Spectral sensitivity, spatial resolution, and temporal resolution and their implications

for conspecific signalling in cleaner shrimp

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SUMMARY STATEMENT:

We show that cleaner shrimp—colourful, finely-patterned animals—are likely colour blind and have coarse spatial resolution, and thus cannot resolve their own appearance or that of conspecifics.

ABSTRACT

Cleaner shrimp (Decapoda) regularly interact with conspecifics and client reef fish, both of which appear colourful and finely-patterned to human observers. However, whether cleaner shrimp can perceive the colour patterns of conspecifics and clients is unknown, because cleaner shrimp visual capabilities are unstudied. We quantified spectral sensitivity and temporal resolution using electroretinography (ERG), and spatial resolution using both morphological (inter-ommatidial angle) and behavioural (optomotor) methods in three cleaner shrimp species: *Lysmata amboinensis, Ancylomenes pedersoni*, and *Urocaridella antonbruunii*. In all three species, we found strong evidence for only a single spectral sensitivity peak of (mean ± s.e.m.) 518 ± 5 nm, 518 ± 2 nm, and 533 ± 3 nm, respectively. Temporal resolution in dark-adapted eyes was 39 ± 1.3 Hz, 36 ± 0.6 Hz, and 34± 1.3 Hz. Spatial resolution was 9.9 ± 0.3°, 8.3 ± 0.1°, and 11 ± 0.5°, respectively, which is low compared with other compound eyes of similar size. Assuming monochromacy, we present approximations of cleaner shrimp perception of both conspecifics and clients, and show that cleaner shrimp visual capabilities are sufficient to detect the outlines of large stimuli, but not to detect the colour patterns of conspecifics or clients, even over short distances. Thus, conspecific viewers have likely not played a role in the evolution of cleaner shrimp appearance; rather, further studies should investigate whether cleaner shrimp colour patterns have evolved to be viewed by client reef fish, many of which possess tri- and tetra-chromatic colour vision and relatively high spatial acuity.

INTRODUCTION

Animal visual capabilities are diverse, and understanding how an organism perceives visual scenes requires knowledge of that animal's spectral sensitivity (wavelength sensitivity of the photoreceptors), temporal resolution (ability to visually track rapid changes in a scene; Frank, 1999; Frank, 2003), and spatial resolution (ability to perceive detail). Numerous studies have shown that it is inaccurate to use human perception of colour and luminance to assess how an animal perceives a visual scene (Bennett et al., 1994; Safran and Vitousek, 2008); however, studies that quantify and incorporate receiver-appropriate measures of temporal or spatial resolution are rare (but see Baldwin and Johnsen, 2011; Johnsen, 2006; Marshall, 2000).

Broadly, visual physiology may be adapted for certain tasks, such as the detection of relevant visual signals (summarized in Cronin et al., 2014; Land and Nilsson, 2002). For example, the multiple chromatic channels in some primates assist in foraging for fruit against a leafy background (Osorio and Vorobyev, 1996; Regan et al., 2001), and "acute zones" in the eyes of *Polistes* paper wasps allow individuals to resolve the facial patterns of conspecifics (Sheehan et al., 2014). However, existing work on the relationship between visual capabilities and visual signals has focused on how a single aspect of vision relates to the detection of a signal. In reality, multiple aspects of an animal's visual physiology, including spectral sensitivity and both temporal and spatial resolution, all play a role in whether or not an animal can detect a given signal.

The interactions of cleaner shrimp with client reef fish are well studied (reviewed in Côté, 2000; Losey et al., 1999a), but their visual ecology is poorly understood. Cleaner shrimp attract clients to cleaning stations, which they usually occupy in groups or pairs (Bauer, 2006; Briones-Fourzán et al., 2012; Bshary et al., 2007; Chapuis and Bshary, 2010; Fletcher et al., 1995; Huebner and Chadwick, 2012b), and then provide cleaning services to clients by removing ectoparasites, in turn receiving a meal. Thus, cleaner shrimp interact on a regular basis with conspecifics and client fish, both of which, to human observers, display brilliant colours and fine patterns. Evidence from the cleaner shrimps *Lysmata debelius* and *L. amboinensis* suggests that the formation and maintenance of conspecific groups relies at least in part on visual cues (Fletcher et al., 1995; Rufino and Jones, 2001; Simoes and Jones, 1999), which could include colour patterns as an intraspecific signal. Additionally, to initiate cleaning, clients approach cleaners and adopt a characteristic solicitation pose, a stationary position with opercula and/or fins flared out, occasionally accompanied by a colour change (Côté et al., 1998; Hobson, 1971; Huebner and Chadwick, 2012a; Losey, 1972; Stummer et al., 2004); therefore client colour patterns may serve as an interspecific signal. Here, we

quantify cleaner shrimp visual capabilities to ask whether or not cleaner shrimp can reliably perceive the colours and patterns of conspecifics and/or clients.

We examined the visual physiology of three cleaner shrimp species: *Lysmata amboinensis, Ancylomenes pedersoni,* and *Urocaridella antonbruunii*. To quantify spectral sensitivity and temporal resolution, we used electroretinography (ERG). Electroretinography measures the physiological response of photoreceptors in situ, and is the method of choice for comparative studies, and for examining spectral sensitivities in animals whose sensitivities have not been previously quantified. Crustacean electroretinograms are monophasic; thus, the ERG response mirrors the response of the photoreceptors alone (Chapman and Lall, 1967; Goldsmith and Fernandez, 1968). To quantify spatial resolution, we used both morphological and behavioural methods. We then used those visual parameters to provide a general sense of how cleaner shrimp might view the patterns and colours of conspecifics and client fish.

These three cleaner shrimp species were selected because they are known fish cleaners (Becker et al., 2005; Chen and Huang, 2012; Côté, 2000), are colourful and finely-patterned, operate cleaning stations with conspecifics, and live on shallow coral reefs (often < 20 m deep), which are spectrally-broad, high light-intensity environments. We assumed that cleaner shrimp, which display a variety of colours, have colour vision. Additionally, based on the high-light environment in which they live and their regular interactions with finely-patterned conspecifics and client fish, we predicted that cleaner shrimp have high temporal resolution and fine spatial acuity that would allow them to perceive conspecific and client colour patterns over ecologically-relevant distances.

MATERIALS AND METHODS

Animals

The three species used in this study were selected to represent species from different geographic locations and different genera that contain cleaner shrimp. *L. amboinensis* is found throughout the Red Sea and tropical Indo-Pacific, operating cleaning stations in pairs (Fletcher et al., 1995). *A. pedersoni* is found throughout the Caribbean living in association with anemone hosts, in groups of two to 17, but occasionally more, individuals (Briones-Fourzán et al., 2012). Lastly, *U. antonbruunii* inhabits reefs in the Indian Ocean, and lives in groups of at least 10 individuals (Chen and Huang, 2012).

L. amboinensis and *U. antonbruunii* were purchased from Consistent-Sea Inc. (Gardena, CA, USA), and *A. pedersoni* from Dynasty Marine Associates, Inc. (Marathon, FL, USA). All individuals were wild-caught. Electroretinography was performed at the Nova

Southeastern University Oceanographic Center (Dania Beach, FL, USA). Animals were housed in aquaria using flow-through seawater drawn from an inlet to the Atlantic just off the coast of Dania Beach, FL. Animals used for recording electroretinograms were kept in the dark, to keep them as dark-adapted as possible prior to use in experiments. During optomotor experiments, animals were housed in aquaria at Duke University (Durham, NC, USA) on a 12:12 light-dark cycle, and in artificial seawater made from Instant Ocean (United Pet Group, Blacksburg, VA, USA). Temperature was kept at approximately 23-24°C, with salinity maintained between 28 and 31 ppt. Animals were fed one stick of Crab Cuisine (Hikari®, CA, USA) three times per week.

