina. Though the volumes of these retinas are reduced, their opacity remains conspicuous to an observer. The light reflected from structures overlying the retinas of stomatopod crustacean larval eyes, referred to here as eyeshine, is hypothesized to further reduce the visibility of opaque retinas. Blue or green wavelengths of light are most strongly reflected in stomatopod larval eyeshine, suggesting a putative spectral matching to the light environment against which the larval eyes are viewed. We tested the efficacy of stomatopod crustacean larval eyeshine as an ocular camouflaging mechanism by photographing larvae in their natural light environment and analysing the contrast of eyes with the background light. To test for spectral matching between stomatopod larval eyeshine and the background light environment, we characterized the spectrum of eyeshine and calculated its performance using radiometric measurements collected at the time of each photographic series. These results are the first to demonstrate an operative mirror camouflage matched in both spectrum and radiance to the pelagic background light environment.

KEY WORDS: Camouflage, Larva, Stomatopod, Visual ecology

INTRODUCTION
For small animals in the pelagic environment, such as marine crustacean larvae, survival in such a featureless world often depends on the ability to avoid being seen. Given this strong selection for crypsis, multiple mechanisms have evolved in open-water habitats that facilitate the visual melding of an animal’s body into its surroundings, the most notable being mirrored sides (Denton et al., 1972; Johnsen and Sosik, 2003), counterillumination (Clarke, 1963; Johnsen et al., 2004), and transparent body tissue [reviews of transparency (Breder, 1962; McFall-Ngai, 1990; Johnsen, 2001); reviews of general pelagic camouflaging mechanisms (Nilsson, 1995; Johnsen, 2014)]. Many crustacean larvae provide exquisite examples of the latter mechanism, possessing bodies that are so transparent they are seemingly made of glass. The compound eyes are the only features containing opaque pigments in these animals. Though crustacean larval eyes have evolved to increase their transparency with elongated optical elements and condensed retinas (Nilsson, 1983), opaque screening pigments must remain between photoreceptors in order to preserve resolution of the visual scene. Among crustacean larvae, the stomatopods are particularly effective at maximizing their transparency. Often, the eyes are the only visible features on a stomatopod larval body, which depending on the species, can be up to 40 mm long (Feller et al., 2013). Thus to a human peering into a bucket of plankton, stomatopod larvae may only appear as moving pairs of black dots fixed side-by-side as the animal pirouettes through space.

Undescribed structures within stomatopod larval eyes strongly reflect blue or green wavelengths of light, referred to here as eyeshine (Fig. 1). The eyeshine discussed here is not to be confused with that produced by the light-reflecting tapetum observed beneath the rhodops of some crustacean eyes (such as those of Nephrops norvegicus (Loew, 1976). Careful observation of the reflecting material responsible for stomatopod larval eyeshine suggests that it includes a photonic structure found between the optical and photoreceptor layers of the eye, but absent from the optical pathway (Fig. 1C). The absence of eyeshine from the pathway of light absorbed by photoreceptors (as evidenced by the presence of a robust pseudopupil) suggests that eyeshine does not function as a visual filtering mechanism or otherwise act to influence photoreception in these eyes. We have observed stomatopod eyeshine in all stomatopod larval eyes examined to date, regardless of species or stage (authors’ personal observation) (see also Williams et al., 1985; Cronin et al., 1995; Jutte et al., 1998; Cronin and Jinks, 2001). Outside of stomatopod larvae, the presence of eyeshine has been observed in brachyuran crab larvae (authors’ personal observation) (Cronin and Jinks, 2001), embryonic and larval caridean shrimp [Palaeomonetes pugio (Douglass and Forward, 1989); P. argentinus (Harzsch et al., 1999)], embryos of the American lobster [Homarus americanus (Harzsch et al., 1998)], and the adult isopod Astacilla longicornis (Nilsson and Nilsson, 1983). With no apparent role in photosensitivity, eyeshine is instead hypothesized to serve as a pelagic camouflage for the conspicuous retina over which it is produced (Nilsson and Nilsson, 1983; Douglass and Forward, 1989; Cronin et al., 1995; Jutte et al., 1998; Cronin and Jinks, 2001). Though the role of crustacean larval eyeshine has been posited as a camouflage many times, the hypothesis has never been formally tested. We sought to test the eyeshine camouflage hypothesis by analysing several components of eyeshine in the natural viewing environment of stomatopod larvae. Through imaging and analysis of spectral reflectance of stomatopod larval eyeshine both in the laboratory and in the natural light environment, we tested the efficacy of eyeshine at reducing the inherent contrast of the larval retina at different depths, orientations and periods of the day. These data provide sound support for the hypothesis that stomatopod larval eyeshine functions as an ocular camouflaging mechanism.
RESULTS

Post hoc animal identification

Three species of stomatopod larvae were sampled in this study, representing three of the seven superfamilies presently described in Stomatopoda: *Pullosquilla thomassini* Manning (*N*=6; Lysiosquilloidea), *Pseudosquillana richeri* Moosa (*N*=1; Pseudosquilloidea), and an unknown species of *Harpiosquilla* (*N*=1; Squilloidea). Each sampled individual was an early stage larva that did not molt between nocturnal capture and daytime imaging. We could not assign individuals to a specific developmental stage since there are no published descriptions of the larval stages available for either of the identified species. It can be inferred, however, that given the similarities in size and morphology, the six sampled *P. thomassini* individuals were most likely the same developmental stage.

