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RESEARCH ARTICLE

Transparent anemone shrimp (*Ancylomenes pedersoni*) become opaque after exercise and physiological stress in correlation with increased hemolymph perfusion

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ABSTRACT

Whole-body transparency, an effective camouflage strategy in many aquatic species, can be disrupted by environmental and/or physiological stressors. We found that tail-flip escape responses temporarily disrupt the transparency of the anemone shrimp *Ancylomenes pedersoni*. After as few as three tail flips, the previously transparent abdominal muscle became cloudy. Eliciting additional tail flips to the point of exhaustion (16±1 s.e.m.; *n*=23) resulted in complete opacity, though the original transparency returned after 20–60 min of inactivity. We hypothesized that an exercise-induced increase in blood volume between muscle fibers creates regions of low refractive index fluid between high refractive index muscles, thereby increasing light scattering. We documented pre- and post-contraction perfusion by injecting Alexa Fluor 594 wheat germ agglutinin that labeled sarcolemmal surfaces and endothelial cells in contact with hemolymph and found more hemolymph perfused through the abdominal tissue post-exercise, presumably owing to more capillaries opening. In addition, we altered salinity (to 55‰ and 8‰), perforated the abdomen and injected a vasodilator. All three treatments increased both perfusion and opacity, lending further support to our hypothesis that increased hemolymph perfusion to the abdomen is one mechanism that can disrupt a shrimp's transparency. The fact that transparent shrimp at rest have little to no evidence of perfusion to their abdominal musculature (unlike the opaque shrimp *Lysmata pederseni*, which had more perfusion even at rest) indicates that they may experience significant physiological trade-offs in order to maintain their transparency; specifically, limiting blood flow and thereby reducing oxygen delivery may result in reduced performance.

KEY WORDS: Camouflage, Crustacean, Muscle, Light scattering

INTRODUCTION

Cryptic organisms employ various strategies to avoid being detected by predators, including matching the background (Endler, 1978). Background matching is often mediated by pigmentation patterns (e.g. melanism in the peppered moth; reviewed in Ruxton et al., 2004) that allow prey to blend in with their surroundings. However, certain color patterns may match the background of only one specific microhabitat, thus restricting pigmented prey from exploiting larger portions of their environment (Johnsen and Sosik, 2003). Transparency is a cryptic strategy that solves these problems by allowing organisms to match any background. However, unlike colored camouflage, that only involves the body surface, transparency must be maintained throughout the volume of an animal (reviewed in Johnsen, 2001). To maintain transparency, animals may need to limit exercise or other forms of physiological stress, as these may alter the internal ultrastructure and thus increase light scattering.

In any substance, variation in refractive index on size scales greater than a half wavelength of light can lead to observable light scattering and thus increase opacity (Benedek, 1971). For example, snow, while non-absorbing, is opaque because of its complex structure that strongly scatters light. In the case of muscle, any variations in tissue density and composition that can occur during periods of physiological stress may lead to an increase in opacity.

Whole-body transparency is not an unchangeable or static physical state. The siphonophore *Hippopodius hippopus* becomes opaque when touched because proteins are thought to precipitate in its mesoglea and increase light scattering (Mackie and Mackie, 1967). Opacity also increases in other transparent animals as a result of physiological or environmental stressors. Clear ghost shrimp (*Palaemonetes pugio*) lose their transparency because of changes in temperature and salinity that occur over the tidal cycle (Bhandiwad and Johnsen, 2011). For example, *P. pugio* transmits approximately 50% of incident light through its abdomen at salinities of 0 to 20 ppt at 20°C, but nearly 0% at salinities of 30 ppt at 30°C. Surviving shrimp returned to their pre-stress levels of transparency within 24 h of being returned to the control conditions (15 ppt, 20°C) (Bhandiwad and Johnsen, 2011).

Because light scatters when it encounters a medium of a different refractive index, Bhandiwad and Johnsen (2011) hypothesized that the loss in transparency was related to the pooling of hemolymph in the intramuscular space, thus creating regions of low-index fluid between the regions of high-index muscle. An approximate calculation demonstrates how even a seemingly small amount of reflection at one interface can add up to a significant reduction in the amount of light that can be transmitted (Fig. 1) (Bhandiwad and Johnsen, 2011). Using Fresnel's equations, and assuming perpendicular incidence of the light and refractive indices of ~1.5 for muscle and ~1.35 for hemolymph, 0.3% of light is reflected at each interface [$=(1.5–1.35)^2/(1.5+1.35)^2$]. Over the approximately 2 mm thickness of the shrimp tissue there can be hundreds of such interfaces, substantially reducing the amount of transmitted light (Fig. 1C). This admittedly simplified analysis provides a conservative estimate of light scattering as the interfaces are not always perpendicular to the incident light and thus would scatter even more light. It demonstrates, however, that even small amounts of hemolymph surrounding hundreds of individual fibers can opacify an animal (Fig. 1).

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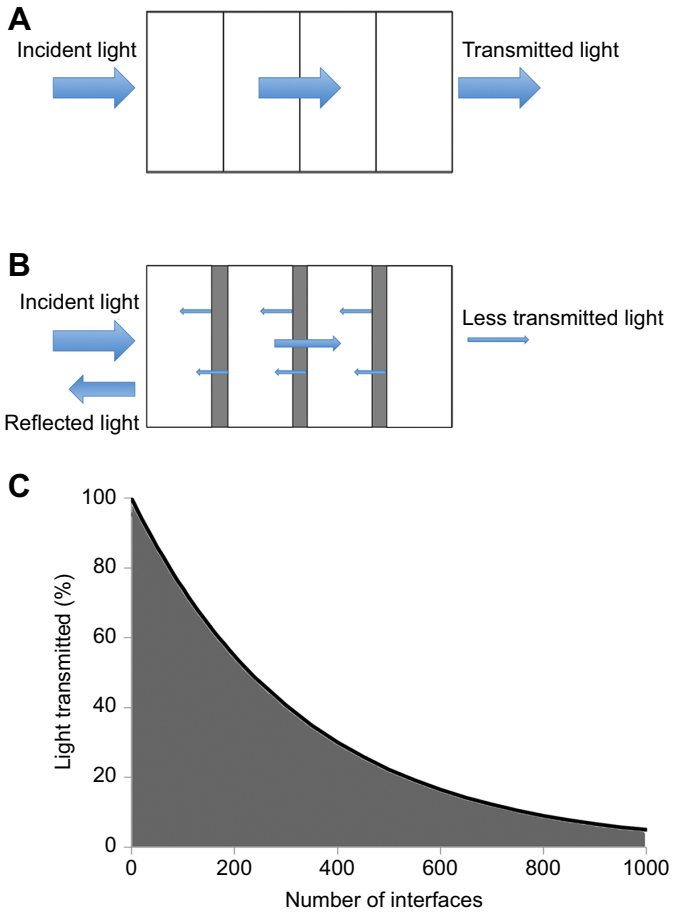

