Nanoscale ultrastructures increase the visual conspicuousness of signalling traits in obligate cleaner shrimps

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Abstract

Signal theory predicts organisms should evolve signals that are conspicuous to intended receivers in natural signalling environments. Cleaner shrimps remove ectoparasites from reef fish clients and many signal their intent to clean by whipping long, white antennae. Since white is a reliably conspicuous colour in aquatic environments, we hypothesized that selection has acted to increase broad-spectrum antennal reflectance in cleaners. Using Scanning Electron Microscopy, optical models, and reflectance measurements, we found that the antennae in three obligate cleaner species from two families (Palaemonidae and Lysmatidae) had thick (~6µm) chitinous layers or densely-packed high refractive index spheres (300-400nm diameter), which models show increase reflectance (400-700nm). Two facultative and non-cleaning species had no visible antennae ultrastructure beyond chitinous exoskeleton. Antennae reflectance was significantly higher in obligate cleaners than facultative and non-cleaning species. Our results suggest that some obligate cleaners may have evolved ultrastructures that increase the conspicuousness of the antennae as signals.

Introduction

Organisms use visual signals for many functions, including attracting mates, warding off predators, and communicating with mutualistic partners (e.g., [1–3]). Often, visual signals are colourful and created using pigments (e.g. carotenoid-based signals found in various taxa [4,5]) or nanostructures, such as those mediating the iridescent colours of butterflies and birds [6,7]. Other signals are achromatic (e.g. white or...
black), and much of the work on achromatic signals focuses on the role of melanin pigments and ultrastructure in dark brown or black colouration (e.g., [8,9]). White as a signal colour, however, is also found across taxa, although less attention has been given to its structural basis (but see, e.g., [10–12]). Revealing the structural basis of white signals in animals can help us understand how selection has shaped this widespread signal type.

Cleaner shrimp are exclusively marine decapod crustaceans that remove and eat ectoparasites from dozens of species of coral reef fish clients (reviewed in [13]). Several species of cleaner shrimp from different families use white body parts to signal their intent to clean [14–17]. For example, Ancylomenes pedersoni (Palaemonidae) initiates cleaning interactions by whipping its white antennae [14], and several members of the genus Lysmata (Lysmatidae) advertise cleaning services by both rocking their white legs and waving their white antennae [15,17] (Figure 1, left). White signals can be particularly effective when signalling to multiple species, as cleaner shrimp do, because they should appear conspicuous in most aquatic settings regardless of the receiver’s spectral sensitivity (colour vision capability) [18]. Furthermore, white signals, unlike coloured signals, should be effective across depths, water types, and most viewing angles, since they create a reliably high achromatic contrast with most backgrounds. One challenge cleaner shrimp face, however, is that their antennae are only ~150µm in diameter, potentially limiting their detectability. One way to offset the limitation of these narrow diameters could be to evolve nanostructures that maximize reflectance from the antennae, similar to the pterin nanoparticles found on the wings of white pierid butterflies [10].

Here, we tested the hypothesis that cleaners are under selection to increase the visibility of their signals in a way that is effective for a range of visual systems across diverse visual environments, as is consistent with cleaners engaging with a large diversity of client fish types, across a range of depths and against a range of natural backgrounds. We use Scanning Electron Microscopy (SEM), optical modelling, and measurements of the reflectance of cleaner shrimp antennae to explore antennae ultrastructure and its effects on reflectance, in three obligate cleaner species in the families Palaemonidae (Ancylomenes pedersoni) and Lysmatidae (Lysmata amboinensis, L. debelius) [19]. For comparison, we conducted the same analyses in a species of facultative, or occasional, cleaner (Palaemonidae: Periclimenes yucatanicus; [20]) and one non-cleaner (Lysmatidae: L. boggessi [21]) (Figure 1).
Methods

Specimens

Wild-caught specimens of the Caribbean *Ancylenes pedersoni* (n=2), *Periclimenes yucatanicus* (n=3), and *Lysmata boggessi* (n=3) were purchased from Dynasty Marine (Marathon, FL, USA), and wild-caught specimens of the Indo-Pacific *Lysmata amboinensis* (n=3) and *Lysmata debelius* (n=3), sourced from Indonesia, were purchased from LiveAquaria (Rhineland, WI, USA). Shrimp were shipped within one to two days of being sourced from the wild, and within six hours of arrival in the lab, were humanely euthanized by freezing, the antennae were removed, and the bodies were processed for DNA preservation for use in other projects.