Image contrast analysis

Animals were photographed under water, in their natural environment at a variety of positions, depths, and illumination conditions selected to most broadly sample views of an animal in the upper hemisphere of three-dimensional space from a fixed observer on the benthos (Fig. 2, see ‘In situ imaging’ in Materials and methods). Within each photograph a small, opaque dark standard and/or a dead animal with degraded eyeshine was also imaged as a control. From each of these photographs, Michelson contrast values were calculated between the larval eyespot (or control) and the background. Michelson contrast is a metric that contains information regarding the relative brightness or darkness of an object against a background. Per the definition of Michelson contrast (see Materials and methods), values range between −1 and 1 with a value of zero equal to no contrast.

All Michelson contrast values of larval eyeshine with the background light environment are reasonably close to 0.0 (value of no contrast). These results are consistent among the three species measured and at all depths, azimuth directions, elevation angles, rotations, and refracted solar elevations (Fig. 3). Data in Fig. 3 are presented using a gray scale to visually represent individual contrast values from each azimuth angle, viewing angle above the horizon, axial orientation, and rotation measured from four individuals that represent all studied species and depths, as well as the corresponding dark standard (depicted in Fig. 2).

Fig. 1. Stomatopod larval eyeshine. Photographs of (A) *Pullosquilla thomassini* and (B) *Pseudosquillana richeri* individuals used in these experiments. Lateral, dorsal and ventral images of *P. thomassini* in A show the change in eyeshine brightness and color across the eye, a characteristic of this species. (C) Diagram depicting the putative location of photonic structures responsible for producing stomatopod larval eyeshine. PRL, photoreceptor layer; Rh, rhabdom; L, lens; CC, crystalline cones; OL, optical layer. Scale bars, 500 μm.

Fig. 2. In situ imaging and radiometric experimental design. Depicted here are all directional variables included for underwater photographic and environmental radiometric data collection. Data were collected at different depths (*d*) and at different times of day or solar elevations (*θ*s). Photographs and radiometric probe directions were oriented in four directions relative to the sun (Into sun 0 deg, Right sun 90 deg, Down sun 180 deg, Left sun 270 deg). At each sun direction, measurements were made with the animal positioned with its body axis horizontal (H) or vertical (V) in three angles relative to the horizon (0, 45 and 90 deg). At each position in space, each animal was rotated in four positions (Ventral, Left, Dorsal and Right). Inset photographs depict these four rotational positions, the dark standard (Dark), and H versus V body axes.
Data points, or dots, in Fig. 3 are shaded such that the contrast value of 0.0 (the value of no contrast) equals the gray circular background and contrast values above or below 0.0 are lighter or darker gray, respectively. Thus the closer a value of contrast is to 0.0 at a given viewing co-ordinate, the less discernable the dot is on the plot.

