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Spiny lobsters detect conspecific blood-borne alarm cues exclusively through olfactory sensilla

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SUMMARY

When attacked by predators, diverse animals actively or passively release molecules that evoke alarm and related anti-predatory behavior by nearby conspecifics. The actively released molecules are alarm pheromones, whereas the passively released molecules are alarm cues. For example, many insects have alarm-signaling systems that involve active release of alarm pheromones from specialized glands and detection of these signals using specific sensors. Many crustaceans passively release alarm cues, but the nature of the cues, sensors and responses is poorly characterized. Here we show in laboratory and field experiments that injured Caribbean spiny lobsters, *Panulirus argus*, passively release alarm cues *via* blood (hemolymph) that induce alarm responses in the form of avoidance and suppression of feeding. These cues are detected exclusively through specific olfactory chemosensors, the aesthetasc sensilla. The alarm cues for Caribbean spiny lobsters are not unique to the species but do show some phylogenetic specificity: *P. argus* responds primarily with alarm behavior to conspecific blood, but with mixed alarm and appetitive behaviors to blood from the congener *Panulirus interruptus*, or with appetitive behaviors to blood from the blue crab *Callinectes sapidus*. This study lays the foundation for future neuroethological studies of alarm cue systems in this and other decapod crustaceans.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/211/16/2600/DC1

Key words: chemoreception, Crustacea, olfaction.

INTRODUCTION

Many animal taxa when exposed to hazardous situations such as predatory attack release signals or cues that communicate danger to nearby conspecifics. These alarm signals or cues are often transmitted through chemicals. Release of these chemicals varies considerably across phyla, and depending on whether they benefit the sender and/or receivers they are defined as signals or cues (Smith, 1992). Chemicals that are actively released and signal alarm specifically among conspecifics are known as alarm pheromones (Blum, 1985; Wyatt, 2003). For example, some social insects when attacked by intruders use specialized glands to actively release alarm pheromones that signal to conspecifics to defend their colonies (Blum, 1985). These insect colonies consist of genetically related individuals, and consequently these alarm pheromones benefit signalers and receivers. In contrast, some animals when injured by predators passively leak fluids that induce alarm behavior in neighboring conspecifics, including social groups with little genetic relatedness (Smith, 1992). These chemicals, which benefit receivers but may not directly benefit the sender, are called chemical alarm cues (Seeley, 1995; Viney and Franks, 2004).

Alarm pheromones are widespread across phyla but best studied in arthropods; in particular, insects (Blum, 1985). Social insects release alarm pheromones when the colony is threatened, inducing fighting or fleeing behavior from conspecifics (Wyatt, 2003). Honeybees, for example, release alarm pheromones from their specialized stinging apparatus to recruit conspecifics to fight intruders (Hunt et al., 2003; Breed et al., 2004). This stinging apparatus is autotomized onto the intruder, releasing alarm pheromones that serve as a target for other attacking honeybees. Other insects also use specialized glands to release alarm pheromones that trigger fighting or fleeing behavior by conspecifics (Blum, 1985). The alarm pheromones mediate these behavioral responses primarily through the insect's olfactory pathway (Galizia et al., 1999; Yamagata et al., 2006; Yamagata et al., 2007).

Chemical alarm cues are used extensively by aquatic animals. These cues are especially important during periods of activity such as foraging, when the animal faces the greatest risk of predation (Lima and Dill, 1990). Chemical alarm cues leaked from injured or freshly killed conspecifics indirectly indicate risk from active predators. Vertebrates such as fish release alarm cues passively from fresh wounds in the skin (Pfeiffer, 1977; Smith, 1992; Chivers and Smith, 1998). These alarm cues in fish, like those in insects, are detected by the olfactory system. Some fish have several olfactory pathways, with the one detecting alarm cues being anatomically and functionally separate from those detecting sex pheromones and food odors (Hamdani and Døving, 2007).

The passive release of alarm cues during predation events is also frequent in aquatic invertebrates. For example, sea urchins, sea snails, sea anemones and crustaceans respond with alarm to chemical cues leaked from injured conspecifics. Sea urchins, *Diadema antillarum*, respond to alarm cues by moving away (Snyder and Snyder, 1970). Sea snails similarly respond by crawling or burrowing (Snyder, 1967; Jacobsen and Stabell, 2004). Sea anemones respond to anthopleurine, a chemical that leaks from damaged tentacles, by quickly retracting their tentacles (Howe and Sheik, 1975). Decapod crustaceans also respond with alarm behavior

to fluids leaked from injured conspecifics (Zimmer-Faust et al., 1985; Rittschof et al., 1992; Hazlett, 1994; Fleming et al., 2007). Caribbean spiny lobsters, *Panulirus argus*, have been reported to avoid fluids of injured conspecifics (Parsons and Eggleston, 2005; Parsons and Eggleston, 2006; Bouwma, 2006; Briones-Fourzán et al., 2006; Briones-Fourzán and Lozano-Álvarez, 2008). However, the source of chemical alarm cues from injured conspecifics and the immediate behavioral responses to such cues are poorly characterized. Furthermore, in decapod crustaceans in general and Caribbean spiny lobsters in particular, the sensory mechanisms for detecting chemical alarm cues are unknown. These crustaceans have dual chemosensory pathways in their antennules, a major chemosensory organ (Fig. 1). One of these pathways is analogous to the olfactory pathway; it is based on aesthetasc sensilla, which have dendrites of chemoreceptor neurons that project to the olfactory lobes (Grünert and Ache, 1988; Schmidt and Ache, 1996a; Schmidt and Ache, 1996b). The other is a non-olfactory chemo-mechanosensory pathway; it is innervated by the nine types of 'non-aesthetasc' sensilla, which contain both chemo- and mechanoreceptor neurons that project to the lateral antennular neuropils and median antennular neuropil (Schmidt and Ache, 1996a; Schmidt and Ache, 1996b). The aesthetasc–olfactory lobe pathway may uniquely process detection of pheromones and other conspecific odors, whereas the non-aesthetasc–lateral antennular neuropil pathway and the aesthetasc pathway both detect general odors including food chemicals (Gleeson, 1980; Gleeson, 1992; Steullet et al., 2002; Schmidt and Derby, 2005; Johnson and Atema, 2005; Horner et al., 2008a; Horner et al., 2008b).

