Spiny lobsters use urine-borne olfactory signaling and physical aggressive behaviors to influence social status of conspecifics

Shkelzen Shabani*, Michiya Kamio and Charles D. Derby

Neuroscience Institute and Department of Biology, Georgia State University, Atlanta, GA 30303, USA

*Author for correspondence (e-mail: shabanis@ohsu.edu)

Accepted 30 April 2009

SUMMARY
Decapod crustaceans, like many other animals, engage in agonistic behaviors that enhance their ability to compete for resources with conspecifics. These agonistic behaviors include the release of chemical signals as well as physical aggressive and submissive behaviors. In this study, we report that Caribbean spiny lobsters, *Panulirus argus*, use both urine-borne chemical signaling and physical aggressive behaviors during interactions with conspecifics, and that these agonistic behaviors can influence the behavior and eventual social status of the interactants. Spiny lobsters that engaged primarily in physical aggressive behaviors became dominant, whereas spiny lobsters that received these physical aggressive behaviors responded with avoidance behaviors and became subordinates. Dominant animals frequently released urine during social interactions, more than when they were not in contact with subordinates and more than when they were not paired with another animal. Subordinates released urine significantly less often than dominants, and no more than when not paired. Preventing release of urine by catheterizing the animals resulted in an increase in the number and duration of physical interactions, and this increase was primarily driven by dominants initiating interactions through physical aggressive behaviors. Introducing urine from one of the catheterized animals into an aquarium reduced physical aggressive behavior by dominant animals to normal levels. Urine-borne signals alone were capable of inducing avoidance behaviors from solitary spiny lobsters in both laboratory and field conditions. We conclude that urine serves as a chemical signal that communicates social status to the interactants. Ablation experiments showed that these urine signals are detected primarily by aesthetasc sensilla of the olfactory pathway.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/212/15/2464/DC1

Key words: chemoreception, communication, Crustacea, olfaction, social behavior.

INTRODUCTION
Many animals compete with conspecifics to gain better access to food, shelters, mates and other resources (Rowell, 1974; Drews, 1993; Barroso et al., 2000; Petrilus et al., 2004; Burmeister et al., 2005; Gherardi, 2006; Holvand et al., 2008; Izawa and Watanabe, 2008; Val-Laillet et al., 2008). This competition, which is common among gregarious animals (Drews, 1993), often involves agonistic behaviors, such as aggressive physical acts, as well as ritualized behaviors, which may include signals used in communication. Agonistic interactions between a pair of opponents may start symmetrical, with the two acting equally aggressively, but then progress with one showing primarily aggressive behaviors and winning and the other showing submissive behaviors and losing. Those that engage primarily in aggressive behaviors are referred to as dominants, whereas those engaging in submissive or avoidance behaviors are referred to as subordinates (Drews, 1993). Consequently, dominants often have greater access to resources (Wilson, 1975). Many solitary and gregarious decapod crustaceans express such dynamic social behavior (Scrivener, 1971; Berrill, 1975; Berrill, 1976; Bruski and Dunham, 1987; Issa et al., 1999), and they may use chemical signals to communicate the established social status (Breithaupt and Atema, 2000; Breithaupt and Eger, 2002). Thus, agonists that chemically communicate may end the interaction sooner or not interact at all (Briffa and Williams, 2006; Rutte et al., 2006; Baird et al., 2007).