**Table 1. Results from pair-wise statistical analyses of Michelson contrast data sets**

<table>
<thead>
<tr>
<th>Test</th>
<th>Contrast data set</th>
<th>N</th>
<th>Mean</th>
<th>Median</th>
<th>s.d.</th>
<th>α</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wilcoxon rank-sum</td>
<td>All (8) larval individuals</td>
<td>803</td>
<td>−0.09</td>
<td>−0.09</td>
<td>0.07</td>
<td>0.025</td>
<td>&lt;0.001</td>
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<tr>
<td></td>
<td>All dark standards</td>
<td>205</td>
<td>−0.6</td>
<td>−0.61</td>
<td>0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>All dead eyes</td>
<td>128</td>
<td>−0.25</td>
<td>−0.24</td>
<td>0.08</td>
<td>0.025</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>All Pu th6</td>
<td>150</td>
<td>−0.11</td>
<td>−0.11</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pu th4 (17 m)</td>
<td></td>
<td>−0.25</td>
<td>−0.24</td>
<td>0.08</td>
<td>0.025</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Pu th2 (17 m)</td>
<td></td>
<td>−0.11</td>
<td>−0.11</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wilcoxon signed rank</td>
<td>Pu th1</td>
<td></td>
<td>−0.075</td>
<td>−0.072</td>
<td>0.05</td>
<td>0.025</td>
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<td></td>
<td>1 m/47 deg</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11 m/61 deg</td>
<td>55</td>
<td>−0.05</td>
<td>−0.06</td>
<td>0.05</td>
<td>0.017</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Pu th2</td>
<td></td>
<td>−0.05</td>
<td>−0.12</td>
<td>0.08</td>
<td>0.017</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>1.5 m/52 deg</td>
<td>69</td>
<td>−0.12</td>
<td>−0.13</td>
<td>0.04</td>
<td>0.017</td>
<td>0.029 n.s.</td>
</tr>
<tr>
<td></td>
<td>17 m/60 deg</td>
<td>69</td>
<td>−0.11</td>
<td>−0.11</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pu th6</td>
<td></td>
<td>−0.099</td>
<td>−0.096</td>
<td>0.06</td>
<td>0.017</td>
<td>0.029 n.s.</td>
</tr>
<tr>
<td></td>
<td>6 m/49 deg</td>
<td>76</td>
<td>−0.11</td>
<td>−0.11</td>
<td>0.04</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>6 m/80 deg</td>
<td>74</td>
<td>−0.11</td>
<td>−0.11</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Different data set comparisons visualized in Fig. 4. Depth and solar elevation information given as data set identifiers for paired data from a single individual. N, number of contrast values in data set; s.d., standard deviation; α, significance threshold after Bonferroni correction; P, probability value; n.s., not significant. Uncorrected significance threshold was 0.05.
Overall, 95% of total eyeshine contrast values occurred within a range of −0.346 to 0.225 (Table 1, Fig. 4). All eyeshine contrast values measured from eight individuals were significantly different from those of all dark standards (Table 1, Fig. 4). All eyeshine contrast values were significantly different from those of eyeshine (Table 1). (B) Box plots of all measured eyeshine and control contrast values by individual photographic series. Box, interquartile range; whiskers, 1.5 interquartile range; circular dots, outliers. Median is denoted by a continuous, black horizontal line in each box. Boxes and outliers are shaded as follows: white, live animal eyeshine contrast; solid gray, dark standard contrast; stippled, dead eye contrast. Note that some experimental outliers overlap with dark standard data. Gray and white vertical bars in background visually separate box plots by individual photographic series. Three individuals (Pu th1, Pu th2, and Pu th6) were imaged twice under different illumination conditions (different depths or θRS).

Fig. 4. Summary of image contrast analysis. (A) Histogram of binned contrast values (bin size, 10 deg) from all sampled individuals (gray shading; Includes: P. thomassini, N=4; P. richeri, N=1; unknown Harpiosquilla, N=1) and dark standards (black shading). Each contrast value represents the average of three raw contrast values measured at different image exposures for a given orientation or rotation angle in space. Since dark standards were only measured for axis orientation, solar azimuth and horizontal elevation directions (not for animal rotations), the dark standard data set contains one-quarter as many values as the eyeshine data. Dark standard contrast values were significantly different from those of eyeshine (Table 1). (B) Box plots of all measured eyeshine and control contrast values by individual photographic series. Box, interquartile range; whiskers, 1.5 interquartile range; circular dots, outliers. Median is denoted by a continuous, black horizontal line in each box. Boxes and outliers are shaded as follows: white, live animal eyeshine contrast; solid gray, dark standard contrast; stippled, dead eye contrast. Note that some experimental outliers overlap with dark standard data. Gray and white vertical bars in background visually separate box plots by individual photographic series. Three individuals (Pu th1, Pu th2, and Pu th6) were imaged twice under different illumination conditions (different depths or θRS).

Brackets at the top of the box plot indicate data sets included in separate pair-wise statistical tests summarized in Table 1. Pu th, Pullosquilla thomassini (individuals 1–6); Ps ri, Pseudosquillana richeri; UK Ha, unknown Harpiosquilla; Dead, dead eye animal control (likely P. thomassini) imaged with Pu th6. Dashed line represents 0.0 value of no contrast with the natural environment. θRS, refracted solar elevation of the sun. *March 2014 samples. Number of contrast values represented in the box plot of each data set are as follows: Ps ri, 76; UK Ha, 73; Pu th1 47 deg, 58; Pu th1 61 deg, 77; Pu th2 52 deg, 69; Pu th2 60 deg, 80; Pu th3, 80; Pu th4, 80; Pu th5, 60; Pu th6 49 deg, 76; Pu th6 80 deg, 74; Dead 49 deg, 65; Dead 80 deg, 63.

Spectral properties of larval eyeshine

Prior to analysing the inherent contrast of larval eyeshine in the natural environment, we characterized some of the fundamental properties of stomatopod larval eyeshine reflectance in an independent set of experiments conducted in June 2010 (Fig. 5). These data provide a general characterization of how eyeshine varies with angle of illumination and polarization in P. thomassini and Gonodactylaceus falcatus. Though G. falcatus was not sampled during our contrast analysis experiments, these data (collected June
2010) provide a fundamental understanding of the spectral properties of larval eyeshine necessary for further analyses among a wider range of species.