Because of this wealth of knowledge on the chemosensory system of Caribbean spiny lobsters, we chose this species as a model system to study alarm cues and the predation risk-assessment system in decapod crustaceans. We first asked about the source of alarm cues from injured animals and the types of alarm behavior induced by such alarm cues, under both field and laboratory conditions. To answer this question, we examined the alarm efficacy of hemolymph (blood), since it is the major fluid released from injured conspecifics. We performed the same tests to determine whether Caribbean spiny lobsters respond with alarm only to conspecific hemolymph or whether they respond to hemolymph from other crustaceans of differing phylogenetic relatedness to *P. argus*. Finally, we examined the role of each of the two antennular chemosensory pathways in mediating the behavioral responses to hemolymph, by examining behavioral responses to hemolymph before and after surgical manipulations of different types of sensilla.

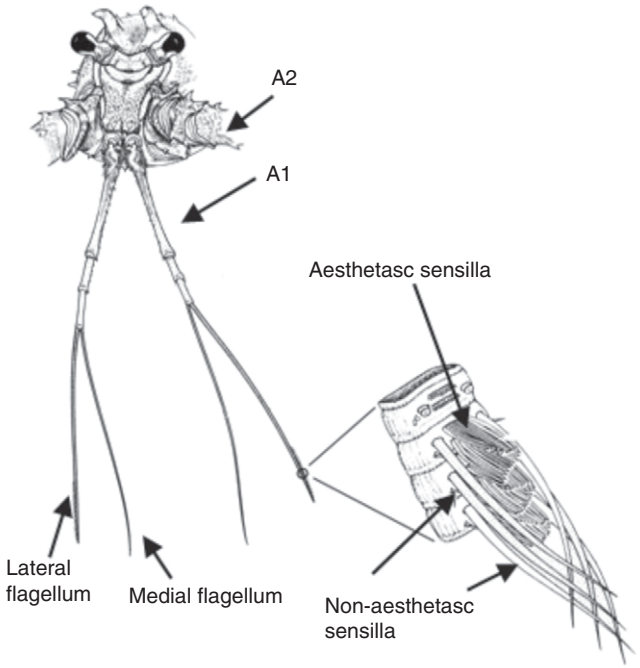

Fig. 1. Chemosensory organs of spiny lobsters. A1, first antenna or antennule; A2, second antenna. A1 bifurcates after the basal segments into the lateral and medial flagella, which share many of the same non-aesthetasc sensilla. However, only the lateral flagellum contains rows of aesthetasc sensilla. Figure modified from Schmidt et al. (Schmidt et al., 2006).

MATERIALS AND METHODS

Laboratory experiments

Animals

Caribbean spiny lobsters, *Panulirus argus* (Latreille), collected from the Florida Keys with a carapace length of 65±7.7 mm (mean ± s.d., *N*=53), were held in an aquarium room at Georgia State University during February and March of 2005 and February of 2007. The aquarium room was kept under fluorescent artificial light in 12 h:12 h light/dark phases. Lobsters were individually held in 80 l aquaria (60 cm×30 cm×45 cm length×width×height) containing filtered sea water (Instant Ocean®, Aquarium Systems, Mentor, OH, USA) at 25°C. Each aquarium contained a hand-made plastic shelter at one end. The shelter was 25.4 cm×24 cm×24 cm (height×width×depth) fabricated from plastic egg crate louvers and CPVC pipes, with one side positioned against the aquarium's back wall and with a ramp for lobsters to move up and hide. These shelters provided a refuge for lobsters, which enabled lobsters to express their alarm behavior and for us to quantify it. Animals were video recorded (Sony DCR PC110; Japan) during the dark phase under low red-light conditions.

Chemical stimuli

Hemolymph was collected from *P. argus*, California spiny lobsters *Panulirus interruptus* (Randall) or blue crabs *Callinectes sapidus* (Rathbun), using a 3 ml syringe and needle (IM $1_{1/2}$: Becton Dickinson, Franklin Lakes, NJ, USA) inserted at the base of the fourth or fifth leg. We used fresh hemolymph diluted 100 times with sea water (SW) for each experimental day for most behavioral tests. Hemolymph diluted 300 times was used only for the behavioral assay on stimulus specificity because it was as potent as hemolymph at 100 times dilution. SW was used as a negative control stimulus in all experiments. Shrimp odor was used as a feeding stimulus. It was prepared by blending shrimp tissue in SW at a concentration of 2 mg ml^{-1}, then filtering.

Ablation of sensilla

To determine whether the aesthetasc pathway is necessary or sufficient to mediate alarm behavior, we performed behavioral experiments before and after ablation of aesthetasc or non-aesthetasc sensilla. To test for the necessity of aesthetascs, we performed behavioral tests during February and March of 2005 on 20 lobsters, first on all animals before treatment ('intact') and then after either ablation of the aesthetasc pathway (nine lobsters) or sham treatment (11 lobsters). Ablation of the aesthetascs was accomplished by shaving the aesthetascs while sparing the non-aesthetasc sensilla on the lateral flagellum and elsewhere. To shave the aesthetasc sensilla, we immobilized the lobster, placed it in a plastic container (15 quart Nalgene sterilisation pan; Lima, OH, USA; 38 cm×47 cm) filled

with SW 15–20 cm deep under a dissecting light microscope, and shaved off aesthetasc sensilla using a miniature scalpel. Control lobsters underwent the same treatment, except that sensilla were left intact (sham treatment). After treatment, both ablated and non-ablated lobsters were allowed to acclimate in their aquaria for 1 week before we performed the same behavioral experiments as before treatment.