Competition for shelters, which provide refuge from predators, is common among decapod crustaceans. Crayfish (*Orconectes rusticus*) with dominant status forcefully evict conspecifics with subordinate status from their shelters (Martin and Moore, 2008). In the absence of shelter or burrow, dominant crayfish (*Procambarus clarkii*) engage in aggressive behaviors and burrow to make shelters, whereas subordinate crayfish engage in submissive behaviors and burrow less (Herberholz et al., 2003). Consequently, subordinate crayfish have less access to shelters and are more likely to be evicted from shelters. Furthermore, subordinate crayfish also have less access to food (Herberholz et al., 2007). Field studies show similar behavior by *Orconectes virilis* and *O. rusticus* during competition for food and shelters (Bergman and Moore, 2003). Subordinate crayfish (*O. rusticus* and *Procambarus acutus acutus*) avoid fights with dominants regardless of familiarity (Zulandt Schneider et al., 2001; Gherardi and Daniels, 2003). Relatively similar results are reported for American lobsters (*Homarus americanus*) and Norway lobsters (*Nephrops norvegicus*) (Karnofsky and Price, 1989; Karnofsky et al., 1989; Katoh et al., 2008). Both species reduce their fighting in consecutive encounters (Karavanich and Atema, 1998b; Johnson and Atema, 2005; Katoh et al., 2008). Spiny lobsters *Panulirus argus*, *Panulirus longipes*, *Panulirus cygnus* and *Jasus lalandei* differ in sociality from crayfish and American lobsters in that they aggregate in and around shelters, yet they compete for shelters and show aggression around them (Fielder, 1965; Chittleborough, 1974; Berrill, 1975; Berrill, 1976; Meyer-Rochow and Penrose, 1976; Lozano-Álvarez, 1996; Childress, 2007). Spiny lobsters are nocturnal animals, and they avoid predators...
Urinary signals influence social status

by spending considerable time inside shelters, especially during the day but also at night (Weiss et al., 2008).

During agonistic interactions, some decapod crustaceans release urine from their nephropores located at the base of their antennae and direct it toward their opponents through water currents generated by their fan organs, gill baiers or other structures (Atema, 1985; Breithaupt, 2001; Herberholz and Schmitz, 2001; Denissenko et al., 2007). Work on snapping shrimp (Alpheus heterochaelis) implied that the fast anteriorly directed gill currents, which are used especially during physical contact with conspecifics, disperse urine-borne signals (Herberholz and Schmitz, 2001). These currents are high velocity, cover distances of more than two body lengths, and are often directed toward the anterior of the opposing animal where its olfactory organ and other prominent chemosensors are located. Furthermore, dominants use these fast gill currents significantly more frequently than do subordinates. Work on American lobster and narrow-clawed crayfish (Astacus leptodactylus) also suggests that gill currents disperse urine (Atema, 1985; Breithaupt and Eger, 2002). Procambarus clarkii also uses its fan organs to draw odors toward the olfactory organs by creating directed water currents (Brock, 1926; Brock, 1930; Denissenko et al., 2007). Both crayfish and American lobsters change their pattern of urine release according to their social status (Breithaupt et al., 1999; Breithaupt and Atema, 2000; Breithaupt and Eger, 2002). During an initial paired encounter, dominants release more urine than subordinates (Breithaupt and Eger, 2002). In subsequent encounters, familiar opponents decrease the number and duration of interactions. These and other results, especially on crayfish and American lobsters, suggest that urine signals contribute significantly to the decrease in physical aggression. Breithaupt and Eger (Breithaupt and Eger, 2002) showed that dominant crayfish increase the rate of urine release, but subordinate crayfish do not. Furthermore, urine is released especially during physical aggressive behaviors and directly in front of the opponent. Zulandt Schneider and Moore (Zulandt Schneider and Moore, 2000) added support to this idea by showing that a pair of crayfish has significantly longer fights when urine release is blocked. Although an absence of urine release prolongs fights in the subsequent encounters, it does not change the established social status. Other chemical, mechanical and visual stimuli may play a role in communicating social status in crayfish (Bruski and Dunham, 1987). Relatively similar results are reported in American lobsters; however, unlike crayfish, American lobsters decrease the duration of fights in subsequent encounters only when paired with familiar opponents, and blocking the release of urine after the familiarization period does not alter the duration of fights in subsequent encounters (Karavanich and Atema, 1998a; Karavanich and Atema, 1998b; Breithaupt and Atema, 2000). Similar results are reported for Norway lobsters (Katoh et al., 2008).