Most eyeshine reflectance spectra, regardless of species, contain two characteristic peaks in reflectivity: a short-wavelength peak (450–550 nm) and a long-wavelength peak (>800 nm; Fig. 5B–D). In order to resolve the contribution of eyeshine to the observed reflectance spectra, we chose to examine the linear polarization properties of stomatopod larval eyeshine reflectance. It is well established that reflective structures often produce spectra that are linearly polarized (Chiou et al., 2007; Douglas et al., 2007; Stavenga et al., 2010; Stavenga et al., 2011); thus we expected only the portions of the reflectance curve contributed by eyeshine structures to respond to polarization filtering. We observed strong changes in polarization in the short-wavelength region of the reflectance curve, which suggests that eyeshine-producing structures most strongly reflect in this region of the reflectance spectrum (G. falcatus data in Fig. 5B). The second, long-wavelength peak in an eyeshine reflectance curve is weakly polarized at most (Fig. 5B), suggesting that it arises from ommochrome screening pigments expressed in the opaque portion of the retina (Marshall et al., 1991).

The amount of light reflected from eyeshine is strongly associated with the stage, and thus eye-size of the animal. The eye diameter of an early stage larva in species such as P. thomassini and G. falcatus is ~500 μm. A last stage larva of either of these species will have a retinal area that is roughly twice that and thus reflect more light to the spectrometer. With the exception of the last stage P. thomassini individual measured in Fig. 5C, all eyeshine reflectances presented are from early stage larvae. The low levels of light reflected from small, early stage eyes results in the spectral noise observed at wavelengths below 410 nm in Fig. 5D and Figs 6–8. It should be noted that while the amount of light reflected changes with ontogeny of the eye, the spectral shape of eyeshine reflectance remains constant across developmental stages.

To determine the illumination angle that would produce the maximum eyeshine reflectance, we measured eyeshine reflectance from four illumination angles (45, 60, 75 and 90 deg relative to the 10X objective). Maximum eyeshine reflectance was measured at an illumination angle of 45 deg of a dorsal-laterally positioned eye, which was also the minimum illumination angle possible within our apparatus (Fig. 5C,D).

Pullosquilla thomassini eyeshine is unlike that of other examined stomatopod larval species, both examined in this study and among all crustacean larval species examined to date (K. D. Feller, unpublished data and personal observations). In both early and late stage larvae, the ventral region of each eye appears blue, and the color transitions to green as one travels across the retina to the dorsal eye region (Fig. 1A). Under 45 deg illumination, the blue ventral eye reflects a narrow-band short wavelength spectrum (~445 nm peak), whereas the green dorsal region produces a higher intensity, broadband longer wavelength reflectance (470–600 nm flat peak). Fig. 6 demonstrates how reflectance changes across the eye of individual Pu th1 while holding the illumination angle at 45 deg and rotating the eye in 15 deg steps from ventral to dorsal.

In situ spectral analysis of larval eyeshine

The analysis of eyeshine spectra in the natural environment was performed using radiometric data collected concurrently with each in situ photographic series (see ‘In situ spectral analysis’ in Materials and methods). The spectral properties of eyeshine were analysed in situ from two individuals representing different species and eyeshine reflectances: Pullosquilla thomassini and Pseudosquillana richeri. Since P. thomassini displays different reflectance spectra across the retina (Fig. 6), we included both a dorsal and ventral reflectance measurement in our spectral analysis. To examine further whether...
**DISCUSSION**

**General**

By systematically testing animals in their natural light environments, we demonstrated that stomatopod larval eyeshine effectively reduces retinal contrast and is spectrally matched to the viewing background at depth. Matches between spectra of background radiances and calculated eyeshine from endemic species suggest that spectral matching is adaptive to particular habitats (Figs 7, 8). Though all eyeshine measurements of Michelson contrast were close to 0.0, the value of no contrast, there was variation in the range of contrast values among data sets. The maximum range of contrast values within a single photographic series was −0.28 to +0.22 (Pu th1, 11 m depth), while the minimum range of contrast values only varied from −0.16 to −0.03 (Pu th4, depth 17 m; Fig. 4). For datasets where a single animal was photographed in two different illumination conditions (Pu th1 and Pu th2), the animal demonstrated a significant change in the ranges of contrast values measured between the two conditions (Pu th1, P<0.0025; Pu th2, P=4.51e-3). Changes in contrast did not vary significantly with the rotation of the animal or viewing angle, leading to the conclusion that eyeshine contrast is influenced by the illumination conditions (such as depth and solar elevation) rather than the physical light-reflecting properties of the reflector.