Scanning Electron Microscopy

To identify underlying structural features that may enhance antennae reflectance we used SEM. Two 3mm long sections of antenna per animal were excised from two specimens per species and fixed for 12 hours in 2.5% glutaraldehyde buffered with artificial seawater. After fixation, each sample went through a dehydration series of 30%, 30%, 50%, 50%, 70%, 70%, 90%, 90%, 100%, 100%, 100% EtOH (15 minutes/step). Samples were then dried using a LADD CPD3 critical point dryer (Ladd Research Industries, Williston, VT, USA) to preserve tissue ultrastructure. Once dried, samples were freeze-fractured to expose the cross-section, mounted on aluminium SEM stubs with copper tape, and sputter-coated with an ~8nm thick layer of gold (Denton Desk V; Denton Vacuum LLC, Moorestown, NJ, USA). The samples were imaged using an Apreo S scanning electron microscope (ThermoFisher Scientific, Waltham, MA, USA) at the Duke University Shared Materials and Instrumentation Facility with an acceleration voltage of 1kV and magnifications of 2500x–15000x. We used Fiji [25] for morphometric analyses of the sphere layer found using SEM.

Optical Modelling

In two of the three obligate cleaner species, SEM images showed a layer of spherical nanoparticles inside the cuticle layer. To investigate how the morphology and optical properties of these particles impact antennae reflectance, we performed finite-difference time-domain simulations (FDTD) using the Lumerical solver version 2020b (Ansys, Canonsburg, PA, USA). Random close-packed aggregations of spheres mimicking the arrangement observed in the antennae were generated using the Uniform Random Particle Distribution (URPD) structure in Lumerical.
We performed three sets of simulations to determine the effects of sphere refractive index, sphere layer thickness, and sphere layer diameter on reflectance. First, to determine the effect of sphere refractive index on reflectance, we simulated a 2µm thick layer of non-absorbing spheres underneath a 5µm layer of homogenous chitin, varying sphere refractive index from 1.57 (approximately that of chitin) to 2.00 (a lower estimate of that of the pteridine granules found in white Pierid butterflies [11]) in increments of 0.01. Notably, this range encompassed the refractive index of isoxanthopterin (1.78), which prior work has identified as the molecular basis of the spheres underlying white body colouration in *Lysmata amboinensis* (one of the obligate cleaners studied here) [12]. Second, we simulated layers of spheres (randomly assigned diameters between 300-400nm, based on what was found in *A. pedersoni* and *L. amboinensis*) between 1µm and 10µm thick in 300nm thickness intervals to determine how reflectance changes with layer thickness. Third, we simulated the effects of sphere diameter on the reflectance of a 2µm thick layer of spheres (again based on what we observed in *A. pedersoni* and *L. amboinensis*) ranging between 100nm and 1000nm in diameter, in 30nm intervals; within each simulation, all spheres had the same diameter (to within 5nm of one another). For investigating the effects of both layer thickness and sphere diameter, we simulated three different sphere refractive indices, 1.57 (chitin), 1.78 (isoxanthopterin), and 2.00 (pteridine), with a background matrix equal to the refractive index of seawater (1.34).