Electrophysiology

Equipment

Electroretinographic recording was used to measure spectral sensitivity (n=4-8 individuals/species) and temporal resolution (n= 4-6 individuals/species). During trials, a plastic post was attached to the dorsal surface of the animal using cyanoacrylate glue (Super Glue, Pacer Technology, Rancho Cucamonga, CA, USA), and the animal was suspended in seawater, which was maintained at room temperature (approximately 23°C). The animal was placed such that its eyes were out of the water, but the rest of the body was submerged, leaving the pleopods free to move and circulate water.

A Tungsten microelectrode (FHC Inc., Bowdoin, ME, USA) was inserted beneath the cornea of the left eye under dim red light (610 nm cut-off filter), which minimized light adaptation of the eye. This electrode was used to record electrical responses to light stimuli; a differential reference electrode was placed in the surrounding seawater, which was grounded by an AgCl-coated wire. The eye was exposed to light stimuli, and AC recordings of the eye's response were amplified, digitized, and stored using a custom program written in LabView (National Instruments, Austin, TX, USA). Cutoff frequencies were 1Hz and 15KHz. We used a Spectral Products (Putnam, CT, USA) CM110 monochromator (175W Xenon lamp) to shine a stimulus light of controlled wavelength on the eye of the animal, via one branch of a bifurcated, randomized fibre light guide (EXFO, Quebec City, Quebec, Canada), which was placed so as to bathe the entire eye in a diffuse circle of light (rather than exposing the eye to a pinpoint source). Bathing the entire eye in light allowed as many ommatidia as possible to contribute to the response, and maximized the likelihood that we would be able to detect any secondary receptor types if present. A computer-controlled shutter (Model VS25, Uniblitz, Rochester, NY, USA) set the stimulus duration. The

irradiance of the stimulus light was dictated using a neutral density wheel controlled by the computer, and irradiance was calibrated in units of photons $cm^{-2}s^{-1}$ for data analysis.

Spectral Sensitivity

Following placement of the electrode, organisms were dark adapted for 1-2 hours, and then exposed to a standard flash of set wavelength and intensity (test flash). Once the response to test flashes had remained steady for half an hour, spectral sensitivity measurements were initiated using 100 ms flashes of monochromatic light; irradiance of the flash was adjusted until a criterion response was achieved (defined as the minimum response distinguishable from background noise, either 50 or 100 µV) at each wavelength. Animals were tested at 10 nm intervals from 350 to 610 nm. Standard flashes of set wavelength and irradiance were presented to the animal approximately every five flashes, to ensure that the animal remained dark adapted throughout the experiment.

Once the criterion response had been reached at each wavelength, chromatic adaptation experiments were performed, wherein adapting light was presented to the eye via the second branch of the bifurcated light guide. The adapting light was a white light from a halogen lamp (LS-1, Ocean Optics, Inc., Dunedin, FL, USA), filtered with a 381 nm filter (FWHM 14 nm), a 487 nm filter (FWHM 10 nm), or a 550 nm longpass filter. The irradiance of the adapting light was adjusted with neutral density filters so that the irradiance required to obtain the criterion response at a given wavelength was at least one log unit brighter than when dark-adapted. Animals were chromatically adapted until standard flashes remained steady for ten minutes (this usually occurred within 30 minutes of adaptation), before beginning spectral sensitivity measurements. Animals were then tested on the same series of wavelengths as above, including test flashes, until the criterion response was reached at each wavelength. For each individual, we recorded a dark-adapted spectrum and a chromatically-adapted spectrum, then allowed the animal to dark-adapt once again, and recorded another chromatically-adapted spectrum. We repeated this process with each individual shrimp for as many chromatic adaptations as was possible before the ERG degraded.

To visualize the spectral sensitivity data, we plotted the reciprocal of the irradiance (photons $cm^{-2}s^{-1}$) required to produce the criterion response at each wavelength. We fitted absorbance curves to the spectral sensitivity data using visual pigment templates (Stavenga et al., 1993). To statistically compare dark-adapted and chromatically-adapted curves within each species, we split the spectral sensitivity curves into four categories: UV (350 – 400 nm), Blue (410 – 470 nm), SensMax (the region of maximum sensitivity, 480 – 540 nm), and Red

(550 – 610 nm) (following Cohen and Frank, 2007; Frank et al., 2009). For each individual, we calculated the mean inverse irradiance required to generate a criterion response to test flashes within each category. We then calculated ratios for UV:SensMax, Blue:SensMax, and Red:SensMax for dark-adapted and chromatically-adapted curves. For example, for each species, the UV:SensMax ratio for dark-adapted eyes was compared with the UV:SensMax ratio for each chromatic adaptation, and so on with Blue:SensMax and Red:SensMax. A Shapiro-Wilk test was used to determine if data were normally distributed. As in Frank et al. (2009), to compare a given ratio between dark-adapted and each chromatic adaptation, we used a one-way ANOVA for parametric data sets and a Kruskal-Wallis test for non-parametric data sets (Zar, 1999). We used the Bonferroni method to correct for multiple comparisons, with n=9, because we performed nine ratio comparisons per species.

Temporal Resolution and V/LogI

We quantified temporal resolution using two metrics. First, we used the maximum critical flicker fusion frequency (CFF_{max}) to quantify temporal resolution in both dark- and light-adapted conditions. The CFF is the highest stimulus rate for which the eye can still produce responses to each flash, but is dependent upon irradiance. The CFF_{max} is not dependent upon irradiance, and is reached when the eye cannot follow increasing rates of stimulus flashes, even with increases in irradiance. CFF_{max} can be compared between species, and is thus well-suited for comparative studies (see for example Cohen and Frank, 2006; Frank, 1999; Frank, 2000; McComb et al., 2013). In many photoreceptors, light adaptation shortens the response time, resulting in faster temporal dynamics and higher temporal resolution (Frank, 2003); to account for this, we first calculated CFF_{max} in dark-adapted eyes (eye exposed only to the flickering stimulus light), and then in light-adapted eyes (eye bathed in adapting light of controlled wavelength and also exposed to the flickering stimulus light).

To measure dark-adapted CFF_{max}, animals were allowed to dark-adapt until standard flashes remained constant in magnitude for 30 minutes (1-2 hours depending on the individual). A 15Hz flickering stimulus pulse (510 nm, with a 50% dark cycle, 50% light cycle) was presented to the eye for two seconds. If the eye produced the same number of response peaks as the number of stimulus flashes in the pulse train, the animal was considered able to resolve a stimulus of that speed. The rate of the stimulus pulse was then increased until the animal could no longer produce responses to every flash in the pulse train. At this point, the irradiance of the stimulus light was increased by approximately half a log unit. When, after three increases in light irradiance, the eye could not produce responses to

every flash in the pulse train, the CFF_{max} was considered achieved. In between each pulse train, the eye was allowed to dark-adapt, which was confirmed by monitoring the magnitude of the response to the standard flash that was given once a minute until the eye was dark-adapted.

For light-adapted CFF_{max}, the eye was bathed in a white adapting light, and the flickering light stimulus was transmitted through the other end of the bifurcated light guide. The same protocol as for dark-adapted CFF_{max} was followed, except that animals did not dark-adapt in between pulses. To statistically compare dark- and light-adapted CFF_{max} both within and between species, we used a two-way ANOVA. Because the two-way ANOVA showed a significant interaction between Light/Dark and Species ($p<0.001$), we performed post-hoc pair-wise tests, using one-way ANOVAs for normally-distributed data, and non-parametric Wilcoxon tests for data that were not normally distributed.