The effect of chemical signals on the duration of physical interactions was also assessed through manipulation of the olfactory organ of crayfish and American lobster. Ablating the olfactory organ (i.e. the olfactory sensilla, or aesthetascs, on the antennular lateral flagella) of crayfish P. clarkii prevents the decrease in duration of fights in subsequent encounters (Horner et al., 2008a). Olfactory ablation in American lobsters has a similar effect (Johnson and Atema, 2005). In both ablation studies, however, the direct effect of urine or any other source of chemical signals on behavior was not tested.

The olfactory organ mediates detection of urine signals in some social and sexual contexts of decapod crustaceans. Crustaceans have dual chemosensory pathways in their antennules (Schmidt and Ache, 1996a; Schmidt and Ache, 1996b). One is an olfactory pathway, whose receptors are aesthetasc sensilla containing olfactory neurons with axons projecting to the olfactory lobes. The other is a non-olfactory pathway, whose receptors are the bimodal ‘non-aesthetasc’ sensilla, which contain both chemoreceptor and mechanoreceptor neurons with axons projecting to the lateral antennular neuropils and median antennular neuropil. Although both antennular pathways detect general odors including food odors, only the aesthetasc/olfactory pathway carries information about conspecific odors (Gleeson, 1980; Gleeson, 1982; Steullet et al., 2002; Johnson and Atema, 2005; Schmidt and Derby, 2005; Horner et al., 2008a; Horner et al., 2008b).

Caribbean spiny lobsters (P. argus), unlike crayfish and clawed lobsters, are gregarious animals that also engage in agonistic interactions when competing for shelters and food (Fielder, 1965; Berrill, 1975; Berrill, 1976; Childress, 2007) (and S.S., personal observation). Aggregation behavior is partly communicated by chemical cues released in the urine of conspecifics (Nevitt et al., 2000; Ratchford and Eggleston, 1998), which in crayfish and clawed lobsters are used for communicating social status. Caribbean spiny lobsters prefer shelters scented with the urine of conspecifics, and they lose this preference if their olfactory pathway is ablated (Horner et al., 2008b). The choice of shelter is complex and depends heavily on the context in which these chemical cues are transmitted from conspecifics. Spiny lobsters will often aggregate in large numbers but aggregations vary widely depending on environmental and physiological factors.

According to Fielder’s observations (Fielder, 1965), spiny lobsters often engage in agonistic physical interactions when competing for shelters, similar to crayfish and clawed lobsters. Beyond these observations, however, it is not known whether any gregarious crustaceans, including spiny lobsters, use urine to communicate social status in the way that solitary crustaceans such as crayfish and American lobsters do. Therefore, we investigated whether urine is used to communicate social status in the Caribbean spiny lobster P. argus. We used a series of behavioral experiments to test the hypotheses that spiny lobsters communicate social status by releasing urine-borne signals, that urine is a signal of threat in the context of social communication but not as a disturbance signal, and that these urine signals are detected by the olfactory pathway.

MATERIALS AND METHODS

Animals

Caribbean spiny lobsters, P. argus Latreille, collected from the Florida Keys, with a carapace length between 50 and 80 mm were held in an enclosed aquarium room at Georgia State University. The aquarium room was kept under fluorescent light in 12 h light/dark phases. All animals used in behavioral experiments were intermolt (Lyle and MacDonald, 1983). Animals were individually held in 80 l aquaria (60 cm L × 30 cm W × 45 cm H) containing filtered sea water (Instant Ocean®, Aquarium Systems, Mentor, OH, USA) at 25°C. They were fed three times a week with one piece of shrimp (1–2 g). Each aquarium contained a shelter at one end to provide a refuge. The shelter was 24 cm L × 24 cm W × 25.4 cm H fabricated from plastic egg crate louver and chlorinated polyvinyl chloride pipes, with one side positioned against the aquarium’s back wall and with a ramp to enable the spiny lobsters to move up and hide. In one experiment, the shelter was a concrete rectangular block (23 cm × 23 cm × 23 cm). All behavioral experiments were run and video recorded (Sony DCR PC110) during the dark phase between 2 and 8 h under reduced red light conditions.
Catheterization

A catheterization method was used for visualizing urine release, collecting urine and preventing urine release. The method is described in detail in supplementary material Fig.S1, and its applications are as described in the following experiments.