Fig. 1. Increasing interfaces increase light scattering and thus reduce light transmission. (A) A schematic demonstrating that when a material (muscle) has no gaps or fluid-filled interfaces large enough to reflect light, light transmission is high. (B) A schematic demonstrating that light reflects at the surfaces of the fluid-filled interfaces, resulting in less light being transmitted through the entire tissue. (C) Using the Fresnel equations for reflection assuming perpendicular incidence of light and refractive indices of 1.5 for muscle and 1.35 for hemolymph, we calculate that 0.3% of light is reflected at every interface. There are a large number of these interfaces throughout the width of the muscle, and the total amount of light transmitted is 0.997 to the power of the number of interfaces, indicated by the black line. The shaded area below the black line indicates the fact that less light would be transmitted than is predicted by this simple reflection model.

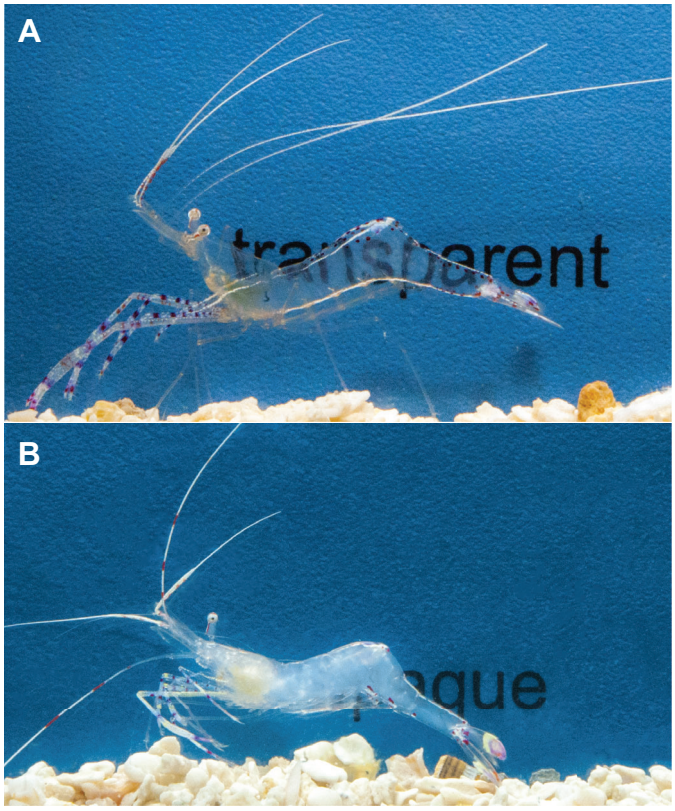

Fig. 2. Changes in transparency after exercise. (A) At rest, under normal conditions, *Ancylomenes pedersoni* was transparent enough to read text through its body. (B) After an average of 16 tail flips, *A. pedersoni* became opaque.

After observing that several species of transparent shrimp (*Ancylomenes pedersoni*, *Periclimenes yucatanicus* and *Periclimenes rathbunae*) become opaque after exercise, we devised a set of four experiments that allowed us to test directly and expand upon the Bhandiwad and Johnsen (2011) hypothesis. In our study, we use the transparent shrimp *Ancylomenes pedersoni* (Chase 1958) (Crustacea, Decapoda), which inhabits Caribbean reef anemones (*Bartholomea annulata*, *Condylactis gigantea*). This shrimp is normally so transparent that when placed on a text background, one can read through its abdomen (Fig. 2A). When threatened, *A. pedersoni* performs a tail-flipping escape response that propels it backwards multiple times. We noticed, though, that multiple escape responses resulted in both exhaustion and a significant decrease in transparency (Fig. 2B). Although it is clear that maintaining transparency is critically important for camouflage (e.g. Zaret, 1972; Zaret and Kerfoot, 1975; Tsuda et al., 1998; Utne-Palm, 1999), less is known about whether and how transparency can be disrupted. Here, we use several experimental methods to test whether changes in perfusion owing to exercise, as well as three other physiological and environmental stressors, can reversibly disrupt transparency in *A. pedersoni*.

MATERIALS AND METHODS

Animals

Individual *A. pedersoni* were collected at 2–20 m depth on coral reefs surrounding Carrie Bow Cay, Belize (16°46′N 88°04′W). Additional specimens were collected in the Florida Keys, USA, by Dynasty Marine Lab (Marathon, FL, USA). Animals were maintained in full-strength, filtered seawater at room temperature (FSW; 35‰ salinity, 21°C) in aerated, recirculating aquaria and were fed fish meal pellets (‘Crab cuisine’, Hikari, Hayward, CA, USA) three times weekly. Animals were not fed in the 24 h prior to any experiments.

Experimental treatments

Exercise

Prior to documenting hemolymph flow to tissues, we first determined how much exercise was necessary to turn the shrimp opaque and how long it took them to recover their transparency. Each shrimp (n=23) was placed in an individual aquarium (water maintained at room temperature), and a stimulus (forceps) was placed in front of the rostrum to generate the tail-flip escape response (this response consistently led to the tail-flip escape behavior until the shrimp was too fatigued to move). The number of tail flips was recorded until the animals were fatigued, defined as no longer making escape responses, swimming movements or attempts to evade the forceps. In this preliminary analysis, shrimp were photographed next to a trans-illuminated transparency standard

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(EIA Halftone Gray Scale, Rochester, NY, USA). For all shrimp, the time to recovery was measured by recording the point at which the shrimp regained their transparency and began to move. At this point, the shrimp were again photographed. We analyzed these photographs before and after treatment, but the all-or-nothing response in opacity (either highly transparent with values greater than 60%, the highest transmission value on the standard, or nearly 100% opaque with light transmission less than or equal to 3%, the lowest transmission value on the standard) led us to simplify the results, which we report as either presence or absence of opacity. This presence or absence of opacity is shown in photographs taken with the shrimp in front of a colored or a black background to best visualize the cloudy-white appearance of the opaque tissue.