All simulations were performed in a 4µm x 4µm x 12µm domain with periodic boundary conditions in the x and y directions, broadband (400nm – 700nm) plane wave source propagating in the z direction, and perfectly-matched layer boundary conditions in the z direction (a computational representation of open boundaries that does not reflect any light at the edges of the domain). Although we simulated one sphere layer, antennae are effectively one layer wrapped into a cylinder, meaning that most incident light upon the antennae passes through two layers. Therefore, we report reflectance as: \( R + RT^2 \), where R is the reflectance of one layer and T is the transmittance, as this equation accounts for reflectance of the front layer, plus reflectance of the back layer after having passed through the front layer twice.

**Reflectance Measurements**

Based on the results of the SEM and optical modelling (above), we made predictions about relative antennal reflectance in each of our focal species. To quantify reflectance and test these predictions, we used calibrated photography under a stereomicroscope to measure reflectance from the antennae following methods in [22]. Calibrated photography was used instead of spectroradiometry because cleaner shrimp antennae are cylindrical rather than flat, causing light to scatter in directions that
are not captured by the fibre optic sensor, likely underestimating reflectance. In brief, photographs of antennae were taken with an iPhone (model 13, Apple Inc., Cupertino, CA) and included a reflectance standard comprising five grey paint swatches of measured reflectance. We measured the 0-255 value from each swatch of the grey standard (using the image analysis software Fiji [23]), using only the green channel of the sRGB image. The green channel was used both to approximate as closely as we are able brightness vision in particular, and because of the three colour channels, it is most closely aligned with the ambient illuminant on a coral reef [24]. We then plotted the green values of the grey standards against their known reflectance values, and fit exponential equations to these values to create a calibration equation for each photograph. Then, in Fiji, we measured green-channel values from four to ten regions on each antenna; the number of regions sampled did not affect our results, as randomly sampling only 4 regions from each specimen and re-running our analyses did not change our conclusions in any instance. We then converted these antennae values to reflectance using the calibration equations generated from the grey standards. We averaged reflectance values across individuals to yield a single measure of antennal reflectance for each specimen, and averaged across specimens to yield average values for each species, for use in analysis.

To determine if cleaner “status” predicts reflectance, we used two approaches. First, we classified species as either an “obligate” or “non-obligate” (which included both the facultative P. yucatanicus and the non-cleaner L. boggessi) cleaner and used an unpaired two-samples Wilcoxon test to determine if mean reflectance differed significantly between these two groups. Second, we built a linear model using the \textit{lm} function in R [25] in which reflectance was the response variable and cleaner status (“obligate,” “facultative,” or “non-cleaner”) was a fixed effect. We assessed the significance of the fixed effect by using an Anova to compare the fit of a full model, which included the fixed effect, to that of a model without the fixed effect using the ‘afex’ package [26].

We then investigated whether, among shrimps with white antennae, the presence of ultrastructures is associated with significantly higher antennal reflectance, using the same two methods as above. First, we compared antennal reflectance in the three obligate cleaner species with the facultative P. yucatanicus using an unpaired two-samples Wilcoxon test, and second, we used the same model as above but with cleaners labelled as either “ultrastructures present” (A. pedersoni, L. amboinensis, and L. debelius) or “ultrastructures absent” (P. yucatanicus). All analyses were performed in R version 4.0.3 [25].
Results and Discussion

Obligate Cleaners Have Visible Antennae Ultrastructure

The facultative cleaner *P. yucatanicus* and the non-cleaner *L. boggessi* had no visible structures in their antennae that increase reflectance beyond a homogenous chitinous exoskeleton. However, antennae in all three obligate cleaners contained visible structure. The antennae of both *A. pedersoni* and *L. amboinensis* contained a layer of spheres (Figure 1). Spheres were slightly larger in *A. pedersoni* (diameter of $395 \pm 40\text{nm}$, mean $\pm$ sd, $n=207$ spheres from two specimens) than *L. amboinensis* ($363 \pm 42\text{nm}$, $n=212$ spheres from two specimens; Figure S1). It was difficult to measure the exact thicknesses of the sphere layers due to distortions from freeze fracturing, but based on measurements in Fiji they appeared to be between 1µm and 3µm in both species. Antennae in the obligate cleaner *L. debelius* had extremely regular layers of chitin stacked tightly together in a structure approximately 6 µm thick.