Though solar elevation and depth were the only illumination factors within our dataset to affect eyeshine contrast, we could not reconcile the two factors from one another. The individuals photographed in two different series in June 2012 (Pu th1 and Pu th2) were each photographed at different depths and solar elevations. Given these data, the influence of each variable could not be ascertained. We revisited this experiment in March 2014 and successfully imaged an individual (Pu th6) at different solar elevations, controlling for depth, and found no significant change in the variation of eye contrast. While these results may suggest that
depth is a primary influence in eye contrast variation, it is difficult to interpret without further analysis. Future experiments that control for depth and solar elevation are necessary for resolving the influence on eye contrast with the background. Due to our small sample size, our observations of changes to eye contrast with solar elevation and depth may also be an artifact of individual variation, which cannot be resolved without further studies. Regardless, the evidence suggests that overall eyeshine is an effective mechanism of reducing contrast in any natural illumination conditions.

**Ecological significance**

Predation is a strong selective force that may drive the evolution of stomatopod larval eyeshine. Previous work demonstrates that rates of fish predation on transparent cladocerans increase when the size and pigmentation of the eyespot is increased via dietary uptake of India ink (Zaret, 1972). Though predation is a likely force driving the evolution of stomatopod larval eyeshine evolution, this hypothesis remains untested. Future behavioral experiments analogous to those with transparent cladocerans will provide an interesting test of the relationship between eyeshine contrast and predation rates. Such experiments, however, would not address whether the range of contrast values observed from stomatopod larval eyes are detectable by predators. To determine if eyeshine contrast is ecologically significant in its worst illumination conditions (most contrast), the lower threshold limits of contrast detection for a predator must be determined through sensitivity modeling experiments.

Stomatopod larvae perform diel vertical migrations, whereby they are found at depth during the day and rise to the surface at night to feed (Ohtomi et al., 2005). Our findings that most eyeshine spectra are well matched to light environments at deeper depths agrees with this aspect of stomatopod larval ecology, since larvae are expected to be found away from the surface at depth during the day. Poor matching between larval eyeshine and broad-spectrum environmental radiances at shallow, 1 m daytime depths may be of little consequence in nature, since larvae would rarely occur in such a habitat. Evidence of spectral matching between shallow (1 m) radiances and the dorsal green eyeshine in *P. thomassini*, however, suggests that this species
may be camouflaging at shallower depths per an aspect of their behavioral ecology that remains to be characterized.

The unusual blue and green regionalized eyeshine seen in *P. thomassini* larval eyes suggests some interesting hypotheses regarding its function in this particular species. It is possible that the two spectrally different regions of the eye (green, dorsal hemisphere and blue, ventral hemisphere) are a mechanism for counter-shading. The green region of eyeshine shows better spectral matching against shallow, background radiances, suggesting that it may be adaptive for camouflage in a shallower habitat. Until more is known regarding the daytime behavior and location of *P. thomassini* larvae, we cannot evaluate the functional significance of this regionalized eyeshine. However, given that the blue-ventral, green-dorsal eyeshine of *P. thomassini* is present throughout the entire range of larval stages (authors’ personal observations in early and last stages), we can assume this novel structure plays a specific role in the ecology and is not related to developmental changes to the eye.

**Eyeshine-producing structures**

The structures responsible for producing stomatopod larval eyeshine are not currently understood, though photonic structures are widely used for camouflage in the aquatic environment. Broad-band reflectors, or silvery mirrors, are a common and effective solution to camouflaging in the pelagic environment (Johnsen and Sosik, 2003). The size of the object being camouflaged may provide insight as to why stomatopod larvae do not use a silver reflector to hide their opaque retina. Though the stalked eyes on a larval stomatopod seem prominent, they are in fact quite small by comparison with the flanks of a silvery fish, which are understood to produce broad-band reflection via multiple layers of guanine crystals with varying quarter-wavelength thicknesses (Denton and Land, 1971). The space required for a typical silver reflector is perhaps not available in a stomatopod eye, which is only ~500 μm in maximum diameter (Denton and Land, 1971); therefore, eyeshine must either be produced by a simple quarter-wavelength multilayer reflector or by a relatively thin coherent scattering photonic structure. The terrestrial diamond weevil, *Entimus imperialis*, has been shown to use spectrally matched reflections from diamond-type photonic crystals for camouflage in forested environments (Wilts et al., 2012). While it is unlikely this specific type of photonic structure is found in stomatopod larval eyes, a comparable large-domain-type photonic crystal may be responsible for producing such finely tuned reflectance spectra.