Following temporal resolution measurements, eyes were allowed to fully dark-adapt, and then V/LogI curves were generated. V/LogI curves plot an eye's electrical response (in Volts) versus the base 10 logarithm of the irradiance (LogI) required to generate that response, and they indicate an eye's dynamic range, the range of irradiances over which a photoreceptor operates. V/LogI curves allowed us to calculate V_{max}, the maximum response that the eye is capable of generating at any irradiance, or the point at which the photoreceptor response is saturated. To generate V/LogI curves, we used 100 ms stimuli of 490 nm monochromatic light (following Frank, 2003), increasing stimulus irradiance step-wise in half log unit steps. Dark-adaptation between each stimulus was confirmed by monitoring the magnitude of the response to the standard flash. V/LogI curves were fit with the Zettler modification of the Naka Rushton equation (Naka and Rushton, 1966a; Naka and Rushton, 1966b; Zettler, 1969) for the irradiance response function of photoreceptors (as in Frank, 2003). In several preparations, the maximum response was not achieved experimentally, but rather was calculated using the Naka-Rushton equation; in these cases, if the highest response recorded in the eye using ERG reached 90% of the calculated V_{max}, those data were used in the analysis. Data were then normalized with respect to the peak response (V_{max}), and averaged across individuals to obtain a species average V/LogI curve.

Also from V/LogI curves, we calculated (1) log(k), with k defined as the irradiance required to generate a response of 50% V_{max}, and a relative measure of an eye's sensitivity; and (2) response latency, defined as the time between stimulus onset and the beginning of the photoreceptor response, measured at 50% V_{max}, which is an indicator of the speed of transduction in the photoreceptors of each species.

Spatial Resolution

We used two methods to quantify spatial resolution, as represented by the minimum resolvable angle (α_{min}). Minimum resolvable angle is inversely related to spatial acuity, so smaller α_{min} values indicate higher visual acuity. First, we used a morphological measure, the interommatidial angle ($\Delta\Phi$), which is the separation between adjacent ommatidial axes (n=6 individuals/species) and an indicator of the spatial fineness with which an image is sampled (Land and Nilsson, 2002). Interommatidial angle can be estimated based on external eye morphology, in particular the radius of curvature of the eye and the average diameter of the eye facets. Second, we used a behavioural optomotor assay (n=5 individuals/species), which estimates spatial resolution by measuring an individual's response to a rotating grating of black and white stripes.

We used a fluorescence microscope to photograph the eyes of six individuals per species. Using Image J, we measured eye diameter in 10 facets per individual, though facet diameter varied little across the eye (Table 1). For the same eyes, we estimated local curvature of the eye by fitting circles to images of the eyes (following Baldwin Fergus et al., 2015); because the eyes were not perfectly spherical, we fitted several circles to each eye image. Both facet diameter and radius of curvature were averaged across individual, and then across species. We then calculated interommatidial angle by dividing the facet diameter (D) by the local radius of curvature of the eye (R). Minimum resolvable angle (α_{min}) was then calculated by multiplying $\Delta\Phi$ by two and converting to degrees (Land and Nilsson, 2002).

Visual acuity can also be measured behaviourally using the optomotor response, a stereotypical behaviour elicited by placing an animal inside a rotating drum lined with vertical black and white stripes. If an animal can resolve the stripes, it will rotate its body in the same direction as the drum rotation (McCann and MacGinitie, 1965; Reichardt, 1961). The thinnest stripe width at which an animal still turns in response to the rotating drum is the point beyond which behavioural responses did not reach a criterion response; two times that width (i.e. the width of one black and one white stripe together) can be interpreted as the limit of the animal's spatial resolution, or minimum resolvable angle (α_{min}).

During optomotor trials, an animal was placed inside a water-filled 30 cm diameter cylindrical acrylic tank. The tank was then lowered into a drum that measured 56 cm in diameter, and which was lined with vertical black and white stripes. The drum and the striped stimuli were rotated from below using a computer-controlled stepper motor (STP-MTRH-23079, AutomationDirect, Atlanta, GA, USA). Stimuli were printed using a laser printer and

covered a range of widths from 4.1-16.3 degrees at intervals of 0.4 degrees. We also printed a control that was uniformly 50% grey. The optomotor tank was illuminated from directly overhead by an LED light panel (Fotodiox Pro LED 312-DS, Fotodiox Inc., IL, USA). Light was held constant at 5600K.

Spatial resolution varies with the brightness of ambient light, and marine animals regularly experience large changes in brightness. For animals that live at roughly 20–30 m depth, variation in sky condition and clarity of water can result in ambient brightness varying over 3–4 orders of magnitude (for example a sunny day with clear water versus a cloudy day with murky water). For this reason, we performed the optomotor assay under three different lighting conditions: Bright (light panel at full brightness), Medium (10% of full brightness), and Dark (0.01% of full brightness). These three lighting conditions, ranging over four orders of magnitude in irradiance, roughly approximate the range of brightness that is normally experienced by animals that live underwater.

Individuals were tested on each lighting condition once a week for four consecutive weeks. Prior to the beginning of the experiment, individuals were placed in the test arena for a 15-minute acclimation period. Just before testing began, an 8 cm diameter acrylic cylinder was lowered around the animal to restrict its movements towards or away from the drum during testing, minimizing changes in distance between the animal's eyes and the rotating stimulus. We then rotated the drum at a constant speed of approximately ten rotations/minute, an optimal speed determined in preliminary experiments. If an individual responded to the rotation by spinning in the direction of rotation, the drum was rotated in the opposite direction to ensure that movement was occurring in response to the stripes. If an animal successfully completed one half-rotation in each direction, we considered it a positive response. If no rotation occurred within one minute, we considered that a negative response. Stimuli were presented in random order, i.e. not consistently from narrower to wider or vice versa. The grey control was used once during each trial; over the course of 156 trials, no individuals responded to the control.

To describe the spatial resolution data, we used the median and interquartile range of α_{min}, because the data were not normally distributed. We used a non-parametric Kruskal-Wallis ANOVA to determine if lighting condition significantly affected spatial resolution both within a species, and across species. We also used the Wilcoxon rank sums method to compare the morphological estimate of best spatial resolution and the best spatial resolution found behaviourally, to determine whether morphologically predicted spatial resolution differed significantly from the best resolution observed behaviourally.

Perception of Conspecifics and Client Fish by Cleaner Shrimp

To investigate perception of conspecifics and clients by cleaner shrimp, we used Fourier methods to create images that approximate how cleaners perceive visual scenes, based on our spectral sensitivity and spatial resolution data (following Baldwin and Johnsen, 2011; Baldwin Fergus et al., 2015). We began with colour images (1024 x 1024 pixels), and extracted one colour channel, resulting in a grayscale image. The Fourier transform of the grayscale image was then multiplied by a Modulation Transfer Function (MTF) whose contrast at the maximum resolvable spatial frequency was less than 2%, a value that was chosen because it approximates the minimum contrast threshold in organisms in bright light conditions (Douglas and Hawryshyn, 1990), and data regarding these specific species were lacking. We then applied an inverse Fourier transform to recover the image as it would appear to the cleaner shrimp eye. The end result was an image in which spatial information spanning angles less than the α_{min} measured behaviourally had been eliminated.

Actual size of each image was estimated based on size data for adults of each species, and distance to the viewer was held constant; thus, different images subtend different angles. In approximations of intraspecific appearance, the viewer was assumed to be 2.5 cm away from the subject, a short but ecologically-relevant distance over which intraspecific signalling could occur between individuals at the same cleaning station. To model perception of clients, the viewer was assumed to be 10 cm away, a value based on published measures of cleaner-client interaction distance (Becker et al., 2005; Chapuis and Bshary, 2010).

RESULTS

Spectral Sensitivity

In all three species, dark-adapted spectral sensitivity curves (Fig. 1A) showed strong evidence for only one sensitivity peak, with peak sensitivity (mean ± s.e.m.) of 518 ± 5 nm (*L. amboinensis*), 518 ± 2 nm (*A. pedersoni*), and 533 ± 3 nm (*U. antonbruunii*). However, dark-adapted curves also showed a shoulder between 420 and 450 nm in *L. amboinensis* and *A. pedersoni*, and a peak in the ultraviolet (around 350 nm), in all three species. To explore whether these are due to additional photoreceptor classes, we performed several chromatic adaptations. Chromatic adaptations are a powerful method for revealing the presence of secondary sensitivity peaks, even those that result from uncommon photoreceptor types or low levels of expression of a certain pigment.