To test for the sufficiency of aesthetascs to mediate alarm responses, we performed behavioral tests during February of 2007 on 20 lobsters, first on all animals before treatment and then after either ablating the non-aesthetasc sensilla (10 lobsters) or sham treatments (10 lobsters). To remove non-aesthetasc sensilla, we immobilized lobsters as for aesthetasc ablation and then removed non-aesthetasc sensilla in multiple steps. First, we shaved all visible non-aesthetasc sensilla on the medial flagella and covered the remaining non-aesthetasc sensilla on the medial flagella with Superglue (Pacer Technology; Rancho Cucamonga, CA, USA; Superglue gel). Next, we shaved all visible non-aesthetasc sensilla on the lateral flagella, except for those in the aesthetasc tuft region, and then covered those surfaces with Superglue. We then shaved the remaining non-aesthetasc sensilla in the aesthetasc tuft region. Sham-treated lobsters were similarly immobilized but were only glued on antennular basal segments. Both ablated and non-ablated lobsters were then acclimated in their aquaria for 1 week before we resumed behavioral testing. In these experiments, concrete rectangular blocks (23 cm×23 cm) were used as shelters.

To determine the effectiveness of our sensillar ablations, we collected the lobsters' antennular flagella after completing the behavioral assays according to Schmidt and Derby (Schmidt and Derby, 2005). Flagella were removed and then fixed in 4% paraformaldehyde (in 0.1 mol l^{-1} Sorensen phosphate buffer + 15% sucrose, or SPB) for 24 h. Flagella were then rinsed with SPB and stored in SPB with 0.02% sodium azide until analyzed. To make 0.1 mol l^{-1} SPB, we dissolved 6.8 g KH_2PO_4 and 21.3 g Na_2HPO_4 in 1 l deionized H_2O, adjusted the pH to 7.4, and filtered the solution. For aesthetasc-ablated lobsters, we counted the number of intact aesthetasc and asymmetric sensilla and damaged guard sensilla. (Asymmetric and guard sensilla are in close proximity to the aesthetasc sensilla and are thus sometimes damaged when shaving aesthetascs.) For non-aesthetasc-ablated lobsters, we counted the number of intact aesthetasc and non-aesthetasc sensilla on the lateral and medial flagella. For both treatments, we calculated the percentage of intact and damaged sensilla of the relevant types. Our analysis demonstrated the efficacy of the sensillar ablations. In the aesthetasc-targeted group, 99.7±0.1% (mean ± s.e.m.) of aesthetasc sensilla were ablated. In the process of ablating aesthetascs, 52.1±3.4% (mean ± s.e.m.) of the asymmetric sensilla and 3.2±0.6% (mean ± s.e.m.) of the guard sensilla were damaged. In the non-aesthetasc-targeted group, we ablated 99.7±0.1% (mean ± s.e.m.) of the non-aesthetasc sensilla, and 97.7±0.7% (mean ± s.e.m.) of the asymmetric sensilla. In the process of ablating the non-aesthetasc sensilla, 49.8±6.6% (mean ± s.e.m.) of the aesthetasc sensilla were damaged.

Behavioral tests

The behavioral assay consisted of two phases: acclimation and test. The acclimation phase consisted of giving lobsters at least 3–5 days to become accustomed to the aquarium and behavioral testing protocol. This included feeding lobsters a piece of shrimp using tongs and delivering SW or diluted appetitive stimuli with glass pipettes. Lobsters learned to move forward to appetitive stimuli (a small piece of shrimp or 1 ml of 2 or 200 mg ml^{-1} shrimp odor) but not to negative controls (tongs without shrimp or pipettes releasing 10 ml SW).

Following this acclimation phase, the test phase began, in which we measured appetitive and alarm behavioral responses to chemical stimuli, as defined below. In the test phase, we delivered 1–10 ml of 2 mg ml^{-1} shrimp odor, observed for 45 s, delivered 10 ml of an experimental stimulus (hemolymph) or control stimulus (SW), observed for 120 s, and then again delivered 1–10 ml of shrimp odor and observed for 30 s. All experimental events were video recorded (Sony DCR PC110) under low-intensity red light during the dark phase, and analyzed later.

We used three dependent measurements to assess the alarm response to hemolymph. The first measurement was a quantification of the occurrence and intensity of the alarm response of each animal during the 120 s period following delivery of a chemical stimulus. Alarm responses include retreat, antennae whipping, high frequency shaking, leg shuffling and tail flipping (supplementary material Movies 1 and 2). The typical alarm response to physical threat or to alarm chemicals in our assay was retreat, in which a lobster curled its tail and walked backwards and away from the stimulus to a corner of the aquarium or inside the shelter. When backing into a corner, a lobster often raised its tail up against the aquarium wall while standing high on its front legs or firmly tucked its tail against the substrate while shuffling its front legs. Since retreat behavior was the most stereotypical and consistent alarm behavior, we used it as one dependent measurement of alarm response. The intensity of the alarm response according to our first dependent measurement was assessed using an ordinal scale, from –3 (most alarming) to 0 (no alarm; Table 1). Differences in intensity of the alarm responses toward control and experimental stimuli were tested for significance using non-parametric Wilcoxon matched-pairs tests. We also evaluated the data on a nominal scale, only evaluating whether or not an alarm response occurred. This allowed a simpler presentation of the data, and it is warranted because the conclusions based on analyses using ordinal and nominal measurements were very similar. According to the nominal scale, a response intensity of –1 or lower (Table 1) was rated as a 'yes' for alarm response. Differences between nominal measurements were tested for significance using McNemar tests.