Existing evidence suggests that eyeshine-producing structures may be composed of three-dimensionally arranged vesicles. Transmission electron microscopic images of eye structures in
caridean shrimp (*Palaemonetes*) larvae, which possess a greenish-gold reflecting eyeshine, show layers of tightly packed, spherical vesicles at the junction of the optics and the photoreceptor layer – the hypothesized location of crustacean eyeshine-producing structures (Douglass, 1986; Douglass and Forward, 1989). Some species of adult Squillloid and Lysiosquillloid stomatopods also possess green light-reflecting pigments on the outer margin of the retina (Marshall et al., 1991). As with the *Palaemonetes* larvae, these structures are composed of spherical vesicles, indicative of an amorphous solid with short-range order. We hypothesize that similar structures may exist in the eyes of stomatopod larvae. Investigations into the ultrastructure that is the source of stomatopod larval eyeshine are currently underway.

Our diverse taxonomic sampling suggests that eyeshine is widespread within Stomatopoda as a mechanism of larval ocular camouflage. Though eyeshine has been observed in all examined stomatopod larval species, it is unclear as to whether the mechanism used to produce eyeshine is identical across species, since different eyeshine reflectance spectra exist. Future investigations that characterize the photonic structures responsible for producing eyeshine should include a diverse taxonomic sampling of species so as to examine how these novel light-reflecting structures vary among stomatopods.

**MATERIALS AND METHODS**

**Animal collection**

The majority of larvae were captured in June 2012 at Lizard Island Research Station (Queensland, Australia), with a smaller subset of samples collected at the same location in March 2014. All collections were performed at night either while wading or scuba diving. During night dives at depths of 6–18 m we took advantage of the innate, positive phototactic behavior of early pelagic stage stomatopod larvae (Dingle, 1969) and attracted larvae to an underwater light source (Underwater Kinetics AquaSun eLED; Poway, CA, USA). Larvae were collected in gallon-size, resealable bags and later sorted in the laboratory. All deep-water collection periods began within 30 min of sunset and had a maximum duration of 40 min. Collections in shallow water (~1 m depth) were routinely performed while wading near shore between 20:00 and 22:00 h. Animals were captured using hand-held dip nets and the same light source was used during collections at depth. Larvae were maintained individually in plastic cups in the laboratory at ambient temperatures (~26°C) in seawater without food. All experiments were conducted within 48 h of capture. Larvae were identified post hoc via DNA barcoding at the University of Maryland, Baltimore County (Baltimore, MD, USA) (see Feller et al., 2013).

**In situ imaging**

Live-mounted animals were photographed in their natural environment (*in situ*) using a Canon G12 camera (Lake Success, New York, NY, USA) and a calibrated rotational chuck mounted on a tripod. Prior to imaging, we established that the spectral properties of larval eyeshine change with illumination angle. Consequently, we tested eye-spots contrast from a variety of viewing angles and directions that best represented the upper hemisphere of view for an observer positioned under water (Fig. 2). We imaged targets from a fixed, benthic position that provided views of the animal from below. Each selected viewing angle and larval posture can be broken down into four components: solar azimuth, viewing angle above the horizon, larval long axis orientation, and larval rotational angle (Fig. 2). The four solar directions included: into the sun’s azimuth (‘Into sun’, 0 deg), right of the sun (‘Right sun’, 90 deg), away from the sun (‘Down sun’, 180 deg), and left of the sun (‘Left sun’, 270 deg). In each direction relative to the sun, the animal was viewed at three viewing angles relative to the horizon (0, 45 and 90 deg) while the long axis of the animal was held either vertically (V) or horizontally (H). As the 90 deg viewing angle position produces redundancy between H and V animals (e.g. [Into sun, 90, H] is the same as [Right sun, 90, V]) only H images were collected for all 90 deg views relative to the horizon. To further view eyeshine reflectance at various orientations, at each position the animal was rotated in four 90 deg turns so as to image eyeshine from the ventral, left, dorsal and right aspects (Fig. 2, inset photographs). In each series, a total of 80 unique views of each animal were thus produced from the described components.

The changes in lighting conditions that accompanied the viewing angles and directions of our images necessitated the use of the camera’s automatic shutter speed selection. Using a fixed F-stop of 8, the automatic camera generated three successive exposures for every raw format image captured: the automatically selected exposure, an underexposed image one stop below, and an overexposed image one stop above the automatic exposure. Thus a total of 240 images per series was collected for image contrast analysis. As a control, a small, black-painted spherical object was used as an opaque dark standard. This 1-mm diameter standard was included in each photographic measurement, placed within a centimeter of the larval target. After photographing, each animal was fixed in absolute ethanol for DNA barcoding and identified post hoc per the methods described in Feller et al. (Feller et al., 2013). Specimens were considered positively identified if the genetic distance relative to a reference sequence of the cytochrome oxidase I (COI) gene was ≤5% (Barber and Boyce, 2006). The only unknown larval specimen in this study was tentatively assigned to the genus *Harpiosquilla* because the smallest sequence distance to a COI gene reference (10%) was to that of *Harpiosquilla harrux*. Since no molecular or morphological barcode record of any adult *Harpiosquilla* species presently exists at Lizard Island, it is likely that this larva represents an undescribed squilloid species from this region. Alternatively, the individual could represent a larva that is passing through from a distant location on a local current. Until DNA barcodes are documented for a greater selection of stomatopod species, the true identity of this individual will remain unclear.