In a subsequent experiment using new individuals, the shrimp were divided into either the control group (no exercise, n=5) or the experimental group (exercise/tail flips, n=5). Individual shrimp from both groups were first anesthetized via exposure to 1°C seawater for 10 min. Each shrimp was then injected with 100 µl of Alexa Fluor 594-labeled wheat germ agglutinin (WGA; Molecular Probes) in FSW (1 mg l^{-1} ml) into the pericardial cavity using a 32 gauge needle. WGA is a lectin that binds to the sialic acid and *N*-acetylglucosaminyl residues present on the basement membrane of the muscle fiber sarcolemma and blood vessel endothelium (Wright, 1984). When injected into the pericardial cavity of a shrimp, WGA moves through the circulatory system and tissues, revealing regions that are in contact with hemolymph.

After injection, the shrimp recovered for 15 min at room temperature. The control group was immediately euthanized (without performing a tail-flip response), and the abdominal muscle was immediately fixed in 4% paraformaldehyde in FSW. The experimental exercise group was induced to tail-flip in the same manner as the initial experiment by placing a stimulus (forceps) in front of their rostrum to generate an escape response. After 16 tail flips (all specimens were opaque; duration of 2 min or less), the shrimp was subjected to the same treatment as the control: immediately euthanized, and the abdominal muscle preserved.

Salinity

Similar to the exercise experimental treatment, this experiment and all subsequent experiments involved anesthetizing each shrimp via chilling to 1°C, injection of 125 µl of Alexa Fluor 594-labeled WGA in FSW into the pericardial cavity followed by recovery for 15 min. Shrimp were then divided into three groups: hyposaline (diluted seawater at 8‰; n=3), control (normal seawater at 35‰; n=6) and hypersaline (concentrated seawater at 55‰; n=3). Exposure was for 30 min before being photographed, euthanized and preserved.

Perforation

After the WGA injection and recovery period, we perforated the abdomen of the shrimp in this experimental group (n=3) using forceps (holding the shrimp in place with dental wax so that they could not tail flip) in order to trigger localized perfusion as part of the wound healing/inflammation response. We then photographed them and preserved the punctured segment.

Proctolin injection

Although circulatory regulation in crustaceans is poorly understood, there is evidence that peptidergic hormones have effects on isolated hearts and cardioarterial valves as well as *in vivo* (McGaw et al., 1994a). For instance, infusion of the crustacean peptide hormone proctolin resulted in a 'vasodilation-like' effect of increased cardiac output and a shunting of blood to the sternal artery of *Cancer magister* (McGaw et al., 1994a), thereby increasing blood flow to the abdomen.

Fifteen minutes after injecting WGA, we injected 50 µl of proctolin (1 mmol l^{-1}) into the pericardial cavity in the experimental animals and a saline solution in the control animals. In eight out of 10 proctolin injections, the shrimp did not survive the infusion; therefore, we only present the results from the two shrimp that survived. These were photographed, euthanized and preserved.

Histology

All tissues were fixed for at least 8 h in 4% paraformaldehyde in FSW, rinsed overnight in 25% sucrose in deionized water, and then mounted with Tissue-Tek® O.C.T. Compound (Sakura® Finetek, USA). Frozen sections were cut at 20 to 30 µm with a Leica Cryocut 1800. Sections were rinsed in PBS, incubated for 30 min in 300 nmol l^{-1} DAPI, and then rinsed again for 5 min in PBS before being viewed.

Image and statistical analysis

Imaging was performed with an Olympus FluoView 1000 laser scanning confocal microscope. Sections from the control specimens were imaged first, and thus served to set the threshold levels required for viewing the red WGA channel, which resulted in some image saturation in the subsequent experimental specimens that were viewed under the same conditions. All confocal micrographs were analyzed using the software package FIJI (Schindelin et al., 2012). For each individual shrimp, at least five sections of muscle tissue were examined and 10 micrographs randomly taken (using systematic uniform random sampling; Howard and Reed, 1998) at the same magnification were analyzed. A stereological point-counting method (Howard and Reed, 1998) was used; a point grid was applied to the image, and all points touching a perfused area (red stain=perfused blood vessel or sarcolemmal area) were tallied and divided by the sum of points to determine the fraction (0 to 1) of perfused tissue, which are reported as mean values per individual. Non-parametric Mann–Whitney *U*-tests were performed to test for significant (P<0.05) differences between the control and experimental conditions in each of the four treatments: exercise, salinity, perforation and proctolin injection.

Micro-CT scans

To visualize the shrimps' overall muscle morphology and circulatory system, one control (at rest, transparent) and one experimental (tail flip, opaque) *A. pedersoni* shrimp were scanned at ultra-high resolution using the micro-CT scanner (model: Nikon XTH 225 ST) at the Shared Materials Instrumentation Facility at Duke University. Because previous histological examinations of sectioned tissue from shrimp fixed in 4% paraformaldehyde in phosphate buffer did not differ from that of shrimp fixed in 2.5% glutaraldehyde in phosphate buffer (L.E.B., unpublished data), shrimp from both fixation preparations were utilized. First, the control shrimp was anesthetized via exposure to 1°C seawater for 10 min and then, in its undisturbed, transparent state, fixed in 2.5% glutaraldehyde in phosphate buffer for 24 h before being transferred to a tube containing 10 ml of Lugol's iodine diluted down to 3.4% concentration. The shrimp soaked in this iodine solution for 7 days before being mounted and scanned at a resolution of 6.74 voxels, at 2500 projections with an exposure time of 500 ms, a voltage of 125 Kv, a current of 104 µA and no filter. Frame averaging was set to 4 for this scan. The second shrimp specimen was induced to tail flip 15 times until opaque, was anesthetized via exposure to 1°C seawater for 10 min, and then was fixed in its opaque state in 4%

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phosphate-buffered paraformaldehyde for 24 h before being transferred to a tube containing 10 ml of Lugol's iodine diluted down to 3.4% concentration. The shrimp soaked in this iodine solution for 7 days before being mounted by being placed in a tube of the original 4% paraformaldehyde fixative and scanned in two parts (and later stitched together) at 7.47 voxels at 2200 projections with an exposure time of 500 ms, a voltage of 120 Kv, a current of 114 µA and no filter. Frame averaging was set to 2 for this scan. Stacks of virtual sections produced by the micro-CT were used for 3-D visualization in the software Avizo version 9.1.1 (FEI Visualization Sciences Group) and the volume rendering function was chosen to maximize contrast between muscular tissue and arterial vessels.

RESULTS

We found that all four experimental treatments – exercise, salinity, perforation and proctolin injection – temporarily disrupted transparency (Figs 2B and 3). Additionally, our stereological analysis of confocal microscope images found that all four experimental groups of shrimp showed substantially more hemolymph perfusion in the abdominal musculature than any of the control groups, though only the exercise and salinity treatments had large enough sample sizes to be statistically significant (Fig. 4).