Behavioral assay of stimuli influencing urine release

This assay tested whether spiny lobsters, either in isolation or paired with a conspecific, release urine when presented with any of several stimuli. To measure urine release, we visualized urine release by catheterizing the animal’s nephropores. These lobsters are referred to as ‘VU lobsters’. Supplementary material Fig.S1A renders a detailed description of the catheterization for VU lobsters. Identical behavioral tests and measurements of urine release were performed on one group of VU lobsters (N=7) first in isolation and then later paired with another spiny lobster. Supplementary material Fig.S2 provides a graphic representation of this assay. All behavioral tests were video taped (Sony DCR PC 110) and analyzed later. During a test, VU lobsters were presented with 10 ml of sea water and then 5 min later with 10 ml of one of the following stimuli: conspecific urine, conspecific hemolymph or shrimp juice. Each lobster was tested with a maximum of two stimuli in 1 day for 2 consecutive days. Conspecific urine was collected as described below, and conspecific hemolymph was collected and used fresh, according to our previous study (Shabani et al., 2008). Shrimp juice was prepared by blending shrimp tissue in sea water (2mg ml\(^{-1}\)) and filtering, according to Shabani et al. (Shabani et al., 2008). Approximately 30–60 min after these tests, VU lobsters were fed a piece of food (ca. 1–2 g shrimp or squid); this served as a positive control to demonstrate that the spiny lobster was capable of releasing urine and that we could detect it, as ingestion of food is always followed by release of long pulses of urine (see supplementary material Movie 1). On day 3, each spiny lobster was presented with a physical disturbance, in which the spiny lobster was prodded vigorously with plastic tongs for 1 min. In response to this prodding, spiny lobsters retreated, tail flipped, and on occasion stridulated. Five days later, each lobster was paired with another spiny lobster and, after 2 days of pairing, the testing procedure was repeated. Hemolymph and physical disturbance were previously shown to evoke submissive or avoidance responses that include ‘retreat’, ‘tail flipping’ and ‘stridulating’ (Lindberg, 1955; Meyer-Rochow and Penrose, 1976; Mulligan and Fischer, 1977; Cobb, 1981; Nauen and Shadwick, 2001; Shabani et al., 2008) (see also supplementary material Movie 2). We quantified urine release as the number of pulses, with the pulses being categorized as short and long. Short pulses lasted for 2–9 s (see supplementary material Movie 3) and formed small puffs. Long pulses (see supplementary material Movies 1 and 6) lasted on average 100 s and formed clouds. Long pulses were also characterized by a peak, identified by a more intense fluorescein color, within 1–2 s of the onset of release (see supplementary material Movies 1 and 6). The durations of long and short pulses did not have a normal distribution, and the two distributions were significantly different (Kolgomorov–Smimov test, \(P<0.05\)).