First, to account for the small shoulder between 420 and 450 nm in both *L. amboinensis* and *A. pedersoni*, we performed chromatic adaptations using a 487 nm (FWHM 10nm) filter and a 550 nm cut-off longpass filter (Fig. 1B,C). If this shoulder were due to a secondary photoreceptor class, it should be significantly higher under 550 nm adaptation than under 487 nm adaptation. However, if not due to a secondary photoreceptor class, then we would expect no differences to arise during chromatic adaptations, and thus we would see no significant differences in the ratio of Blue:SensMax between the 487 nm adaptation and 550 nm cut-off adaptation. As expected, in *L. amboinensis*, the average Blue:SensMax ratio during 487 nm adaptation (0.40 ± 0.05) differed only slightly from the ratio during 550 nm adaptation (0.38 ± 0.03), and this difference was not significant (one-way ANOVA, p=0.62). In *A. pedersoni*, the difference in average Blue:SensMax ratio during 487 nm adaptation (0.57 ± 0.18) compared with the 550 nm adaptation (0.48 ± 0.01) was greater, but this difference was still not significant (one-way ANOVA, p=0.40). Thus, these two chromatic adaptations provide evidence that the shoulder present between 420 nm and 450 nm is not due to a secondary photoreceptor class.

To investigate the peak in the ultraviolet wavelengths, we performed a chromatic adaptation using a 381 nm (FWHM 14 nm) filter (Fig. 1D). We saw no significant differences in any of the ratios when comparing the 381 nm adaptation with any other chromatic adaptation (one-way ANOVA, every p >0.06). Thus, these results did not yield any support for the presence of a secondary photoreceptor class. The ultraviolet peak may be the β peak of the primary visual pigment, although its magnitude is much greater than expected from the β peak. Potentially, however, this peak may be due to a secondary, UV-sensitive photoreceptor class that we were unable to uncover using electroretinography.

Overall, the response waveforms during dark adaptation appeared virtually identical to those under chromatic adaptation to 381 nm light, 486 nm light, and 550 nm light. To compare dark-adapted spectral sensitivity curves with those under chromatic adaptation, we compared the UV:SensMax, blue:SensMax, and red:SensMax ratios during chromatic adaptation with those from dark-adapted eyes, and found no significant differences in any ratio after Bonferroni correction (one-way ANOVA, every p > 0.10). Thus, the lack of significant differences in sensitivity between dark-adaptation and any of the chromatic adaptations, provides further evidence for the presence of only one photoreceptor class (Cohen and Frank, 2007; Frank et al., 2009). Fig. 2 shows results from individual preparations where spectral sensitivity curves from two chromatic adaptations, as well as

dark-adapted curves, were recorded, and demonstrates that the shapes of the sensitivity functions were not preparation-specific (i.e. were not dependent upon electrode placement).

Temporal Resolution, Sensitivity, Response Latency, and V/LogI

All three species showed higher temporal resolution under light-adaptation than dark-adaptation (Table 2). Light adaptation resulted in a significant increase in maximum critical flicker fusion for both *A. pedersoni* ($F_{1,8} = 120$, p<0.001) and *U. antonbruunii* ($F_{1,8} = 9.66$, p=0.01); in *L. amboinensis*, the same trend was seen, but the difference between light- and dark-adapted CFF_{max} was not significant ($F_{1,8} = 3.27$, p= 0.11). Between-species comparisons showed that light-adapted CFF_{max} differed significantly between *A. pedersoni* and *L. amboinensis* (Wilcoxon test; Z=-2.54, p=0.01), as well as between *A. pedersoni* and *U. antonbruunii* (Wilcoxon test; Z=-2.53, p=0.01). However, dark-adapted CFF_{max} did not differ significantly between any species.

All V/LogI curves were sigmoidal in shape; dynamic ranges, log(k), and V_{max} are shown in Fig. 3. Response latency and overall sensitivity of the eye were similar for all species (Table 2). Both CFF_{max} and response latency are characteristics of a photoreceptor's temporal function, and previous studies have demonstrated that they are correlated (Frank, 2003). A Pearson correlation showed a good correlation between CFF_{max} and response latency across species (n=15 individuals; correlation coefficient R=-0.59, p=0.046), indicating that as response latency increases, flicker fusion rate decreases, as expected.

Spatial Resolution

Measures of eye morphology (Table 1) estimated that theoretical best spatial resolution would be 5.6 ± 1.0 deg (*L. amboinensis*), 8.2 ± 0.9 deg (*A. pedersoni*), and 7.2 ± 0.7 deg (*U. antonbruunii*), and thus predicted significant differences in spatial resolution between *L. amboinensis* and both *U. antonbruunii* (Wilcoxon test; Z=2.16, p=0.03) and *A. pedersoni* (Wilcoxon test; Z=-2.64, p=0.008). In each species, the finest spatial resolution measured behaviourally occurred at the brightest lighting condition (Fig. 4): 9.9 ± 0.3 deg (*L. amboinensis*), 8.3 ± 0.1 deg (*A. pedersoni*), and 11 ± 0.50 deg (*U. antonbruunii*). Behaviourally, *A. pedersoni* exhibited significantly finer resolution than both *L. amboinensis* (Wilcoxon test; Z=2.19, p=0.03) and *U. antonbruunii* (Wilcoxon test; Z=2.35, p=0.02).

Because spatial resolution can vary with the irradiance of ambient light, we performed the optomotor assay under three lighting conditions. As predicted, spatial resolution became coarser with a decrease in ambient light (Fig. 4). The difference between average α_{min} at the

brightest condition and the darkest condition was smallest in *A. pedersoni* (12% decrease, Z=-2.19, p=0.03), intermediate in *L. amboinensis* (29% decrease, Z=-2.17, p=0.03), and greatest in *U. antonbruunii* (30% decrease, Z=-2.53, p=0.01). Thus, as expected, decreased light levels resulted in decreased spatial resolution in all three species, so the ability for cleaner shrimp to resolve detail declines under darker conditions, such as deeper depths, darker times of day (e.g. dawn and dusk), cloudy days, or in murky water.

Cleaner Shrimp Perception of Conspecifics and Clients

Based on both our dark-adapted and chromatically-adapted spectral sensitivity curves, we assumed monochromacy in our focal cleaner shrimp species. Thus, we modified achromatic photographs using our behavioural spatial resolution data to approximate perception of conspecifics and clients by cleaner shrimp. These photographs suggested that cleaner shrimp cannot resolve the fine spots and stripes that comprise conspecific appearance, even over distances of 2.5 cm (Fig. 5). Similarly, our results showed that cleaners can resolve the outlines of client fish, particularly against featureless backgrounds, but that cleaners cannot perceive the colours or fine-scale patterns of client fish (Fig. 6).

DISCUSSION

Cleaner Shrimp Visual Capabilities

Here, we present the first study of spectral sensitivity, temporal resolution, and spatial resolution in any species of cleaner shrimp. Using ERG, we found support for only a single photoreceptor class in the three cleaner shrimp we examined, with peak sensitivity between 515 and 535 nm; if only one photoreceptor class is indeed present, then these cleaner shrimp are monochromatic, or colour blind (Fig. 1A). The majority of crustaceans whose spectral sensitivities have been studied (primarily crabs, deep-sea shrimps, or stomatopods) are either mono- or di-chromatic (reviewed in Marshall et al., 1999). However, previous research on crustacean spectral sensitivity has found that shallow-water coastal decapods are often dichromatic, with one photoreceptor maximally sensitive to blue-green wavelengths (480 nm – 540 nm), and a second UV/blue-sensitive photoreceptor with peak sensitivity near 400 nm (Cronin, 2006; Goldsmith and Fernandez, 1968; Johnson et al., 2002; Marshall et al., 1999; Marshall et al., 2003), although monochromacy among shallow-water decapod crustaceans is not unprecedented (see Marshall et al., 1999 for a review).