Three-dimensional reconstructions from CT scans of the shrimp show the abdominal musculature and larger vessels; we use them here as a model to describe the shrimp's general anatomy. Transverse sections taken through the abdomen (Fig. 5) demonstrate the tissue plane appearing in all subsequent confocal images.

Exercise experiment

First, we observed that some shrimp became opaque after as few as three tail flips. Eliciting additional tail flips to the point of exhaustion (16±1 s.e.m.; n=23) resulted in complete opacity (Fig. 2B) for all shrimp, with original transparency and mobility recovering after 20–60 min; there was no correlation between number of tail flips and time to recovery.

The control group of non-exercised shrimp remained transparent and micrographs of cross-sections revealed that the injected WGA had not perfused to the abdominal muscle tissue, demonstrated by the lack of staining (Fig. 6A) or staining of the main artery only (0.005±0.004, mean±s.d. fraction of tissue perfused; Fig. 4). The experimental (exercised) group showed an obvious difference (0.55 ±0.06, mean±s.d. fraction of tissue perfused; Figs 4 and 6). After 16 tail flips, the shrimp became opaque and micrographs revealed hemolymph staining of the endothelia and the sarcolemmal boundaries of the abdominal musculature (Fig. 6B). Perfusion was statistically greater in the exercised group than the control, non-exercised group (U=25, P=0.01).

Salinity experiment

Shrimp placed in hyposaline (8‰) and hypersaline (55‰) water became opaque in fewer than 2 min (Fig. 3A), but recovered transparency within 10 min in FSW.

The control shrimp (in 32‰ seawater) remained transparent, and micrographs revealed little to no hemolymph had perfused through the abdominal muscle tissue; only the main artery showed staining (Fig. 7B). Micrographs of hyposaline and hypersaline treatments showed staining around individual muscle fibers (Fig. 7A,C). The fraction of tissue perfused in both the hyposaline and hypersaline treatments had similar means (0.60±0.08 versus 0.59±0.07 mean± s.d., respectively), thus we lumped the salinity treatments together (n=6) for statistical analysis and found that the hemolymph perfusion after exposure to changed salinity was significantly greater than in the control (U=36, P=0.005; Fig. 4).

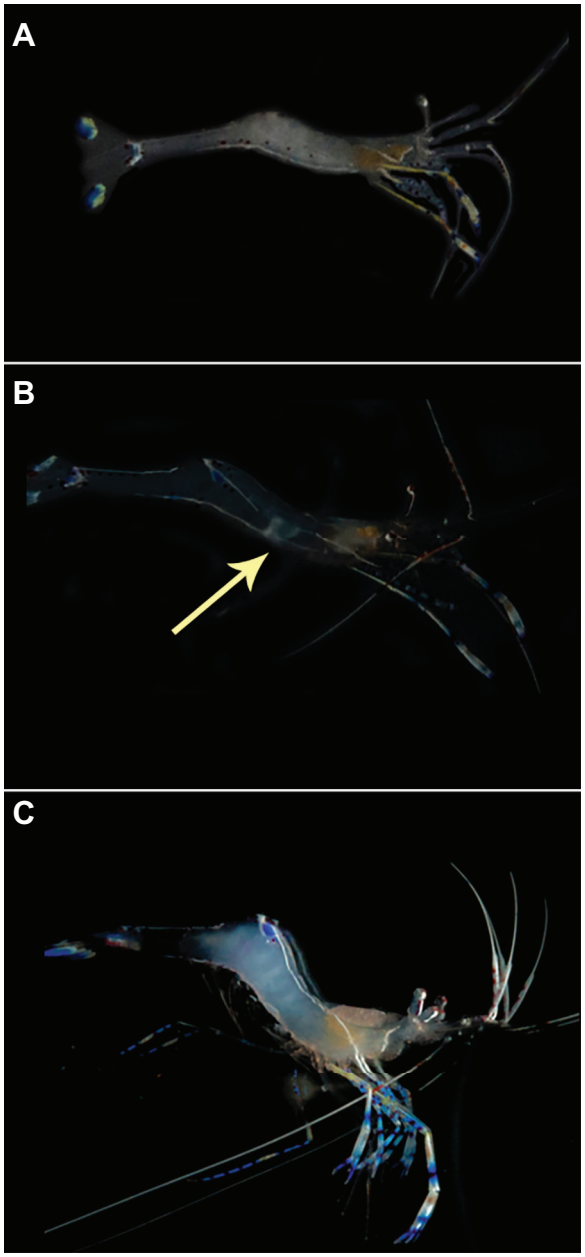

Fig. 3. Physiological stressors result in increased opacity. (A) Salinity changes (both hypertonic and hypotonic) resulted in *A. pedersoni* turning opaque. (B) Inciting a wound-healing response by perforating the abdomen resulted in localized increases in opacity (yellow arrow). (C) Injecting with 50 µl proctolin (1 mmol l^{-1}) resulted in opacity everywhere but the distal tip of the telson. N.B. all shrimp were photographed in front of a black background and any debris in the tanks was cleaned up in image processing but the shrimp themselves remain un-retouched.

Perforation experiment

Incisions in the abdomens of three shrimp resulted in opacity surrounding the puncture site (Fig. 3B). Micrographs of the WGA staining revealed staining of the tissues surrounding the abdominal vessels at the site of the wound (Fig. 7D). The fraction of tissue perfused at the localized site of the punctures (0.41±0.04 mean±

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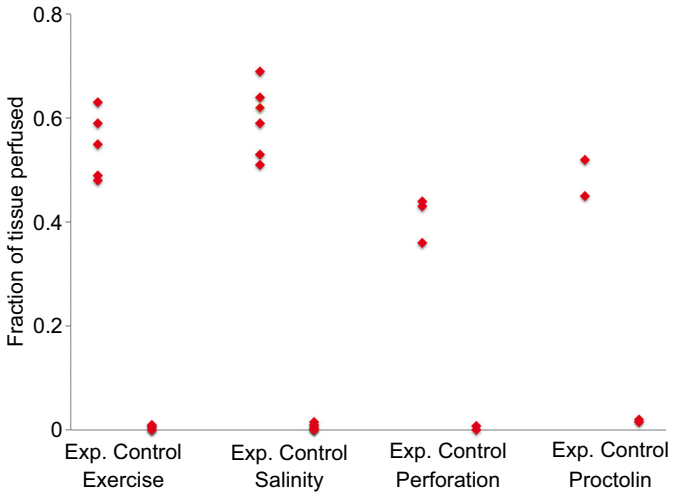

Fig. 4. Experimental shrimp show more perfusion than control shrimp. Each data point represents the mean fraction of perfused abdominal muscle tissue of an individual shrimp calculated using unbiased stereological point counting methods (Howard and Reed, 1998) on >5 muscle sections and >10 micrographs. In all four treatment groups, the amount of perfused tissue in the experimental groups ranged between 36% and 70% compared with between 0% and 2% in the control groups. Mann–Whitney *U*-tests showed statistically significant ($P<0.01$) differences between experimental and control groups for the exercise ($n=5$) and salinity ($n=6$) treatments, but statistical power was lacking for the perforation and proctolin treatments owing to small sample sizes ($n=3$ and $n=2$, respectively).

s.d.) was greater than that of the undisturbed control shrimp (0.003±0.004; Fig. 4), though the sample size of only three individual shrimp meant that the ranked, non-parametric Mann–Whitney *U*-test could not show significance beyond the $P=0.1$ level ($U=9$, $P=0.1$).