Behavioral assay of urine release during interactions

This assay was performed to determine the pattern of urine release during interactions. We used pairs of VU lobsters (N=7 pairs). Members of a pair showed 4.9±1.4% (data are means ± s.e.m., here and below unless noted otherwise) difference in carapace length. Supplementary material Fig.S3 provides a graphic representation of this assay. Before pairing, these animals were isolated for at least 1 week during which time they were fed every other day, the last feeding being 2 days before pairing. We video recorded the interaction during the first hour of pairing and measured the number of pulses of urine release and the duration of each, and the occurrence of aggressive and submissive behaviors. To determine dominance status during the first hour of interaction, we used a dominance score, \(D\), according to Song et al. (Song et al., 2006). Briefly, dominance score was determined according to the number of behaviors during interactions included in the formula, \(D=[100(2\text{Att}+\text{App}+\text{Ret}–2\text{Esc})]/(2\text{Att}+\text{App}+\text{Ret}+2\text{Esc})\), where Att is the number of attacks, App is the number of approaches, Ret is the number of retreats and Esc is the number of escapes. Attacks are defined as the initiation of aggressive behaviors of three types: grabbing the antenna or legs from the side using the front legs (‘antenna grabbing’ and ‘leg grabbing’, respectively; see supplementary material Movies 4 and 6), and poking their legs underneath the abdomen (‘abdomen poking’), which often induced tail flips (escapes) by the opponent (see supplementary material Movie 5). Approaches included spiny lobsters locking front-to-front with their antenna or their front legs (‘antennae locking’; see supplementary material Movie 3) with their opponent. Antennae locking is similar to claw-lock in American lobsters (Johnson and Atema, 2005). Submissive behaviors included retreat and escape tail flipping (see supplementary material Movies 4–6). Retreat involved spiny lobsters walking backward away from the opponent (see supplementary material Movies 4 and 6). Tail flips, which sometimes were associated with stridulation, were induced when the opponent attacked the spiny lobster by grabbing its legs or antennae, and/or poke its abdomen. A positive dominance score indicates a ‘dominant’ animal, and a negative score indicates a ‘subordinate’ animal.

These paired spiny lobsters were then offered food on the first and second days of pairing. Urine release was quantified the same way as described above. We also determined whether urine was released when the spiny lobsters were: engaged in aggressive physical interactions, i.e. approach, aggressive and/or submissive behaviors (‘phys-int’); touching or less than half a body length away from each other but not engaged in approach, aggressive and/or submissive behaviors (‘in contact’); or more than half a body length away from each other and not physically interacting (‘distant’) (see supplementary material Movie 6).

Behavioral assay of the role of urine in communicating social status

To test whether urine contributes to the communication of social status, we manipulated the release of urine from two groups of spiny lobsters and compared their behavior with each other and with controls. Supplementary material Fig.1B shows the method of preventing urine release and collecting urine, and supplementary material Fig.S4 provides a graphic representation of this assay. We compared interactions of paired spiny lobsters from three groups: (1) catheterized spiny lobsters, which could not release urine (‘Cath’); (2) catheterized spiny lobsters paired with experimenter-controlled presentation of urine (‘Cath+Urine’); and (3) uncatheterized control spiny lobsters, which released urine normally (‘Control’). Spiny lobster pairs had less than 2% difference in carapace length to minimize the effects of size in determining social status. We based this matching on studies on American lobsters where the eventual social status of paired animals was random if carapace length difference was within 5% (Scrivener, 1971; Karavanich and Atema, 1998a).

Spiny lobsters of all three groups were initially placed individually into 801 aquaria for at least 1 week prior to pairing. During this
time they were fed every other day, with the last feed 2 days before pairing. Urine released from catheterized animals was collected and stored at –20°C. We measured the volume of urine released during the following conditions: (1) within 1 h of feeding; (2) 24 h after feeding; (3) 24 h after a second feeding; and (4) every hour for 5 h during one dark cycle. These data provided baseline measurements of urine release for isolated animals, to compare with release during pairing.

Spiny lobsters from all groups were paired for 24 h, the first hour of which was video recorded and analyzed later. After the first hour and 24 h later, all spiny lobsters were offered a piece of food (shrimp or squid) directly over their legs. Physical interactions (number and duration of aggressive and submissive or avoidance behaviors, as described above) were scored during the first hour of interaction. Other measurements were volume of urine released and food intake, depending on the group. The volume of urine released was measured during the first hour of interaction in both the Cath and Cath+Urine group. Urine release during interactions was measured and separated according to the three conditions, as before: phys-int, in contact and distant. The total volume of urine released was also measured after 24 h of pairing in the Cath group. Food ingestion was scored after the first hour and after 24 h of interaction in all groups.