All barcode sequences of the cytochrome oxidase subunit I mitochondrial gene were deposited in GenBank (GB-KJ828804, KJ882805, KJ828806, KJ828807, KJ828808, KJ828809, KJ828810, KJ828811). Since eyeshine often disappears when a larva dies, only animals that survived to the fixation procedure were used for the experimental analysis. We utilized the reduction in eyeshine post mortem as a second control by including a dead larva in the *Pu sbb* photographic series (March 2014). Both the dead larva (which died prior to experimentation) and a dark standard were mounted within a centimeter of the living specimen. Two photographic series at different solar elevations were collected using the dead eye control.

**Image analysis**

Before analysing the *in situ* images, we calibrated the digital camera for its brightness sensitivity across the 8-bit dynamic range of each red (R), green (G) or blue (B) pixel. To do so, we photographed a series of six gray standards (white, gray 8, gray 6.5, gray 5, gray 3.5 and black; Macbeth Colorchecker; Baltimore, MD, USA) illuminated under diffuse, natural sunlight. In Adobe Photoshop, both the average green and blue channel pixel values were measured from each gray standard in each exposure in the series. This produced values between 0 and 255, the maximum range for any 8-bit pixel channel. For every exposure, five error ratios were calculated between neighboring gray standards and plotted versus pixel value to determine how pixel value changes with exposure using our camera system. A linear regression of this plot was then calculated from both the blue and green channel measurements and used to produce the following correction formula:

\[
y = \frac{x}{0.0004x + 0.8}
\]  

(1)

Using this formula, a corrected pixel value (*y*) of each experimental image could be calculated for each raw 8-bit measurement (*x*) from a photograph. Calibration measurements from the blue and green channels produced the same correction formula. Due to the attenuation of different wavelengths of light under water with depth, only the blue and green 8-bit channels provide reliable data for the range of depths measured in these experiments. Measurements from either of these channels were sufficient for our analysis since there was no difference in the correction formula. Because green wavelengths attenuate at a slightly faster rate than blue, we selected the...
green 8-bit channel for our analysis so as to minimize potential saturation of pixel values at shallow depths.

For analysis of experimental images, a 15 deg circular template was centered on the mounted larva in each raw format image using Adobe Photoshop. The corrected average green channel pixel value within this circle was taken to be the background radiance. Near-field objects, such as the larval mount or reef substrate, were excluded from this measurement by selecting the averaged area of scattered pelagic light within the circle using a drawing tablet (Wacom Intuos; Vancouver, WA, USA). The 15 deg region was selected to duplicate the acceptance angle of the probe used for radiometric data collection (see ‘In situ spectral analysis’ below). Eye radiance was determined by averaging all pixels within the larval retina. The same procedure was performed for radiance measurements of the dark standard. Photographs in which the retina was not in focus were not included in the final analysis.

Contrast is a quantitative measurement of how bright or dark an object is relative to a given background and can be measured using either the Michelson or the Weber formula for contrast (Johnsen, 2012). When addressing questions of inherent contrast of an object at a fixed distance, as was the case with this study, either Michelson or Weber contrast formulae may be used (Johnsen, 2012). Weber contrast is often preferred to calculate attenuation of contrast with distance of small objects (Johnsen, 2012) or for sensitivity modeling of animals viewing eyeshine (O’Carroll and Wiederman, 2014). Since our objective was not designed to model the visibility of larval eyes to hypothetical viewers, but rather to test the inherent contrast of stomatopod larval eyes with the background environment, we chose to calculate the Michelson contrast (CM) of our subject with the background:

\[
CM = \frac{L_{\text{eye}} - L_{\text{background}}}{L_{\text{eye}} + L_{\text{background}}},
\]

where \( L_{\text{eye}} \) is the eye radiance and \( L_{\text{background}} \) is the background radiance. In this way, we were better able to analyse inherent contrast, since the values produced from this formula range between −1 and 1, with the value of no contrast equal to zero.