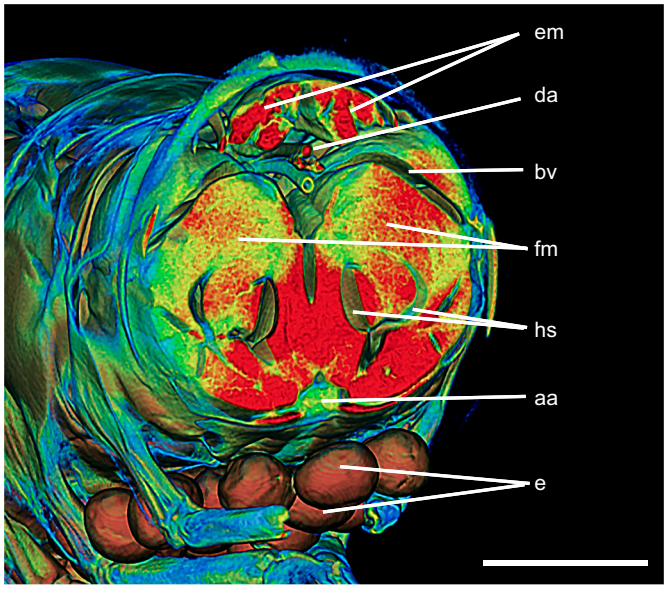

Fig. 5. Three-dimensional reconstruction from a micro-CT scan of *A. pedersoni*, virtually dissected at a transverse plane through the posterior abdomen. This section plane represents the location where all subsequent sections were taken and examined via confocal microscopy. Notable muscular and vascular features are labeled: em, extensor muscle; da, dorsal artery; bv, branching vessels; fm, flexor muscles; hs, hemolymph spaces; aa, abdominal artery; e, eggs. Scale bar, 1 mm.

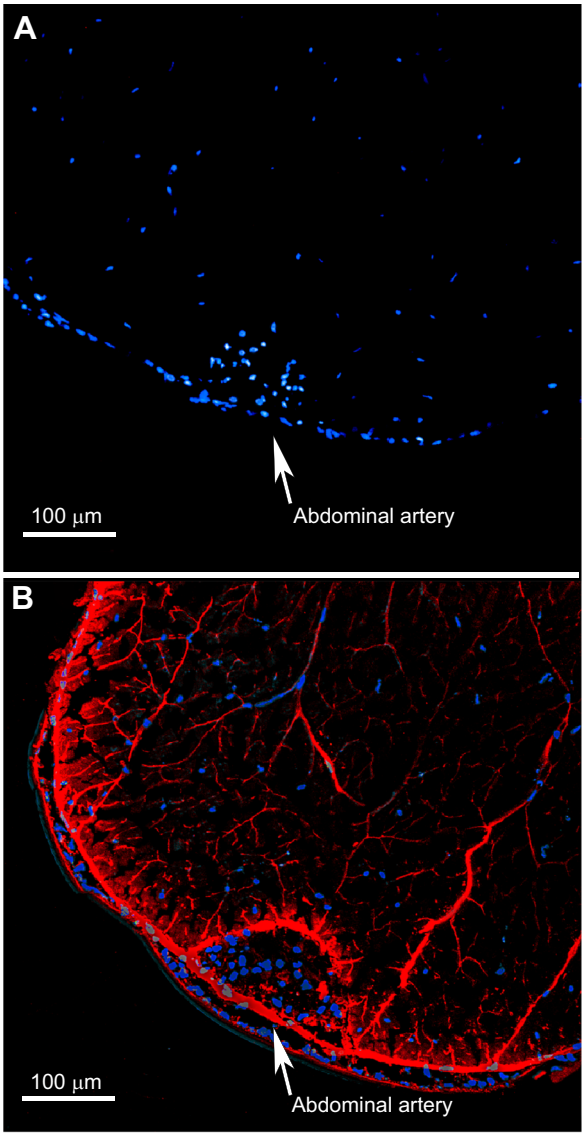

Fig. 6. Exercise experiment. Cross-sections of abdominal muscle from shrimp injected in the pericardial cavity with WGA (red) and tissue sections stained with DAPI (blue). Arrows indicate the abdominal (ventral) artery. (A) The control shrimp (no exercise) remained transparent and showed no evidence of WGA staining, indicating that the WGA had not perfused any vessels or sarcolemmal boundaries in the abdominal muscle. (B) The experimental shrimp (tail-flip/exercise) became opaque and the red staining in the capillaries and around individual fibers indicate that hemolymph perfused these areas. N.B. this original micrograph displayed the nuclei as a teal color, which was later color-replaced with blue to match the other images; however, this does not affect the display of perfusion data.

Proctolin injection experiment

Injection ($n=2$) with proctolin resulted in full-body opacity in one specimen and opacity everywhere but the distal tip of the tail in the second (Fig. 3C). Micrographs from a control (saline-injected) specimen (Fig. 7E) revealed staining confined to the main artery. Micrographs from the proctolin-injected specimens showed staining of additional vessels and around muscle fibers and what appears to be an increase in diameter of the artery (Fig. 7F). Following the same trend as the other treatments (Fig. 4), the fraction of tissue perfused in the proctolin-injected shrimp (0.49±0.04 mean±s.d.) appeared greater than in the saline-injected controls (0.018±0.004),