The second dependent measurement of alarm was time spent in the shelter, expressed as a percentage of the 150 s time period after delivery of a stimulus. Statistical evaluation of differences in such ordinal data was achieved using Wilcoxon matched-pairs tests. A significantly greater percentage time in the shelter indicated that lobsters were alarmed by the stimulus.

Table 1. Intensity scale for the responses of *P. argus* to stimuli

Score	Behavior toward stimulus
3	Lobster moves toward the stimulus and forward to the front of the aquarium, shuffling its front legs against the aquarium walls
2	Lobster moves toward the stimulus and forward to the front of the aquarium and at least two body lengths from initial position but does not shuffle its legs against the aquarium walls
1	Lobster moves half a body length forward
0	Lobster does not move forward or backward from the stimulus
–1	Lobster moves backward half a body length and lowers against the substrate
–2	Lobster moves backward at least one body length or inside shelter from outside
–3	Lobster moves backward inside the shelter and moves up the ramp of the shelter

The third dependent measurement was the suppression of the appetitive response to a food odor by hemolymph. An appetitive response is defined as the animal moving forward towards the source of the chemical. The intensity of the appetitive response to shrimp odor was measured on an ordinal scale from +3 (most attractive) to 0 (not attractive; Table 1). The intensity of the suppression of the appetitive response due to presentation of hemolymph was determined by comparing the intensity of the appetitive response before and after delivery of hemolymph. If the response to the first presentation of shrimp odor was significantly greater than the response to the second presentation of shrimp odor (which came after the presentation of hemolymph), then this was considered a suppression of the appetitive response. This statistical evaluation was made using Wilcoxon matched-pairs tests. We also used a nominal measurement of suppression of foraging by hemolymph, again because the conclusions using this simpler measurement were highly similar to those using nominal measurements. A response of 0 or lower to the second presentation of shrimp odor, after a response of +1 or greater to the first presentation of shrimp odor, was rated as suppression of the appetitive response by hemolymph. Differences between nominal measurements were tested for significance using McNemar tests.

Our data were collected in the course of three experiments. Our first experiment tested whether Caribbean spiny lobsters respond with alarm behavior when exposed to conspecific hemolymph before and after ablation of the aesthetasc sensilla. The control stimulus was SW. These two stimuli were delivered 'blindfold' randomly over 2 days, with a maximum of two stimulus presentations per day per lobster. All three dependent measurements were used in this analysis.

Our second experiment tested whether spiny lobsters respond with alarm behavior when exposed to hemolymph before and after ablations of the non-aesthetasc sensilla. This was performed and evaluated in the same way as for the first experiment, except for the nature of the ablation.

Our third experiment tested the stimulus specificity of hemolymph in inducing either alarm or appetitive responses in *P. argus*. We examined behavioral responses to hemolymph from *P. argus*, *P. interruptus* and *C. sapidus* as experimental stimuli and SW as a control stimulus. We performed this experiment using two groups of lobsters, with three stimuli for each group. One group of lobsters was tested with *P. argus* hemolymph (positive control), SW (negative control) and *P. interruptus* hemolymph. A second group was tested with *P. argus* hemolymph, SW and *C. sapidus* hemolymph. This experiment was similar to the first two except that we did not present the second shrimp odor. Thus, in this experiment, we only used the first dependent measurement of alarm response to hemolymph, and did not use suppression of the appetitive response by hemolymph. Statistical significance was investigated using Cochran's Q test followed by McNemar tests for the related samples. To test for a significant difference between the alarm responses to hemolymph of *C. sapidus* and *P. interruptus*, we performed a Chi-squared test for independent measurements.

Field experiments

A field study was performed to determine whether wild Caribbean spiny lobsters show the same alarm responses to hemolymph of conspecifics as animals in the laboratory. The field study was performed in May–August of 2006 in the waters near the Florida Wildlife Commission facility in Marathon, Florida. Lobsters were video recorded using an underwater microvideocamera (Micro Video^{TM} Bobcaygeon, ON, Canada) mounted in a PVC pipe and positioned approximately 1 m from the lobster. Recordings started at dusk, around 19:30 h, and usually ended before 21:30 h. During this time, light levels dropped sufficiently so that most lobsters left their dens and foraged (Herrnkind et al., 1975). Some of the recordings around 21:00 h required artificial illumination through two IR illuminators (IR-200 ProVideo; http://www.surveillance-video.com). The IR illuminators were positioned directly above the surface of the water and crevice site. We recorded from 18 sites, consisting of crevices of various shapes and lobsters of varying numbers, all at approximately 1–3 m water depth. Small crevices contained on average 3.3 lobsters. Stimuli were delivered to lobsters through plastic tubes placed at each crevice site prior to the behavioral experiments. We positioned the delivery end of the two plastic tubes (0.4 mm i.d., 0.5 mm o.d.) approximately 0.3 m away from the opening of each crevice site. The stimulus-loading end of the plastic tubes was outside the water. One of these plastic tubes delivered the experimental stimulus (hemolymph, diluted 100 times with filtered SW) and the other delivered the control stimulus (filtered SW). Hemolymph was collected from healthy lobsters by withdrawing it from the base of the fourth or fifth legs using a syringe.

Our experiments had a paired design, with each lobster presented with two stimuli. SW, a negative control stimulus, was presented first, followed by a 3–4 min observation period. Then hemolymph was delivered, followed by another 3–4 min observation period. For each test, 60 ml of stimulus was delivered over 60–90 s. We chose to use this protocol rather than a randomized design because preliminary tests showed that animals first exposed to hemolymph often moved far enough away from the site of stimulus release that we were unable to present them with a second stimulus, whereas presentation of SW almost never produced this response. Thus, given our aim of using the power of a paired design, we always presented the SW negative control first.