The second group of animals (Cath+Urine) consisted of pairs of animals that, like those in the Cath group, were catheterized and thus could not release urine, but we released urine during the experiment to determine its role in agonistic interactions and in influencing social status. Urine of each of these isolated catheterized spiny lobsters was collected during the previous few days and stored at –20°C. Urine was thawed to room temperature before use. We injected urine of one member of the pair, which was chosen randomly, in three pulses during 1 h of pairing. Each pulse was ~3.3 ml over ~20 s, for a total of 10 ml. We delivered three pulses randomly, in three pulses during 1 h of pairing. Each pulse was ~3.3 ml over ~20 s, for a total of 10 ml. We usually injected urine when spiny lobsters were prodded with plastic tongs, for 2 min every 20 min over a 4 h period, and the urine was pooled. Urine was frozen at –20°C until used. The control stimulus was sea water. All experiments were video recorded (Sony DCR PC110) under reduced red light during the dark phase, and analyzed by individuals unaware of the experimental conditions. Differences between control and experimental stimuli were tested for significance using a non-parametric Wilcoxon matched-pairs test or McNemar test, depending on the nature of the dependent measure.

To determine whether the olfactory (aesthetasc) pathway is necessary or sufficient to mediate responses to urine, we performed behavioral experiments before and after ablations of olfactory (aesthetasc) or non-olfactory (non-aesthetasc) chemosensilla. We performed behavioral tests on 20 spiny lobsters, first on all animals before treatment (‘intact’) and then after either ablation of aesthetasc sensilla (nine spiny lobsters) or sham treatment (11 spiny lobsters). Next we performed behavioral tests on 23 spiny lobsters, first on all animals before treatment and then after either ablating the non-aesthetasc sensilla from antennules (10 spiny lobsters) or sham treatments (10 spiny lobsters). Ablation of the aesthetascas and non-aesthetascas and sham treatments was accomplished as described previously (Shabani et al., 2008).

To determine the effectiveness of the sensillar ablations, we collected the spiny lobsters’ antennular flagella after completing the behavioral assays according to Schmidt and Derby (Schmidt and Derby, 2005) and Shabani et al. (Shabani et al., 2008). Flagella were cut and then fixed in 4% paraformaldehyde (in 0.1 mol l–1 Sorensen phosphate buffer + 15% sucrose, or SPB) for 24 h. We then rinsed the flagella with SPB and stored them in SPB with 0.02% sodium azide until analyzed. To make 0.1 mol l–1 SPB, we dissolved 6.8 g KH2PO4 and 21.3 g Na2HPO4 in 1 l deionized H2O, adjusted the pH to 7.4, and filtered the solution. For aesthetasc ablated spiny 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 aesthetascas.) For non-aesthetasc ablated spiny lobsters, we counted the number of intact aesthetasc and non-aesthetasc sensilla on the lateral and medial flagella. For both treatments, we calculated the percentages 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% of aesthetasc sensilla were ablated. In the process of ablating aesthetascas, 52.1±3.4% of the asymmetric sensilla and 3.2±0.6% of the guard sensilla were damaged, but none of the seven other types of non-aesthetasc sensilla were affected. In the non-aesthetasc targeted group, we ablated 97.7±0.7% of the asymmetric sensilla and 99.7±0.1% of the other eight types of non-aesthetasc sensilla. In the process of ablating the non-aesthetasc sensilla, 49.8±6.6% of the aesthetasc sensilla were damaged.
We also performed the same behavioral assay to test whether behavioral responses to urine are concentration dependent. We only measured appetitive and alarm responses to urine or sea water; shrimp juice was not tested, so no suppression of appetitive responses was measured. Individual spiny lobsters were tested with urine from 1% to 0.001% full strength. Two groups were tested: one with 1% to 0.1% urine and sea water as control, and another with 0.01% to 0.0001% urine and 1% urine and sea water as control. These stimuli were presented randomly over a 3 day period.