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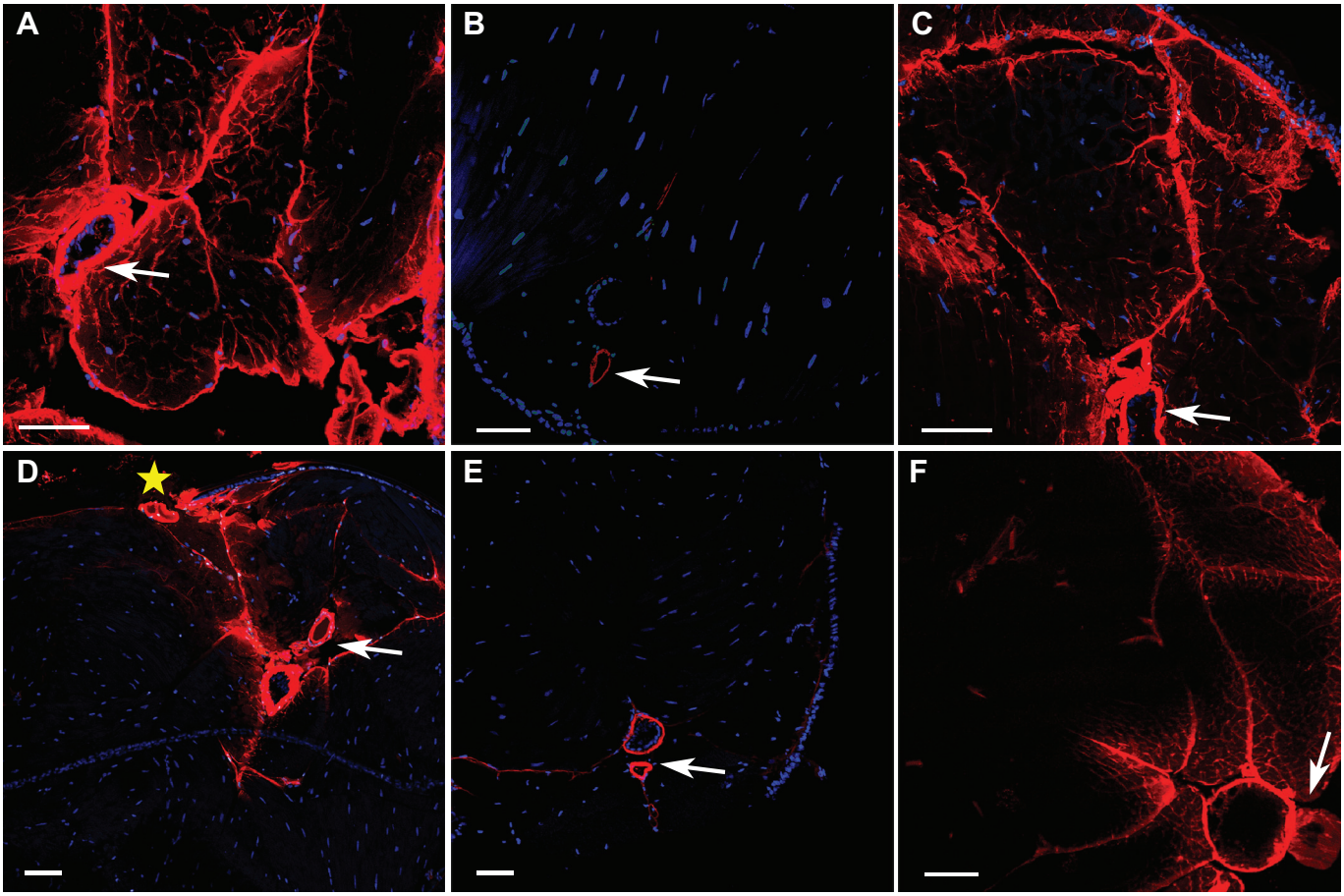

Fig. 7. Additional perfusion experiments. (A–C) Salinity changes; (D) perforation; (E,F) proctolin injection. Transverse sections of abdominal muscle from shrimp that had been injected in the pericardial cavity with wheat germ agglutinin (WGA; red) and then tissues (except for F) stained with DAPI (blue) to help with orientation by visualizing nuclei. White arrows indicate major dorsal artery. (A–C) The experimental shrimp (A: hyposaline 8‰; C: hypersaline 55‰) became opaque, and the red staining in arterioles, capillaries and around individual fibers indicates that hemolymph perfused these areas of abdominal tissue. In contrast, the control shrimp (B: 32‰ normal salinity) remained transparent and showed no evidence of WGA staining except in the main artery. (D) A transverse section of the abdominal muscle from a shrimp that had been perforated in the abdomen to stimulate a wound-healing response (see Fig. 3B). WGA stain indicated that hemolymph perfused through the main artery and through smaller vessels to the site of the wound (yellow star). (E,F) Transverse section of the abdominal muscle of a control (E: saline-injected) shrimp that was also stained with DAPI shows that the main artery was perfused, but there is little perfusion elsewhere. (F) Cross-section of the abdominal muscle of the proctolin-injected shrimp (not stained with DAPI), shows that the main artery as well as branching vessels and the sarcolemmal boundaries of some fibers were perfused.

but with a sample size of only two injected shrimp, the Mann–Whitney *U*-test was not significant (U=4, P=0.3; Fig. 4).

DISCUSSION

The results of these four experimental treatments show that the transparency of *A. pedersoni* can be reversibly disrupted after exposure to physiological or environmental stressors, and that the increase in opacity is associated with a corresponding increase in perfusion of hemolymph to the abdominal musculature. While there may be additional mechanisms that disrupt transparency, the correlation of increased perfusion with increased opacity suggests that increased hemolymph flow (and thus presumably widened interfaces) between muscle fibers increases light scattering.

Scattering, not absorbance

An object or organism is transparent if it neither scatters nor absorbs light. Most biological tissues do not have pigments and do not absorb light, but crustacean hemocyanin has a weak absorbance band centered at 340 nm when de-oxygenated and at 580 nm when oxygenated (Redfield, 1930; Erker et al., 2008). Despite this, even if the concentration of hemocyanin in the hemolymph of *A. pedersoni* is as high as in other crustaceans, the optical path length is too short to affect transparency significantly. Additionally, absorbance makes tissue darker or more strongly colored rather than opaque. Thus, the white opacity we see in Figs 2B and 3 must be a result of light scattering.

Modeling light scattering in the abdominal muscle tissue and circulatory system of a crustacean is fundamentally difficult and computationally expensive owing to the extraordinarily complex relationship between tissue ultrastructure and how it scatters light (Bohren and Huffman, 1998; Johnsen and Widder, 1999). In cases of simple or highly regular structures, scattered light can be calculated by solving Maxwell's equations analytically (e.g. Benedek, 1971; Ameen et al., 1998). However, the abdominal tissues in shrimp (unlike that of a cornea) do not possess the required simplicity. Numerical methods such as the finite element method have been used in calculating light scattering based on the actual ultrastructure for individual cells (e.g. Dunn and Richards-Kortum, 1996; Drezek et al., 2000). However, they are computationally expensive for multicellular tissue. In addition, unless many runs are performed including or

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excluding various components, it is impossible to determine what features are most important for scattering at various angles.