To examine changes in eyeshine contrast with time of day, or solar elevation, the refracted solar elevation at the time of each photographic series was calculated using Snell’s law:

\[
\theta_2 = \sin^{-1} \left( \frac{n_1 \sin \theta_1}{n_2} \right).
\]

Snell’s law describes the angle light is refracted as it enters a medium with a different refractive index (n). For our calculations, we used the n of air (\( n_1 = 1.0 \)) and the n of water (\( n_2 = 1.33 \)). Solar elevation data (\( \theta_1 \)) were acquired from the NOAA Solar Position Calculator (www.esrl.noaa.gov/gmd/grad/solcalc/; accessed 21 April 2014) for the specific date and time of each photographic series at the Lizard Island co-ordinates (−14.673°S, 145.454°E). \( \theta_2 \) was then used to determine \( \theta_1 \), the angle subtended by the sun and the zenith (\( \theta_2 = 90\text{ deg} - \theta_0 \)). The refracted solar elevation of the sun (\( \theta_0 \)) was determined by subtracting \( \theta_1 \) from 90 deg. These calculations provide the position of the sun within Snell’s window, the 96 deg cone through which light in the air is refracted and compressed above an underwater viewer (Lythgoe, 1979; Johnsen, 2012).

**Statistical analyses**

All statistical analyses were performed in R. Each data set was tested for normal distribution using a Shapiro–Wilk normality test. All data sets were normally distributed. To test for differences between contrast data measured from animal eyeshine and the dark standards, a Wilcoxon rank sum test (also known as a Mann–Whitney U-test) was performed. Alternatively, a Wilcoxon signed rank test was used to test for differences between eyeshine contrast data sets measured from a single individual at different depths and/or times of day. A Bonferroni correction method was employed during multiple comparisons of specific data. In our analyses, specific data sets were compared two to three times, thus the significance threshold (α) for a given analysis was either 0.025 or 0.017. All statistical results, as well as corrected significance thresholds, are summarized in Table 1.

**In situ spectral analysis**

Environmental spectra were measured during each photographic series using a calibrated underwater spectrometer, Subspec (Andor Technology/Oriel, Brisbane, Queensland). At the beginning of each series a cosine corrector (CC-3, Ocean Optics, Dunedin, FL, USA) was attached to a 40 cm long by 100 μm diameter optical fiber (UV-Vis; Ocean Optics) to collect downwelling irradiance. Radiance for all angles at which photographs were taken was measured using a 15 deg acceptance angle probe.

Prior to underwater photography, larvae were glued to sticks and affixed to a calibrated angle mount, which was suspended under water and imaged via a submerged 10× microscope objective (Zeiss Dialux 22; Wetzlar, Germany) (Fig. 5A). The front face of the 10× objective was protected from seawater by wrapping it in a plastic film. Larvae were illuminated by a white-light, LS-1 tungsten halogen lamp (Ocean Optics) connected to a 1 μm optical fiber (UV-Vis; Ocean Optics). The optical fiber was submerged and fixed on the calibrated angle mount to illuminate the specimen at one of four fixed angles relative to the optical axis of the 10× objective: 90, 75, 60 or 45 deg. Physical interference between the light source and the objective prevented reflectance measurements from illumination angles smaller than 45 deg. Light was guided to a spectrometer (QE65000; Ocean Optics) using a 1-mm diameter optical fiber (UV-Vis; Ocean Optics) and a custom probe-microscope adaptor attached to the epi-eyepiece of the microscope (in place of the actual eyepiece; Fig. 5A). Reflectance measurements were collected using SpectraSuite software (Ocean Optics) operated on a MacBook Pro (Apple; Cupertino, CA, USA). A measurement of 100% reflectance was obtained at the beginning of each data collection session using a Spectralon diffuse white standard (Labsphere; North Sutton, NH, USA). Due to the ultraviolet opacity of the microscope system, wavelengths less than 400 nm were not included in our analyses. A linear polarizer was rotated to 0 and 90 deg in the path of reflected light to measure the degree of polarized light reflected from the retina. All measurements were acquired from live specimens.

To calculate the eyeshine reflectance spectra in the natural environment, the eye reflectance spectrum from a 45 deg illumination angle (the illumination angle from which maximum reflectance was determined, Fig. 5C,D) measured for each individual was multiplied by the downwelling irradiance measured for an *in situ* condition of interest. Computed eyeshine was compared to radiance spectra collected from all viewing directions for a given *in situ* condition. Since radiance was collected in tandem with the photographic series, this provided real-time environmental spectra present during each image capture. Due to different values of intensities among irradiance, radiance and reflectance spectral data, all spectra were normalized to their maxima for comparison. Using these same methods, we also computed the eyeshine reflectance from larvae of *Squilla empusa*, a Western Atlantic stomatopod species, in the Lizard Island light environment to test whether eyeshine from a species inhabiting a different light environment (such as the Western Atlantic ocean) can provide an adequate spectral match to any background, or if instead it is adaptive to specific environments.

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

The initial concept for this study was proposed by T.W.C. All further designs, methods and analyses were performed by K.D.F. under the mentorship of T.W.C. The manuscript was composed in its entirety by K.D.F., with revisions by T.W.C.

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