We first used FDTD modelling to assess the impact of the sphere layers found in *A. pedersoni* and *L. amboinensis* on reflectance and whether the sphere layers are optimal to increase reflectance. We found a strong, positive, linear effect of refractive index on reflectance, with the reflectance from a 2µm layer increasing from 8.7% at a refractive index of 1.57 to 57% at a refractive index of 2.0 (Figure 2a). Our control simulation, a 5µm chitin layer with no sphere layer had a reflectance of 1.9%. Even the addition of relatively low refractive index spheres ($n=1.57$) to this chitin layer increases reflectance $\approx 4.6x$ compared to the chitin layer alone (1.9% versus 8.7%). With a refractive index of 1.78, spheres comprised of isoxanthopterin increased reflectance over 19-fold (1.9% reflectance for a 5µm chitin layer versus 36.9% reflectance for a chitin layer backed by a 2µm layer of isoxanthopterin spheres).

The second parameter we varied in our simulations was sphere layer thickness. We performed 30 simulations of layers 1µm-10µm thick—with observed layer thickness in cleaner shrimps lying at the lower end of this range, at approximately 2-3µm thick—and found that increasing the layer thickness caused reflectance to increase (Figure 2b). There was a strong linear relationship between thickness and reflectance for thin layers, with a decreasing slope as thickness approached 10µm. The shape of the relationship between layer thickness and reflectance was refractive index dependent. The lower the refractive index, the thicker a layer must be to reach its asymptotic reflectance.

Third, we examined the effect of sphere diameter. Our modelling showed a sharp increase in reflectance at two of the refractive indices used as sphere diameter increased from 100nm to 400nm (Figure 2d), and for all three refractive indices a plateau in reflectance, once sphere diameters reached 400nm. For refractive indices of $n=1.78$ and $n=1.57$, reflectance plateaued at 38% and 10%, respectively,
and for \( n = 2.00 \) at 60%. Thus, our simulations indicate that the size of spheres found in the antennae of \( L. \) amboinensis and \( A. \) pedersoni (Figure 2d, grey shaded area) maximize reflectance.

Notably, the cleaner \( L. \) debelius also had high reflectance but rather than a layer of spherical nanoparticles, had highly regular stacks of chitin layers (Figure 1) in addition to the ordered layering of the chitinous exoskeleton (such as that visible in \( L. \) boggessi). Each layer was approximately 0.27 ± 0.06 \( \mu \text{m} \) (mean ± sd, based on measurements of \( n=10 \) layers) thick, stacked many layers high, for a total layer thickness of ~6\( \mu \text{m} \). Although it is difficult to precisely model the optical implications of this structure based on the SEM images, stacks of sub-micron high index layers are typically associated with high reflectance over at least some portion of the visible spectrum (e.g., [27]).

**Antennae Reflectance is Higher in Obligate Cleaners**

Based on the simulations described above, we predicted that the cleaners \( A. \) pedersoni, \( L. \) amboinensis, and \( L. \) debelius would have higher reflectance than both the non-obligate cleaner \( P. \) yucatanicus and the non-cleaner \( L. \) boggessi. Reflectance measurements using calibrated color photography upheld these predictions. Mean reflectance in the green channel was higher in the three species of obligate cleaner (mean: \( A. \) pedersoni 43.8% and 38.9%, \( n=2 \); mean ± standard deviation: \( L. \) amboinensis 52.1 ± 4.0%, \( n=3 \); \( L. \) debelius 48.1 ±0.40%, \( n=3 \)) than either the facultative cleaner \( P. \) yucatanicus (34.7 ± 1.6%, \( n=3 \)) or the non-cleaner \( L. \) boggessi (21.8 ± 2.3 %, \( n=3 \)) (Figure 2d). Grouping the three obligate cleaners together and the two non-obligate cleaners together showed that reflectance was significantly higher in the obligate cleaners than the non-obligate cleaners (unpaired two-samples Wilcoxon test, \( p=0.004 \)), and a modelling approach showed that cleaner “status” (“obligate,” “facultative,” or “non-cleaner”) is a significant predictor of antennae reflectance (\( p < 0.0001 \)).