There are several parameters, however, that are known to be important, including the number of interfaces between tissues of different densities (e.g. between muscle fibers and hemolymph; Fig. 1). A shrimp that is approximately 2 to 2.5 mm wide with fiber diameters of approximately 20 µm has roughly 250 interfaces across its body, which would reduce light transmission by at least half (Fig. 1). Again, this is a conservative estimate accounting only for light reflected directly back at the interfaces and not the additional scattering that would occur across all angles of incidence. Vessel diameter may also be an important parameter. For example, for arterioles and capillaries that are at least a few microns in diameter and separated by distances of a similar magnitude or greater, light scattering is linearly correlated with vessel diameter (Bohren and Huffman, 1998). Unfortunately, fixation artifacts and the saturation of the stain in some confocal images make calculating an accurate vessel diameter difficult. For example, it is unclear whether any vessel lumena shrink to less than a wavelength of visible light. The micro-CT scans were not able to resolve details of the smaller arterioles and capillaries, but the data from one control and one experimental shrimp do appear to add further support to our hypothesis that vessels widen and more hemolymph spaces appear in experimental (opaque) shrimp (Fig. 8, Movie 1). Our study does not definitively rule out other mechanisms, such as cellular changes (protein precipitation) or changes to the blood itself that may also increase light scattering.

Taking into account the difficulty of accurately determining light scattering from these complex tissues, our simplified model nevertheless demonstrates that the hemolymph surrounding the individual fibers owing to increased blood flow can create hundreds of interfaces throughout the tissue, resulting in increased opacity.

Implications of disrupted transparency?

Our results show that in all experimental treatments that caused an increase in perfusion to the tail, the tissue turned opaque. Just as humans vasodilate (and turn red) during exercise, shrimp apparently perfuse their muscle tissues during tail-flip escape responses and turn white. Similarly, injecting a 'vasodilator', or puncturing the shrimp, is expected to increase blood flow, though only locally in the latter case. Additionally, sudden changes to the shrimp's environmental conditions (increasing or decreasing salinity and temperature) also causes an increase in oxygen consumption and metabolic rate, resulting in higher cardiac output and stroke volume (Bhandiwad and Johnsen, 2011; DeWachter and McMahon, 1996).

In all cases, the control individuals showed little to no evidence of hemolymph perfusion in the abdominal musculature. The main artery was stained with WGA, indicating presence of the labeled hemolymph, but it appeared that hemolymph had not perfused the smaller vessels in the abdomen even 20 to 30 min after injection. This suggests control of perfusion so that blood is shunted away from the abdomen to the thorax. In fact, other studies of decapod crustaceans have found that hemolymph flow in one or more arteries fluctuates independently of flow through the other arteries, suggesting that there are complex underlying control mechanisms of the circulatory system (McGaw et al., 1994a,b). The differential release of peptidergic (e.g. proctolin) or aminergic hormones could function to mediate these kinds of cardiovascular responses either directly or indirectly via their actions with the central nervous system and could divert flow to the anterior vessels and away from the posterior ventral axis (Airriess and McMahon, 1992).

Transparent animals are typically considered to be more fragile and slower than their pigmented congeners (Childress et al., 1990; Johnsen, 2001), but this may be the first documented example of an animal that is diverting blood from its tail muscles presumably to maintain its camouflage, raising questions about the metabolic rate of transparent shrimp and their ability to recover from aerobic activities. The abdominal musculature of these shrimp consists mainly of fast-twitch, anaerobic muscle fibers and few mitochondria (Jimenez et al., 2008). At rest, the metabolic needs of this abdominal tissue are not believed to be great, and it is possible that diffusion or periodically perfusing the abdomen is all that is required to maintain muscle function. The 'visual-interaction hypothesis' (Childress

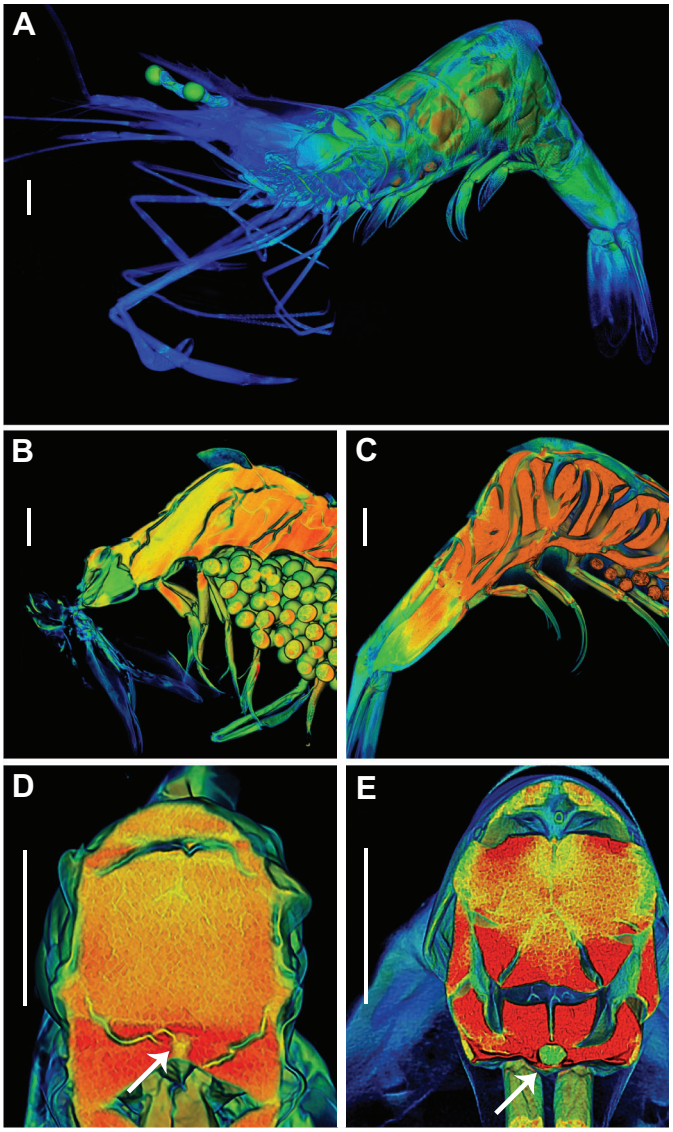

Fig. 8. Three-dimensional reconstructions from micro-CT scans of *A. pedersoni*. (A) Whole shrimp. (B) Sagittal section of the control/transparent shrimp. Volume rendering shows muscle tissue in orange-red. The green-blue lines running through the muscle are major vessels. Compare with (C) sagittal section of the experimental/opaque shrimp. Note the increased hemolymph and vessel spaces as compared with the control. (D) Transverse section of the control/transparent shrimp showing reduced vessel spaces (the green lines), compared with (E) transverse section of the experimental/opaque shrimp showing more obvious vessel spaces and hemolymph sinuses (the blue-green spaces within the red muscle tissue). Arrows point to the abdominal artery. Scale bars, 1 mm. See Movie 1 for animation of a virtual dissection through abdominal muscle of the control/transparent shrimp (left) and experimental/opaque shrimp (right). See Materials and methods for details of fixation and scanning.