We did observe two secondary shoulders or peaks in the dark-adapted curves, one between 420 and 450 nm (in *L. amboinensis* and *A. pedersoni*), and one around 350 nm in all

three species. However, chromatic adaptation experiments suggested that these were not indicative of secondary photoreceptor classes. Rather, the shoulder between 420 and 450 nm was likely due to experimental variation. The ultraviolet peak is likely the β peak of the primary visual pigment, although the sensitivity values we recorded in the UV range are much higher than is expected from a β peak. It is possible that the anomalously high values in the ultraviolet are indicative of a second, UV-sensitive photoreceptor class, though our chromatic adaptation experiments show evidence for only a single photoreceptor class (Fig. 1B,C,D). Crustaceans that are dichromatic tend to have UV/blue-sensitive photoreceptors (Marshall et al., 1999), suggesting that if our cleaner shrimp are in fact dichromatic, it is likely that their secondary spectral sensitivity peak would be in the ultraviolet. Other methods for examining spectral sensitivity (see below) could be used in future studies to search further for a UV-sensitive photoreceptor class. Alternatively, the abnormally high sensitivity values recorded in the ultraviolet may be due to the presence of a UV-leaky screening pigment. Screening pigments are found in many crustaceans (for a discussion see Douglas and Marshall, 1999), and in *Gnathophausia ingens,* previous work has shown that similar anomalous values recorded in the red end of the spectral sensitivity curves were due to the presence of red-leaky screening pigments (Frank et al., 2009).

Behaviourally quantified spatial acuity yielded minimum resolvable angles between 8 and 11 degrees (Fig. 4), while morphological estimates yielded a theoretical best acuity estimate between 5 and 9 deg. A study of 96 species of decapod crustaceans found that interommatidial angle ranged from 2.5 to 11.3 deg (Dobson et al., 2014), putting cleaner shrimp on the higher end of known spatial resolution in decapods. However, compared with other published measures in the animal kingdom, cleaner shrimp spatial resolution is quite low, lower even than that found in sea snails (*Littorina*, 4.5 deg) or scallops (*Pecten*, 1.6 deg) (Land, 1981).

Lastly, we measured several indicators of temporal dynamics, including CFF_{max} and response latency, as well as sensitivity, represented by $\log(k)$ (Table 2). Previous research on temporal resolution in crustaceans has for the most part focused on mesopelagic and deep-sea species (see Frank, 1999; Frank, 2000), so valid comparisons for values of CFF_{max} between our cleaner shrimp and other species are difficult to make. However, a small number of studies have examined response latency and sensitivity in semi-terrestrial and terrestrial crustaceans, which live in high-light environments and thus provide a more valid comparison with cleaner shrimp. In the fiddler crabs *Uca thayeri* and *U. pugilator*, values of $\log(k)$ are 13.9 and 14.8, thus only slightly higher than the values we observed here (reported in Cohen

and Frank, 2006). The beach-dwelling amphipod *Talorchestia longicornis* shows only slightly lower values of log(k) (dark-adapted 11.89/light-adapted 11.97 log photons $cm^{-2}s^{-1}$) than cleaner shrimp, as well as slightly longer response latencies (dark adapted 30.3/light-adapted 27.7 ms) (Cohen et al., 2010). Thus, measures of sensitivity and response latency in our three species of cleaner shrimp were consistent with other studied crustaceans that live in a high-light environment.

Potential Functions of Cleaner Shrimp Vision

Using ERG, we found evidence for only a single photoreceptor class, which is suggestive of monochromacy, and our spatial resolution data show that cleaner shrimp have coarse vision. Thus, it is likely that they cannot resolve either the colours or intricate patterns that comprise their own appearances, even over short distances (Fig. 5). Similarly, cleaners cannot resolve fine-scale features on client fish (Fig. 6). However, our results do suggest that cleaner shrimp are capable of detecting large, looming stimuli—such as the outlines of client fish—and changes in ambient light caused by approaching clients. This is supported by observations that cleaners respond with advertising motions when humans approach the aquaria where they are housed (EM Caves, pers. obs.). Additionally, one behavioural study has shown that cleaner shrimp will exhibit advertising behaviour towards large rectangular pieces of paper, suggesting that perhaps a looming visual stimulus, rather than a stimulus of particular shape, colour, or detail, is the only cue necessary to initiate cleaning (Colin, 1972). Interestingly, predation on cleaner shrimp appears to be rare (summarized in Côté, 2000); thus, although a looming stimulus could also indicate the approach of a predator, it is likely that, on average, it is beneficial to cleaner shrimp to not flee from looming stimuli.

It has been suggested that any cleaning organism—from birds to fish to shrimp—should have good vision, since by definition cleaning organisms must be able to locate small ectoparasites living on other animals (Poulin and Grutter, 1996). Although ectoparasites vary greatly in size, studies of cleaner shrimp diets suggest that cleaners feed primarily on small ectoparasites approximately 0.1–2.5 mm in length (Becker and Grutter, 2004; McCammon et al., 2010). For an ectoparasite to be one 'pixel' across to a cleaner shrimp visual system with spatial resolution of 9 degrees, the shrimp would have to be 15.8 mm away from a parasite 2.5 mm in length, but 0.63 mm away from a parasite 0.1 mm in length. Thus, though larger ectoparasites may be resolvable by the cleaner shrimp visual system, very small ones may not.

The Function of Cleaner Shrimp Colour Patterns

We suggest that selection to resolve the colour patterns of conspecifics, for example during conspecific signalling, has not played a large role in the evolution of cleaner shrimp colour patterns. It may be, however, that selection on other viewers, for example client fish, to see cleaner shrimp has played a role in the evolution of cleaner shrimp appearance. Given that cleaner shrimp receive food from clients (Côté, 2000), it would be beneficial for them to be conspicuous to reef fish, and reef fish have both colour vision and good spatial acuity that may allow them to resolve cleaner shrimp colour patterns (Collin and Pettigrew, 1989; Losey et al., 1999b; Losey et al., 2003; Siebeck et al., 2008). There is evidence from certain reef fish that colour patterns can serve simultaneously as both a conspicuous signal and camouflage, depending on viewing distance and the background against which an animal is viewed (Marshall, 2000). Many cleaner species are symbiotic on host organisms, such as anemones or sponges, and thus different aspects of cleaner shrimp appearance may represent some combination of conspicuousness and host-specific camouflage, depending on signalling behaviour, body position, background, and distance to viewer.

Limits of This Study

Proving monochromacy is inherently difficult, and as discussed above, anomalously high sensitivity values in the UV could indicate the presence of a second, UV-sensitive pigment in cleaner shrimp. There are several methods that could potentially provide additional evidence for mono- or di-chromacy, including histology, genomics, and microspectrophotometry (MSP).

Histologically, the presence of R8 cells can be suggestive of, but does not provide definitive evidence for, UV-sensitivity in crustaceans (Marshall et al., 1999). An organism may be UV-sensitive without possessing an R8 cell (the isopod *Ligia exotica*, Hariyama et al., 1993), or R8 cells may be present but vestigial or small, and thus not functional (as in the eyes of deep-sea oplophorids (Frank and Widder, 1999).

Genetically, opsin gene sequences can help in determining an animal's spectral sensitivity. However, an expressed opsin does not necessarily have any functional significance in vision (for example in the onycophoran *Euperipatoides kanangrensis*, Eriksson et al., 2013). Additionally, many crustaceans express more opsins than would be predicted based on photoreceptor physiology, as has been shown in stomatopods (Porter et al., 2013), brachyuran crabs (Rajkumar et al., 2010; Sakamoto et al., 1996), mysids (Frank et al., 2009), ostracods (Oakley and Huber, 2004), and branchipods (Kashiyama et al., 2009).

Retinal transcriptomes, however, could be used to provide evidence for functional, expressed opsins.

Lastly, MSP can provide information on the absorption characteristics of visual pigments, and could potentially provide further evidence for the existence of a secondary photoreceptor class. However, MSP can have difficulty identifying pigments that exist in small quantities or are situated in small cells, and cannot measure the effects of pre-retinal filtering, self-screening, and reflecting pigments (Goldsmith, 1978; Marshall et al., 1991). Thus, it should be noted that MSP does not always give an accurate picture of the presence of visual pigments or their wavelength of peak sensitivity *in situ*. Ultimately, a combination of the above approaches, in addition to the ERG data presented here, may be necessary to draw firm conclusions regarding monochromacy in cleaner shrimp.