All behavioral responses were recorded, with an emphasis on alarm responses observed in the laboratory experiments. These included moving away from the stimulus or moving into a shelter. Alarm responses were quantified as occurring or not (yes/no), as for laboratory experiments, by an evaluator blind to the nature of the stimulus delivered. Statistical differences between control and experimental stimuli were determined through paired McNemar tests.

RESULTS

Hemolymph evokes alarm behavior

Hemolymph induced several types of response, all indicative of alarm behavior, including retreat, increased sheltering and reduced appetitive responses to food (supplementary material Movies 1 and 2; Table 1). Hemolymph induced an alarm response in a significantly greater percentage of lobsters than did SW (Fig. 2A). Hemolymph also induced significantly more sheltering than control stimuli (Fig. 2B). In addition, hemolymph eliminated appetitive responses to shrimp odor in a significant percentage of lobsters (Fig. 2C).

Aesthetasc ablation eliminates alarm behavior

Lobsters with ablated aesthetascs showed no alarm responses to hemolymph (Fig. 3A), unlike sham-treated lobsters as described above (supplementary material Movie 1). The percentage of experimental lobsters showing alarm responses to hemolymph before ablation was significantly higher than after ablation of the aesthetasc sensilla. However, the percentage of control lobsters showing alarm responses to hemolymph was similar before and after sham treatments (Fig. 3A).

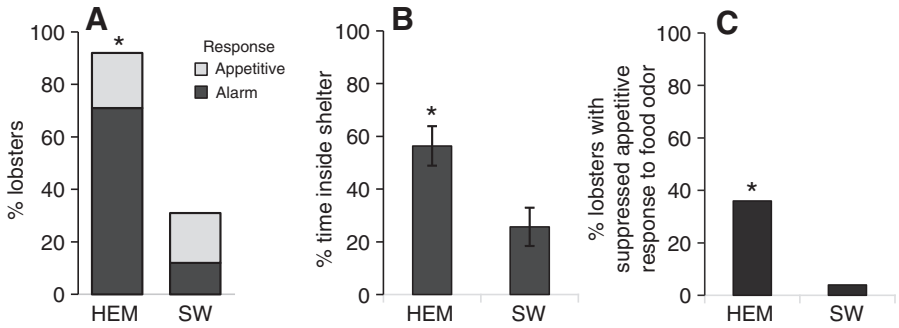

Fig. 2. (A) Hemolymph (HEM) induced alarm responses in significantly more spiny lobsters than did sea water (SW). In contrast, hemolymph and SW induced a similarly low frequency of appetitive responses. (B) Hemolymph caused spiny lobsters to spend significantly more time inside the shelter than did SW. (C) Hemolymph suppressed the appetitive response to shrimp odor in significantly more lobsters than did SW. Results are based on 53 lobsters. *Significant difference between HEM and SW using nominal data and McNemar tests (P<0.05) as described in Materials and methods. Evaluation of the results based on ordinal data using Wilcoxon matched-pairs tests showed similar results.

Lobsters with ablated aesthetascs reversed their behavior in response to hemolymph: instead of showing alarm responses, they showed appetitive feeding responses to hemolymph (Fig. 3A; supplementary material Movie 1). Before aesthetasc ablation, the percentage of lobsters showing appetitive responses to hemolymph was as low as to control stimuli. However, after ablation, this percentage increased significantly, to 100%. For sham-treated lobsters, the low percentage of animals that showed appetitive responses to hemolymph before treatment remained low after sham treatment.

Because aesthetasc-ablated lobsters engaged in appetitive responses instead of alarm responses when exposed to hemolymph, they spent significantly less time inside shelters than they did before ablation (Fig. 3B). After ablation, lobsters spent roughly equal time inside the shelter when exposed to either hemolymph or control stimuli (Fig. 3B). On the other hand, lobsters with sham treatment spent more time inside shelters in response to hemolymph than to control stimuli (Wilcoxon matched-pairs test, P>0.05; Fig. 3B).

Aesthetasc ablation strongly affected the ability of hemolymph to suppress appetitive responses to food chemicals (Fig. 3C). Before ablation, the percentage of lobsters that showed appetitive responses to shrimp odor was significantly higher when shrimp odor was presented after exposure to SW than after exposure to hemolymph. However, this suppression of the response to shrimp odor by