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et al., 1990) states that, in the absence of visually mediated predator–prey interactions, the distances over which predators and prey interact are reduced and selective pressure for rapid locomotory capacity is relaxed. This may explain why transparent shrimp have limited locomotory abilities. As seen in our study, these benthic anemone shrimp have retained their ability to rapidly tail-flip away from a predator, but are capable of only a limited number of contractions before fatiguing, and performing this escape behavior sacrifices their transparent camouflage and leaves them vulnerable to visual predation during a perhaps extended recovery period.

Additional research on the costs and benefits of transparency as a camouflage strategy is needed. For example, we do not know how these animals may deal with pulsatile oxygen delivery, which in most tissues would lead to a burst of reactive oxygen species and tissue damage. Limiting abdominal blood flow may affect their ability to respond to environmental challenges such as salinity or temperature variation, or leave them vulnerable to predation when their transparency is disrupted under these kinds of changes in environmental conditions. *Ancylomenes pedersoni* spend much of their lives stationary, waiting for client fish to approach their cleaning station (Huebner and Chadwick, 2012). They do swim toward the client fish to engage in cleaning services, but prolonged tail-flip behaviors that result in opacity have not been reported. Anecdotally, the first author moved an anemone to a new location, which caused the resident shrimp to tail-flip at least 10 times until it turned opaque. At this point, several wrasse chased and one successfully caught and ate the now-visible shrimp (L.E.B., personal observation). As Bhandiwad and Johnsen (2011) demonstrated, the sighting distance for an opaque animal is greatly increased, making it more vulnerable to predation. Thus, one trade-off to transparency may be a prolonged recovery time from exercise where the animal is vulnerable because it is opaque and not able to undergo another escape response. This begs the question of whether pigmented shrimp occupying the same niche are better able to recover more quickly from exercise and respond to changes in environmental conditions more effectively than *A. pedersoni*, and whether similarly sized pigmented shrimp occupying the same ecological habitat also have little to no perfusion to the abdomen at rest. While more robust testing from a diverse phylogenetic group is currently underway, the results we obtained from one species of pigmented shrimp, *Lysmata pederseni*, at rest (n=2) lend further support to the hypothesis that hemolymph perfusion disrupts transparency and normally opaque shrimp with pigmented cuticles do not have reduced perfusion (Fig. 9).

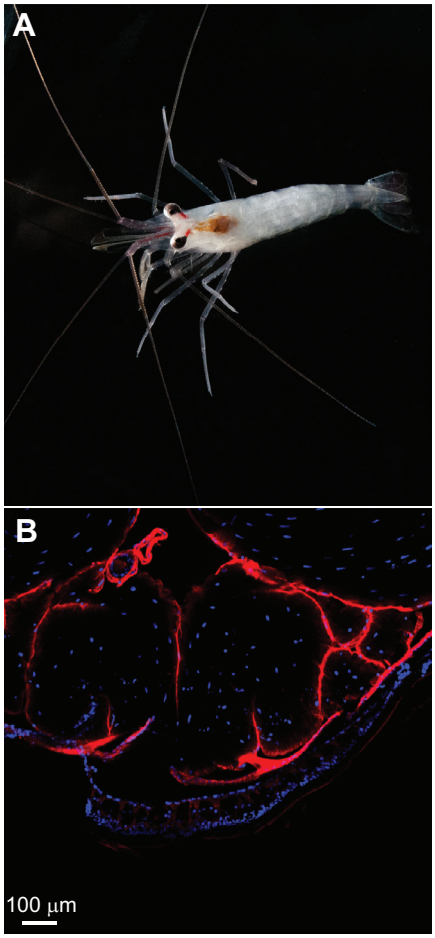

Fig. 9. Opaque shrimp species has different perfusion at rest than transparent shrimp. (A) *Lysmata pederseni* is an opaque shrimp even at rest. This shrimp (no exercise or alteration of the environment) was injected with WGA, and the (B) micrograph shows that even at rest, there is more perfusion to the abdominal muscle than there was in the transparent shrimp species.

Conclusions

Even though we cannot definitively rule out additional mechanisms that may disrupt transparency, the evidence presented here suggests that increased perfusion is one likely mechanism that results in increased light scattering. The fact that transparent shrimp at rest show little to no evidence of perfusion to their abdominal musculature suggests that they may experience significant physiological trade-offs, such as reduced performance and extended recovery from exercise or other stressors.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.E.B.; Methodology: L.E.B., J.G.; Software: J.G.; Formal analysis: L.E.B.; Investigation: L.E.B.; Resources: L.E.B., S.T.K., J.G., S.J.; Writing - original draft: L.E.B.; Writing - review & editing: L.E.B., S.T.K., J.G., S.J.; Visualization: L.E.B.; Supervision: S.T.K., S.J.; Project administration: L.E.B.; Funding acquisition: L.E.B.

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Supplementary information

Supplementary information available online at
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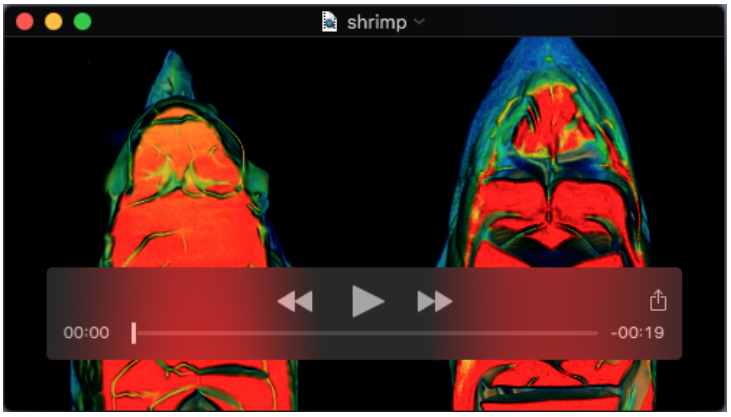

Movie 1. Control shrimp on left, experimental opaque shrimp on right