Conclusions

Whether cleaner shrimp are mono- or di-chromatic, they will be unable to perceive the rich diversity of colours found on conspecifics and clients that are so readily visible to human observers. Additionally, we show here that cleaner shrimp vision is quite coarse, and that low spatial resolution, rather than their colour vision capabilities, is likely the limiting factor on what they are able to view as signals. Ultimately, we can conclude that cleaner shrimp visual capabilities are not sufficient for them to view the colour patterns of conspecifics and client fish as signals, even over short distances. Future studies should consider whether cleaners use chemosensory, tactile, or other sensory abilities in intraspecific communication, interacting with clients, or locating ectoparasites. Additionally, how client fish perceive cleaner shrimp appearance is an avenue of study that may shed light on the selective pressures shaping the colour patterns of cleaner shrimp.

It is now well-accepted among biologists that it is necessary to study the colour and luminance of visual signals from the perspective of relevant receivers (Bennett et al., 1994; Safran and Vitousek, 2008). We show here that the same is true of the spatial component of visual signals. This research underscores the importance of considering more than spectral sensitivity, for example by incorporating receiver-appropriate measures of spatial resolution, before drawing conclusions about the fitness consequences of any visual signal.

List of symbols and abbreviations

ERG: electroretinography

β: The β peak of the primary visual pigment, a secondary peak due to physical structure of the visual pigment

CFF_{max}: Maximum critical flicker fusion frequency

V_{max}: Maximum voltage the eye is capable of producing, maximum electrical response created by the photoreceptors

$\Delta\Phi$: Interommatidial Angle, the separation between adjacent ommatidial axes

α_{min}: Minimum resolvable angle, a measure of spatial resolution

Author competing interests

The authors declare no competing or financial interests.

Author contributions

E.M.C. and T.M.F. designed and executed the electrophysiology experiments and analysed the data. E.M.C. conducted spatial resolution experiments, analysed the data, and wrote the manuscript. T.M.F. wrote the custom software; to obtain the software, interested parties should contact the corresponding author. T.M.F. and S.J. provided intellectual input. S.J. produced images that modelled visual perception by cleaner shrimp, and revised the manuscript. All authors reviewed and corrected the manuscript.

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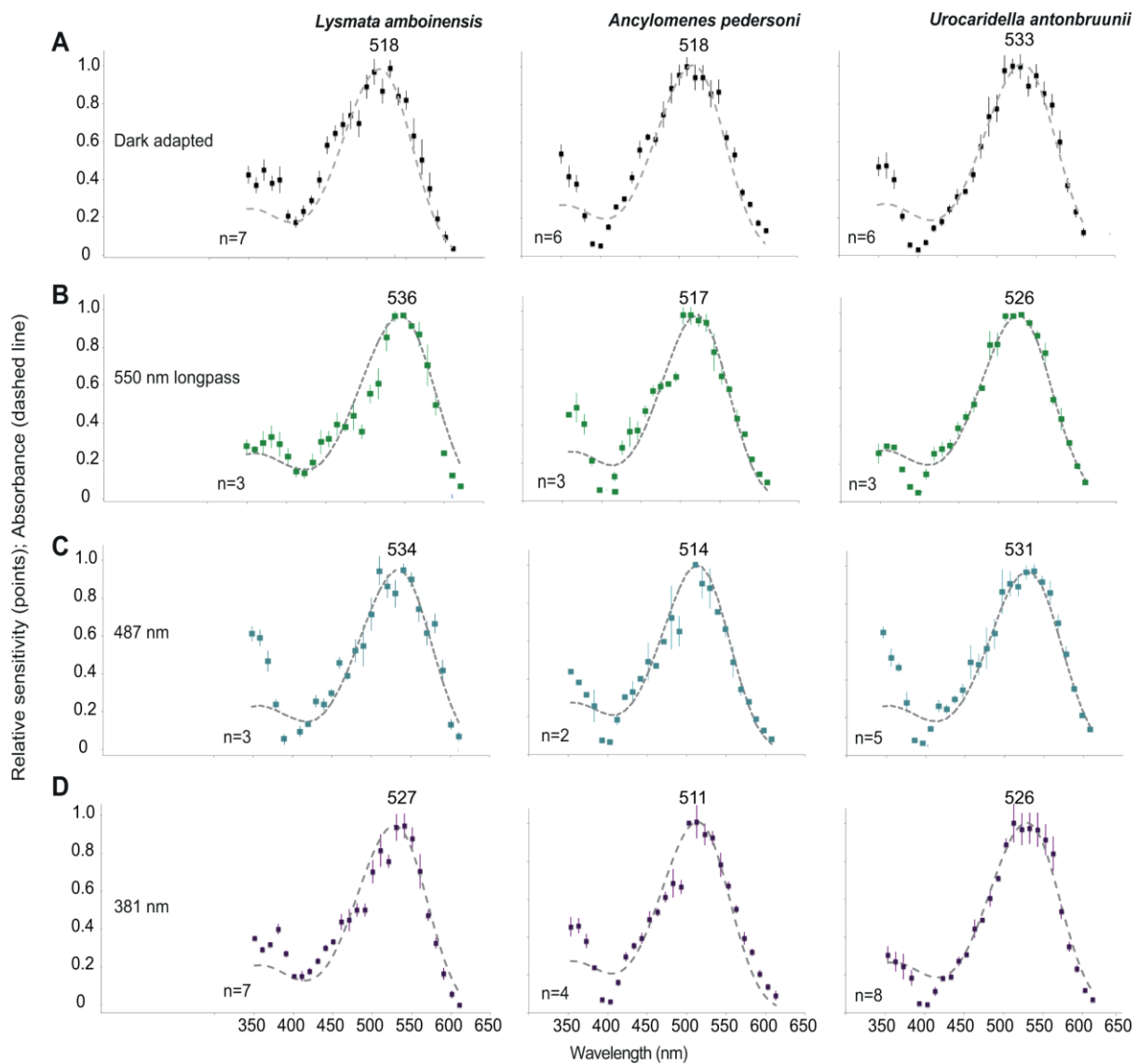
Fig. 1. Spectral sensitivity curves from (a) dark-adapted, and chromatically adapted to (b) 550 nm longpass filter, (c) 487 nm filtered light, and (d) 381 nm filtered light in *Lysmata amboinensis, Ancylomenes pedersoni,* **and** *Urocaridella antonbruunii.* All three species demonstrated only a single peak in each curve. Chromatic adaptation did not significantly shift the peak spectral sensitivity in any case (see text for details of the statistical tests). Data points represent averaged, normalized spectral sensitivity data; dashed lines are best-fit absorbance curves. Numbers over each peak are peak spectral sensitivity (nm).