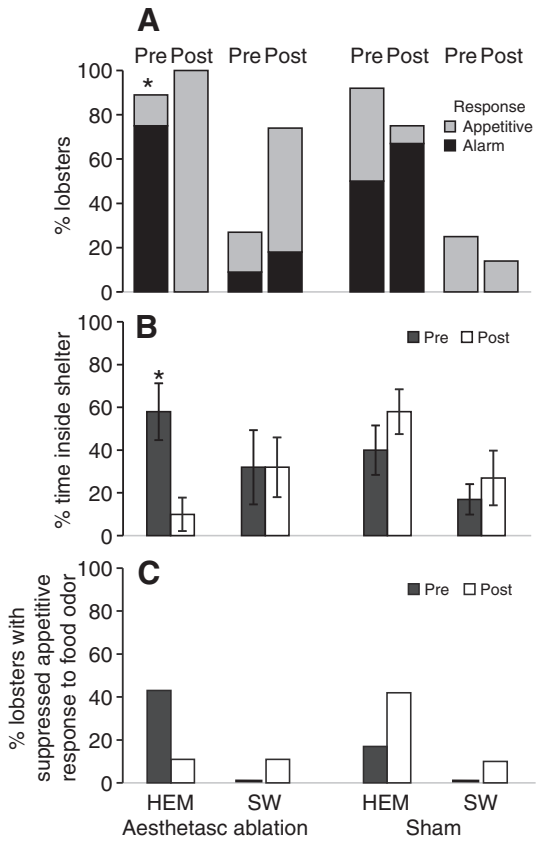

Fig. 3. Ablating aesthetasc sensilla eliminated all forms of alarm response to hemolymph (HEM). (A) Before (Pre) ablation of aesthetasc sensilla, a significantly higher percentage of experimental lobsters (left graph, N=9) showed alarm responses to hemolymph than after (Post) ablation (McNemar test, *P<0.05). The percentage of control lobsters (right graph, N=11) showing alarm responses to HEM before and after sham treatment was the same. The percentage of experimental lobsters showing appetitive responses to hemolymph increased significantly (McNemar test, *P<0.05), to 100%, after ablation. The percentage of control lobsters showing appetitive responses to hemolymph was the same before and after sham treatment. (B) Experimental lobsters spent significantly more time inside the shelter in response to hemolymph before than after ablation (Wilcoxon matched-pairs test, *P<0.05). Control lobsters spent a similar amount of time inside the shelter in response to hemolymph before and after sham treatment. (C) Before ablation, a high percentage of experimental lobsters had suppressed appetitive responses to shrimp odor after hemolymph; however, after ablation, a low percentage of the same lobsters had suppressed appetitive responses. The significance of the results from nominal data above was similar to the significance of results based on ordinal data using Wilcoxon matched-pairs tests.

hemolymph was eliminated following aesthetasc ablation. In contrast to ablated lobsters, sham-treated lobsters showed suppression by hemolymph. However, this suppression was not significantly different from suppression by SW (McNemar tests, $P>0.05$).

Non-aesthetasc ablation does not affect the alarm response

None of the four measurements of alarm responses to sources of alarm chemicals changed after ablating non-aesthetasc sensilla (Fig. 4). First, alarm responses to hemolymph were not eliminated after non-aesthetasc ablation. Following either experimental or sham ablation, responses to these stimuli remained the same (Fig. 4A).

Second, appetitive responses to hemolymph did not change after non-aesthetasc ablation. Both ablated and sham-treated lobsters showed no change in appetitive responses toward hemolymph after treatment (Fig. 4A).

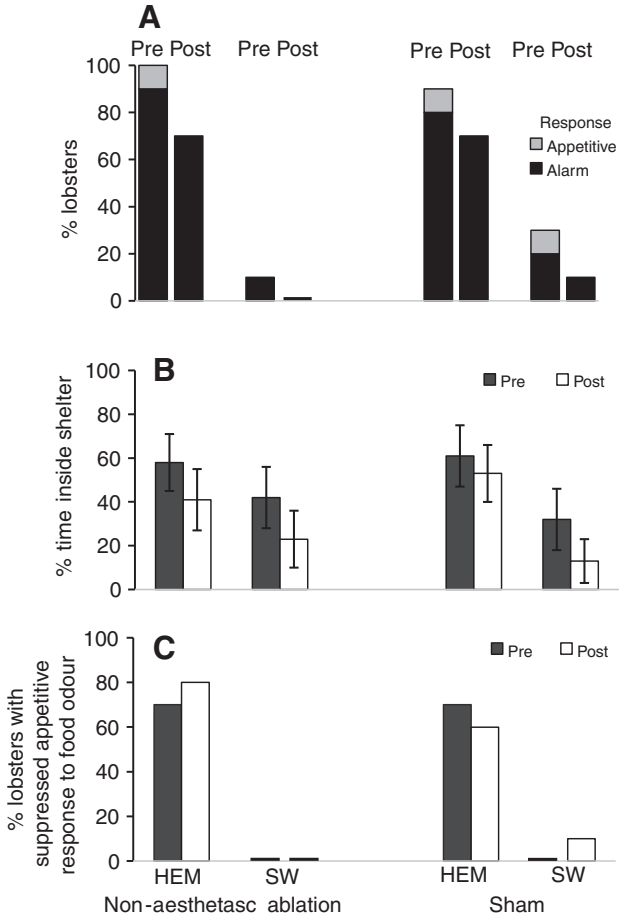

Fig. 4. Ablation of non-aesthetasc sensilla did not affect any form of alarm behavior in response to hemolymph (HEM). (A) The percentage of either experimental (left graph, $N=10$) or control (right graph, $N=10$) lobsters that showed alarm or appetitive responses to hemolymph remained the same after (Post) ablation of non-aesthetascs or sham treatment. (B) Likewise, both experimental and control lobsters spent a similar amount of time inside the shelter in response to hemolymph before and after either treatment. (C) Both experimental and control lobsters before and after treatment showed similar suppression of appetitive responses to shrimp odor when shrimp odor was presented after hemolymph. Nominal data were analysed using McNemar tests ($P<0.05$) as described in Materials and methods. Evaluation of the results based on ordinal data using Wilcoxon matched-pairs tests showed similar results.

Third, the amount of time that lobsters spent inside the shelter in response to hemolymph did not change after ablation of non-aesthetasc sensilla. Neither ablation nor sham treatment significantly changed the percentage of time spent inside the shelter following presentation of hemolymph (Fig. 4B).

Fourth, suppression of appetitive responses to shrimp odor by hemolymph did not change after ablation of non-aesthetasc sensilla. Neither ablation nor sham treatment changed the percentage of lobsters that were attracted to shrimp odor (Fig. 4C).

Stimulus specificity in the alarm response to hemolymph

The alarm response of Caribbean spiny lobsters, *P. argus*, was greatest to hemolymph from conspecifics. The percentage of lobsters that showed alarm responses was significantly higher with hemolymph from conspecifics than with hemolymph from either California spiny lobsters, *P. interruptus*, or blue crabs, *C. sapidus* (Fig. 5). Interestingly, hemolymph from *P. interruptus* evoked alarm responses in a significantly greater percentage of lobsters than SW, whereas hemolymph from *C. sapidus* did not (Fig. 5). Furthermore, appetitive responses of lobsters were significantly lower to hemolymph from conspecifics compared with hemolymph from either *P. interruptus* or *C. sapidus* (McNemar test, $P<0.05$; $N=20$ and $N=19$, respectively; Fig. 5), and *C. sapidus* hemolymph induced appetitive responses in a significantly higher percentage of lobsters than *P. interruptus* hemolymph (Fisher exact test, $P<0.05$, $N=19$) or *P. argus* hemolymph.