We also examined whether, among species with white antennae (\( L. \) debelius, \( L. \) amboinensis, \( A. \) pedersoni and \( P. \) yucatanicus), the presence of ultrastructures is associated with significantly higher reflectance. Comparing reflectance in the species with ultrastructure (\( L. \) debelius, \( L. \) amboinensis, and \( A. \) pedersoni) to the species without (\( P. \) yucatanicus) showed that reflectance was significantly higher in the species with ultrastructures than the species without (unpaired two-samples Wilcoxon test, \( p=0.03 \)). Similarly, a model in which was reflectance was predicted by the presence or absence of ultrastructures in species with white antennae showed that the presence of ultrastructures is a significant predictor of reflectance (\( p=0.002 \)).
Although the reflectance measured here was lower than other bright white colour patches in animals such as *Pieris rapae* wings (60-80%; [10]) or the white stripes of *Sepia officinalis* (60-70%; [28]), reflectance measured here is likely an underestimate due to the cylindrical nature and small diameter of the tissue. Additionally, our calibrated colour photography setup did not allow us to measure reflectance in the ultraviolet, but this would be a particularly interesting future direction, given that ultraviolet-rich blues have been shown to be an important aspect of the “guild coloration” of cleaner fish [3].

**Conclusions**

Our results show that three species of obligate cleaner shrimp that use their white antennae to signal to client fish, *A. pedersoni*, *L. amboinensis*, and *L. debelius*, have structures that increase antennae reflectance. *L. debelius* has a thick chitinous stack of high index layers, in addition to the chitinous exoskeleton also seen in the non-cleaners, that likely serves to increase reflectance, whereas *A. pedersoni* and *L. amboinensis*, despite being in different families, each have evolved 1-3µm thick layers of spheres inside their antennae. Modelling suggests that these spheres are of a diameter that maximizes reflectance, and that this sphere layer increases reflectance by 4.6x to 19x compared to a 5µm chitin layer with no spheres underneath. We assume here that these spheres are made of isoxanthopterin, a material found in reflective layers in other decapod crustaceans, including isoxanthopterin granules forming the bright white stripe down the body of *Lysmata amboinensis* [12] (a species also examined in this study), retinal reflectors of *Cherax quadricarinatus* [29], *Homarus americanus* [30], *Litopenaeus vannamei* [31], *Machrobrachium rosenbergii* [29], *Penaeus setiferus* [32], and reflectors in the photophores of the deep-sea shrimp *Oplophorus spinosus* and *Systellaspis debilis* [33]. Thus, cleaner shrimp may be using a widespread structural toolkit in a novel way to increase antennal reflectance.

By contrast, both the facultative cleaner *Periclimenes yucatanicus* and the non-cleaner *Lysmata boggessi* appeared to have no structures in their antennae that increase reflectance. Interestingly, *P. yucatanicus* still has antennae that appear white, which likely occurs because their antennae have a large number of refractive index interfaces—both at the surface and internally—combined with low absorption, which scatters light across wavelengths and appears white. However, random close-packed spheres of a certain size scatter light over a broad wavelength and angle range very efficiently, in line with our finding that even though *P. yucatanicus* antennae appear white, they do not reflect as much light as the antennae of obligate cleaners that contain specialized nanostructures. Behaviourally, *P. yucatanicus* have been observed signalling to clients with their white antennae, but compared to the related and sympatric *A.*
pedersoni, *P. yucatanicus* are visited by only 1/5th as many clients and they clean only 6% of clients that visit [20], so the ectoparasites consumed during cleaning are likely not a substantial portion of this species’ diet. Thus, the lack of structures that increase reflectance may imply that selection on the signal function of the antennae has not been sufficient to favour the added conspicuousness that spheres or chitin layers can confer on top of chitin alone.