Sample size (individuals) is shown at the bottom left of each graph; differences in sample size arose because, for each individual, we recorded as many dark- and chromatically-adpated spectra as was possible before the response from the eye began to degrade, and the length of time that this allowed differed between individuals. Error bars represent s.e.m.
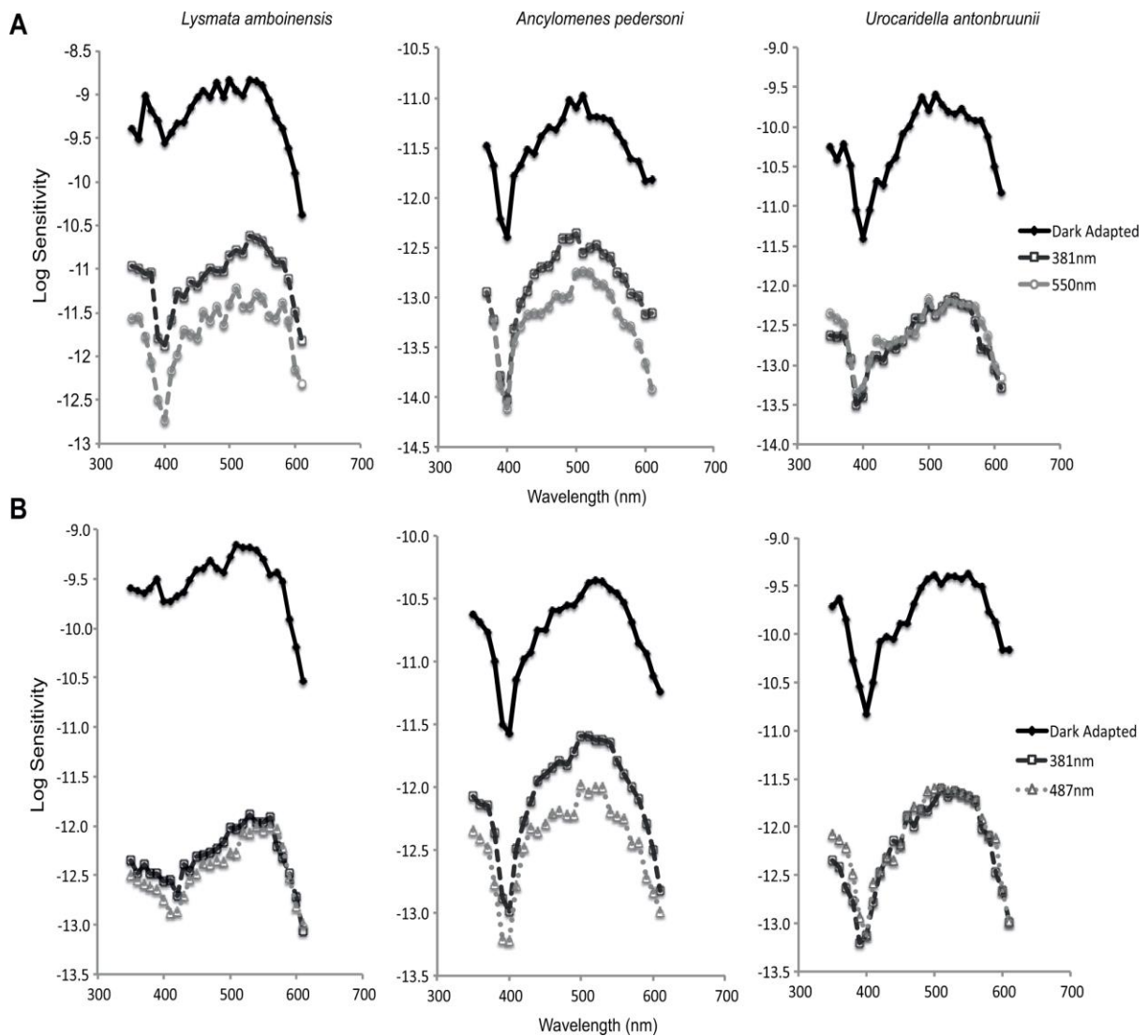
Fig. 2. Representative spectral sensitivity data for preparations in which we measured dark-adapted spectral sensitivity (solid lines) as well as chromatically-adapted spectral sensitivity (dashed lines) under (A) both 381nm and 550nm-longpass chromatic adaptations and (B) both 381nm and 487nm chromatic adaptations. Each panel displays data from one preparation (i.e. specimen and electrode placement, n=1 individual for each panel). Similar results were found in all individuals for which multiple chromatic adaptations were performed.
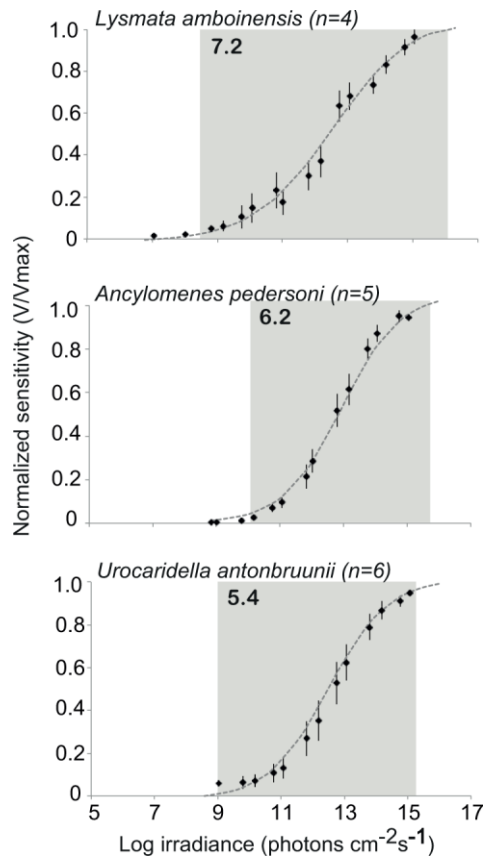
Fig. 3. V/LogI curves for *Lysmata amboinensis, Ancylomenes pedersoni,* and *Urocaridella antonbruunii*. The shaded boxes, and the numbers at the top left of each box, denote the dynamic range. Error bars are s.e.m.
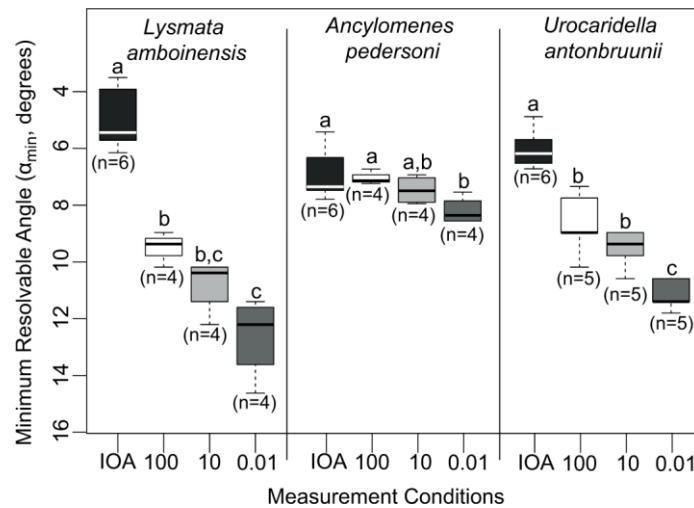
Fig. 4. Spatial resolution, represented by minimum resolvable angle, in *Lysmata amboinensis, Ancylomenes pedersoni,* and *Urocaridella antonbruunii*. We estimated spatial resolution using Interommatidial Angle (black) and a behavioural optomotor assay under full intensity (white), 10% intensity (light grey), and 0.01% intensity (dark grey) lighting conditions. Larger α_{min} values translate to coarser spatial resolution; thus, in this graph, finest spatial resolution is at the top. For each individual, we extracted the single lowest spatial value to which an individual responded during any trial and used those values to calculate species averages; the ranges indicated here thus illustrate the range of minimum resolvable angles expressed across individuals (sample size indicated above each box). Boxes indicate the 25% and 75% quartiles, and the heavy black line indicates the median. Whiskers extend to the highest value that is within 1.5x the interquartile range. Letters above each box represent the results of within-species statistical comparisons; two bars within a species that share a letter did not differ significantly (Wilcoxon paired tests; $p < 0.05$).
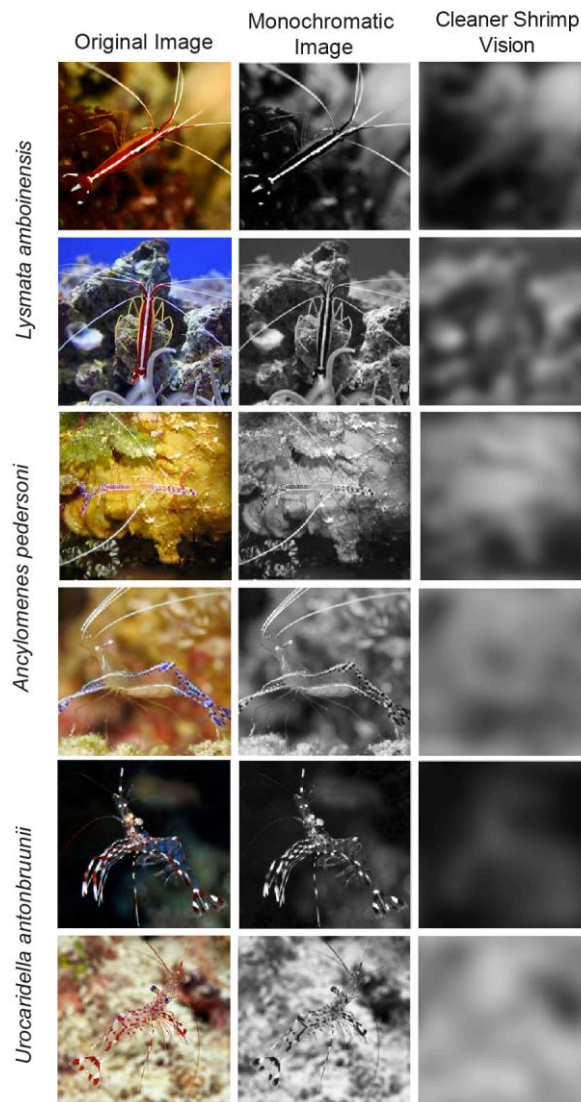
Fig. 5. Accounting for cleaner shrimp monochromatic vision and spatial resolution in intraspecific appearance. The three columns are the original image (left column), an achromatic image created by isolating the green colour channel from the original image (middle column), and simulated perception by a cleaner shrimp from a distance of 2.5 cm (right column). Spatial resolution used to approximate cleaner shrimp vision was that measured using behavioural methods, under the brightest lighting condition. See text for more detailed methods. Images attributed to: www.aquariumslife.com, Florence Charpin, Rokus Groeneveld, and Ülar Tikk.
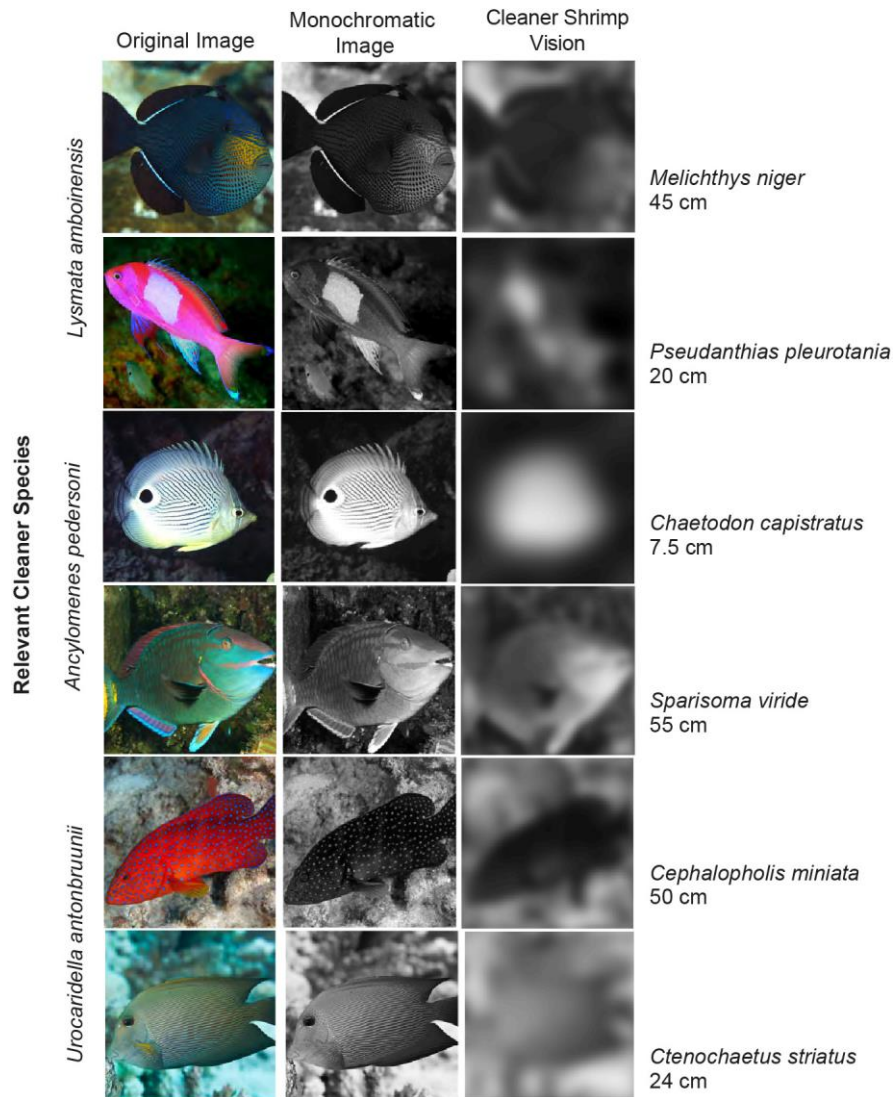
Fig. 6. Approximating client fish perception by cleaner shrimp. The three columns are the original image (left column), an achromatic image created by isolating the green colour channel from the original image (middle column), and simulated perception by a cleaner shrimp from a distance of 10 cm (right column). Client species were chosen based on records of cleaner-client interactions from the literature (Becker et al., 2005; Chen and Huang, 2012; Wicksten, 1995; Wicksten, 2009), and to represent a range of client fish sizes. Size of adult for each species is listed to the right of the photographs. Spatial resolution used to approximate cleaner shrimp vision was that measured using behavioural methods, under the brightest lighting condition. See text for more detailed methods. Images attributed to: Mark Rosenstein, George Ryschkewitcsch, Brian Lasenby, Paddy Ryan, and Bo Davidsson.