Hemolymph induces alarm behavior in wild lobsters

Lobsters in the field showed alarm behavior in response to hemolymph similar to that of animals in our laboratory studies. These behavioral responses included either moving deeper into the den closest to them or moving away from the closest den and moving to another, nearby den (Fig. 6; supplementary material Movie 3). Approximately half of the lobsters tested in the field responded to

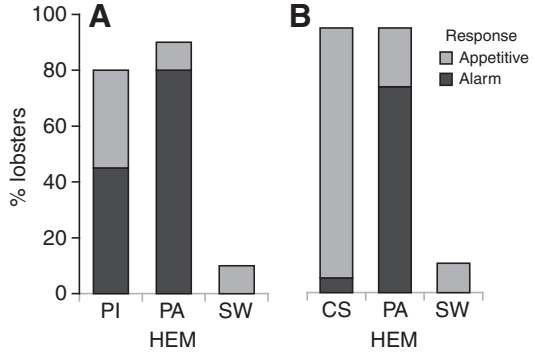

Fig. 5. Spiny lobsters were more likely to show alarm responses to conspecific hemolymph compared with either congeneric hemolymph or hemolymph from a brachyuran crab. Responses of two groups of *P. argus* lobsters are shown in A and B. *P. argus* hemolymph (PA HEM) induced alarm responses in a significantly greater percentage of *P. argus* lobsters than did hemolymph of *Panulirus interruptus* (PI HEM) or the SW control, and PI HEM induced alarm responses in a significantly greater percentage of lobsters than did SW (McNemar test, $P<0.05$, $N=20$) or hemolymph of *Callinectes sapidus* in another group of lobsters (CS HEM; Fisher exact test, $P<0.05$). CS HEM induced appetitive responses in a significantly greater percentage of *P. argus* lobsters than did PI HEM (McNemar test, $P<0.05$, $N=19$) or PA HEM (Fisher exact test, $P<0.05$). Evaluation of the results for ordinal data using Wilcoxon matched-pairs tests showed similar results.

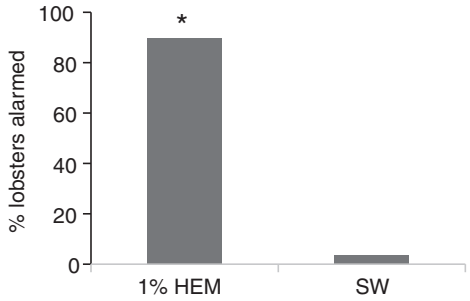

Fig. 6. Field tests of alarm responses of *P. argus* to conspecific hemolymph. Hemolymph induced alarm responses in a significant percentage of wild lobsters (N=59). *Significant difference in the percentage of lobsters showing alarm responses to hemolymph (HEM) compared with SW (P<0.05, by McNemar test).

hemolymph by moving deeper into their den; the other half moved into a different den. Our qualitative observations suggested that whether an alarmed animal remained in the den or moved to another largely depended on the size and shape of the den. Lobsters in deep dens usually remained in the den, whereas lobsters in shallow dens usually vacated them and moved to other dens.

DISCUSSION

Alarm responses of spiny lobsters to hemolymph

Our laboratory and field studies show that Caribbean spiny lobsters, *Panulirus argus*, release blood-borne alarm cues that are detected by the olfactory pathway. These cues induce alarm responses in the form of retreat, increased sheltering, and suppression of the appetitive response to food odors (Figs 2 and 6), but they can also induce antennae whipping, high frequency shaking and tail flipping. Furthermore, blood-borne cues from heterospecific crustaceans can also induce responses in *P. argus*, but the responses are less frequent alarm behavior or even appetitive feeding. Similar alarm behaviors are also induced by physical threat [S.S., personal observations (see Cobb, 1981)].

Our study complements previous studies by showing that spiny lobsters use chemical alarm cues in both field and laboratory conditions. For example, Caribbean spiny lobsters avoid shelters containing damaged conspecifics, both in the laboratory and in the field (Parsons and Eggleston, 2005; Parsons and Eggleston, 2006; Bouwma, 2006; Briones-Fourzán et al., 2006; Briones-Fourzán and Lozano-Álvarez, 2008). Fishermen in Mexico avoid throwing spiny lobster bodies back in the water after removing their tails because this practice leads to a poor catch (Briones-Fourzán et al., 2006). California spiny lobsters avoid dens that contain leaked fluids of fresh conspecific carcasses (Zimmer-Faust et al., 1985). Other crustacean species such as crayfish, hermit crabs and blue crabs also avoid damaged conspecifics (Rittschof et al., 1992; Hazlett, 1994; Acquistapace et al., 2005; Ferner et al., 2005).

Sensory pathways mediating alarm responses of spiny lobsters

This chemically induced alarm response is mediated by the olfactory pathway. In spiny lobsters and other decapod crustaceans, the olfactory pathway is represented by aesthetasc sensilla on the antennules, which contain olfactory receptor neurons whose axons project to the olfactory lobes of the brain (Fig. 1) (Schmidt and Ache, 1996a; Schmidt and Ache, 1996b). In our study, the behavioral responses to hemolymph changed dramatically after ablation of only the aesthetascs but not after ablation of all other antennular sensilla (Figs 3 and 4; supplementary material Movie 1). In fact, aesthetasc-ablated lobsters responded to hemolymph with very different behavior from that of control lobsters: instead of retreating, they walked forward and performed appetitive behaviors. A likely cause of this response is that lobsters without aesthetasc sensilla detect food chemicals but not alarm cues, both of which are present in the hemolymph, through the non-aesthetasc sensilla. Indeed, it has previously been shown that either the aesthetasc or non-aesthetasc pathway can mediate detection, discrimination and orientation toward food odors (Steullet et al., 2001; Steullet et al., 2002). An alternate hypothesis is that aesthetasc-ablated lobsters detect alarm chemicals but the pathways detecting them do not mediate alarm responses.