Signal theory predicts that if signals communicate similar messages to receivers with similar perceptual capabilities, they should be under selection to be similar across species (e.g., convergent floral syndromes [34]). In a system, such as cleaners and clients, where the messages being communicated are advertisements, the particular signal form on which species converge is expected to be one that is particularly conspicuous to the intended receiver (e.g., [35–37]). Cleaner shrimp face a particular challenge in being conspicuous to their intended receiver, because dozens of species of fish with diverse visual capabilities (see, e.g. [38,39]) can all serve as clients, and the visual environment can be highly heterogeneous, varying in water type and depth (which affects light level and spectral composition) and visual background. White (i.e. spectrally neutral) signals are particularly useful compared to colourful signals in these situations because their apparent colour is less affected by ambient light level and spectral composition, the visual system of the viewer, and the visual background, especially when colour constancy is accounted for. Intriguingly, convergence can occur even for non-sympatric species like those studied here (*A. pedersoni, L. amboinensis*, and *L. debelius* do not overlap in range). These three species all presumably need to send similar messages, and all three likely experience similar pressures on signal form from receiver and environment given that all service reef fish and live in the similar optical environments of coral reefs. Thus, convergence in this system may be at a global level, similar to the convergence in signals that advertise cleaner status that has occurred between cleaner wrasses (Labridae) and cleaner gobies (Gobiidae) across the Caribbean and Indo-Pacific [3,40]. The data presented here are consistent with the hypothesis that cleaner shrimp are under selection to increase the visibility of their signals in a way that is effective for a range of visual systems across diverse visual environments.

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Ethics Statement

No formal ethical approvals were required for this work, as Decapod crustaceans are not currently covered under Institutional Animal Care and Use Committees in the United States.

Data Availability

All data and codes for this paper have been made available on Dryad (DOI: 10.5061/dryad.w3r22810d)

References


Figures
Fig. 1. Animal appearance (left) and antennae ultrastructure (middle and right) in cleaner shrimp (*), a facultative cleaner (#), and a related non-cleaner. (Middle) SEM images of antennae show a sphere layer in *A. pedersoni* and *L. amboinensis*; densely-packed chitin layers in *L. debelius*; and chitin exoskeleton only in *P. yucatanicus* and *L. boggessi*; Scale 5µm. (Right) Higher magnification image of structures that confer increased reflectance. Scale 1µm. Photo credits (lefthand column): Eleanor Caves, Frank Gradyan, Haplochromis (CC BY-SA 3.0), and Chprieur (CC BY-SA 4.0).
Fig. 2. Antennae reflectance and FDTD modelling of sphere layers. (A) Simulated reflectance across the visible range (400-700 nm) from a 2µm layer of spheres underneath a 5µm chitin layer as refractive index is varied. FDTD simulations of (B) layers of varying thicknesses and (C) layers 2µm thick but with varying sphere diameters, for refractive indices of chitin (1.57; solid line), isoxanthopterin (1.78; dotted line) and pteridines (2.00; dashed line). (D) Mean
green-channel reflectance of antennae using calibrated colour photography in all five focal species [dots are individuals; antennae are white in *L. amboinensis* (n=3), *L. debelius* (n=3), *A. pedersoni* (n=2), and *P. yucatanicus* (n=3) and red in *L. boggessi* (n=3)]. In (B) and (C) grey shaded regions represent ranges of layer thickness and sphere diameters, respectively, observed in the obligate cleaner shrimp *A. pedersoni* and *L. amboinensis*. 
Fig. S1. Nanosphere diameter. Measurements of the nanospheres found in the antennae in both Ancylomenes pedersoni and Lysmata amboinensis show that both species generally have nanospheres between 300-450nm. The distribution of sizes, however, skews larger in A. pedersoni, but given that measurements came from only 2 individuals per species, we lack power for statistical tests of sphere difference between species.