Tables

Table 1. Measures of eye radius and facet diameter, and morphological and behavioural estimates of spatial resolution (α_{min}) in *Lysmata amboinensis, Ancylomenes pedersoni,* and *Urocaridella antonbruunii.*

	Lysmata amboinensis	*Ancylomenes pedersoni*	*Urocaridella antonbruunii*
Radius of Curvature (mm)	0.93 ± 0.05 (n=10)	0.35 ± 0.01 (n=10)	0.50 ± 0.01 (n=10)
Eye Facet Diameter (μm)	43 ± 1.7 (n=7)	26 ± 0.6 (n=6)	30 ± 0.8 (n=7)
Morphological α_{min} (°)	5.6 ± 0.4 (n=7)	8.2 ± 0.4 (n=6)	7.2 ± 0.3 (n=7)
Behavioural α_{min} (°)	9.9 ± 0.3 (n=4)	8.3 ± 0.1 (n=4)	11 ± 0.5 (n=5)

Sample size indicates number of individuals. In the case of Behavioural α_{min}, we used the single lowest value exhibited by a given individual across all trials and averaged those values to calculate species averages (see methods for details). Values are mean ± s.e.m.

Table 2. Dark- and Light-adapted CFF_{max}, log K, and latency of 50% V_{max} response in three species of cleaner shrimp.

	Lysmata amboinensis	*Ancylomenes pedersoni*	*Urocaridella antonbruunii*
CFF_{max}: Dark-adapted (Hz)	39 ± 1.4 (n=5)	36 ± 0.6 (n=5)	34 ± 1.3 (n=6)
CFF_{max}: Light-adapted (Hz)	42 ± 0.8 (n=5)	48 ± 0.9 (n=5)	40 ± 1.2 (n=6)
K (log photons $cm^{-2}s^{-1}$)	13 ± 0.3 (n=4)	13 ± 0.2 (n=5)	13 ± 0.3 (n=6)
Response latency (ms)	28 ± 2.6 (n=4)	25 ± 2.4 (n=5)	26 ± 2.0 (n=6)

Values are mean ± s.e.m.