Our finding that the aesthetasc pathway is necessary to mediate responses to these intraspecific alarm cues is consistent with previous studies on crustaceans showing that aesthetascs exclusively mediate behavioral responses to conspecific odors. Male blue crabs with ablated aesthetascs show significantly less courtship behavior in response to female urine-borne sex pheromones than males with aesthetascs (Gleeson, 1980; Gleeson, 1982). American lobsters and crayfish with ablated aesthetascs engage more frequently in fights with dominant opponents, which use urine as an indicator of status, than do those with aesthetascs (Johnson and Atema, 2005; Horner et al., 2008a). Caribbean spiny lobsters with ablated aesthetascs show diminished preference to conspecific shelters containing urine-based aggregation cues (Horner et al., 2008b). Our study supports the view that spiny lobsters and other decapod crustaceans have two functionally distinct antennular chemosensory pathways: the aesthetasc pathway, uniquely for conspecific odors; and the non-aesthetasc pathway, which together with the aesthetasc pathway mediates responses to food and other general odors. Our study lays the foundation for future studies of neural processing of alarm cues by the olfactory pathway of spiny lobsters.

Species selectivity of alarm cues

The stereotypical alarm responses of Caribbean spiny lobsters were not entirely specific to hemolymph of conspecifics. While hemolymph of conspecifics induced almost exclusively alarm responses, hemolymph of heterospecifics induced either similar alarm responses, though less frequent or intense, or appetitive feeding responses (Fig. 5). Hemolymph from the more closely related *Panulirus interruptus* was more likely to produce alarm responses from *P. argus* than was hemolymph from *Callinectes sapidus*, which was more likely to evoke appetitive feeding responses. Thus, our results suggest that hemolymph of *P. argus* has a composition of chemicals that can alarm its conspecifics, and the ability of heterospecific hemolymph to induce alarm responses in *P. argus* depends on species relatedness. This idea is supported by recent results (Briones-Fourzán and Lozano-Álvarez, 2008) indicating that fluids of damaged *Panulirus guttatus*, a close relative and sympatric to *P. argus* (Ptacek et al., 2001), induces similar avoidance responses in *P. argus* to those induced by fluids of damaged *P. argus*. Differences in effectiveness of the hemolymph from crustacean species might be due to either the type or concentration of components in the hemolymph. Resolution of this issue must await molecular identification of the alarm cues.

Predation risk assessment in decapod crustaceans

Spiny lobsters, like other aquatic animals, assess risk of predation and use that information in determining their activity. During foraging, animals face the highest risk of attack by predators (Lima and Dill, 1990). Thus, any assessment indicating the presence of

active predators can dramatically change an animal's foraging activity (Wisenden, 2000). Spiny lobsters forage predominantly at night under low light conditions, at which time they rely heavily on their chemical senses for assessing risk while trying to locate food, shelter or mates (Herrnkind et al., 1975; Kanciruk, 1980). If spiny lobsters detect these cues when foraging, they are likely to move away from that area and seek shelter. If they detect these cues when they are already in shelters, they might move deeper into those shelters away from the source of the alarm cues, or they might move to a nearby shelter away from the alarm cues. Thus spiny lobsters tightly regulate foraging and any other activities *via* the risk-assessment pathway – the olfactory pathway that detects the chemical alarm cues.

This risk-assessment system coupled with an escape tactic represents an effective evolutionary mechanism for reducing the risk of predation. Spiny lobsters, like other crustaceans, autotomize their limbs to escape imminent death from predators. Limb autotomy enhances escape and limits fluid loss from wounds (Juanes and Smith, 1995; Fleming et al., 2007), thus benefiting the individual performing it. In addition, limb autotomy might benefit nearby conspecifics, if they can detect blood from the autotomized limb and respond to its alarm cues by pre-emptively defending themselves and avoiding areas containing active predators. This might be considered as a form of predator tagging. We suggest that this might be a mechanism by which this predator risk-assessment system has evolved.

Chemical alarm cues released from injured conspecifics might be transient, as in the case of autotomy, or lingering. For example, some large predators might consume a spiny lobster quickly and without much release of hemolymph, in which case the alarm cue might be short lived. In other circumstances, such as when a spiny lobster is damaged and leaking hemolymph or where a carcass is slowly consumed by a predator, the alarm cue might be present in an area for a longer time (Weiss et al., 2008). In some species, chemical alarm cues can also end up unaltered in predators' bodily excreta, such that conspecifics can detect alarm cues released by the predator (Chivers and Smith, 1998), although such a means of tagging predators has not been demonstrated for spiny lobsters or any other decapod crustacean.

Alarm pheromones or cues are almost exclusively used by animals that live in groups, and since the organization of groups shows interspecific variation, so do their responses to alarm pheromones (Blum, 1985). In response to alarm pheromones, some eusocial insects that form highly organized groups fight aggressively with their chemical weaponry against predators (Blum, 1985). Spiny lobsters live in groups (Herrnkind et al., 1975), but they lack the highly organized social colonies, close genetic relatedness and chemical weaponry that social insects often have. Accordingly, fleeing from blood-borne alarm cues, either into a solitary shelter or towards other intact lobsters to form a group, is a highly adaptive response for spiny lobsters because it reduces their risk of encountering active predators. We suggest that this type of predation risk-assessment system may be much more common and perhaps more complex than previously thought in crustaceans and other arthropods.

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