**Field experiment of responses of spiny lobsters to urine**

A field experiment was performed to determine whether wild Caribbean spiny lobsters show the same avoidance or alarm responses to urine of conspecifics as they do in the laboratory. The experiment was performed in the waters near the Florida Fish and Wildlife Conservation Commission facility in Marathon, FL. Detailed descriptions of the study site and experimental setup have been published previously (Shabani et al., 2008). We recorded from 21 sites, consisting of crevices of various shapes and spiny lobsters of varying numbers, all in water approximately 1–3 m deep. Crevices contained on average 2.7±0.2 spiny lobsters, a total of 56 spiny lobsters. Stimuli were delivered to spiny lobsters through two Silastic tubes placed at a crevice prior to the behavioral experiments. Each tube (i.d. 0.4 mm, o.d. 0.5 mm) had the delivery end positioned ca. 0.3 m from the crevice and the loading end outside the water. We delivered the experimental stimulus (P. argus urine, diluted 100 times with filtered natural sea water collected locally) and the negative control stimulus (filtered sea water) using different tubes. Urine was pooled from three catheterized spiny lobsters (both sexes) in the laboratory and kept at –20°C until used. Our experiments had a paired design, with each spiny lobster presented with two stimuli. Sea water, a negative control stimulus, was presented first, followed by a 3–4 min observation period. Then, urine 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 urine 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 sea water almost never produced this response. Thus, given our aim of using the power of a paired design, we always presented the sea water control first.

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

**RESULTS**

**Context of urine release in VU lobsters**

Spiny lobsters released urine under some specific conditions and not others. One important context of urine release was agonistic interactions, and we focus on this in our paper. Spiny lobsters did not release urine under most other conditions, including when presented with conspecific odors (hemolymph or urine), food odors (shrimp juice), or threat (physical disturbance), whether they were isolated or paired with a conspecific. We also note another context in which spiny lobsters release urine: immediately after ingesting food. This occurred whether animals were isolated or paired with a conspecific (see supplementary material Movies 1 and 8). Isolated VU lobsters released urine within 32±3.2 s (N=7) of eating food in a long pulse that lasted 71.2±4.1 s (N=7; supplementary material Movie 1). VU lobsters paired with conspecifics released urine within 50.5±3.6 s of eating, with pulses lasting 70.3±5.0 s (N=7).

**Visualized urine release during interactions**

Paired VU spiny lobsters released urine during the first hour of interaction, especially when in close contact (Fig. 1). Spiny lobsters stayed in contact without engaging in physical aggressive behavior for most of the first hour (2190±1164 s, or 61% of the hour). During this hour, they engaged in 3.0±0.4 physical interactions that lasted for a total of 249±46.2 s (or 7% of the hour). The animal that eventually was identified as the dominant member of the pair almost always initiated the attacks (93% of the attacks). The eventual dominant animal also used aggressive behavior in these interactions: its dominance score D was 94±2.6. Consequently, dominants engaged in a significantly greater number of physical aggressive behaviors than subordinates (Wilcoxon matched-pairs tests, P<0.05). The animal that eventually was identified as the subordinate member of the pair almost always engaged in submissive or avoidance behaviors in response to the dominant’s aggressive behaviors and on average had a dominance score D of –90±4.5.

Dominant spiny lobsters had greater urine release than subordinates during the first hour of interaction. Dominants released significantly more long pulses of urine than subordinates (Fig. 1A; Wilcoxon matched-pairs tests, P<0.05), and these long pulses by dominants lasted 102±12 s. In fact, all dominants released urine during the first hour of interaction, but only 40% of subordinates released urine. Consequently, the total duration of urine release during the first hour of interaction was significantly higher for dominants than for subordinates (Fig. 1B; Wilcoxon matched-pairs tests, P<0.05). Animals released urine for a significantly longer time when in physical interaction or in contact compared with when distant (Fig. 1C; Wilcoxon matched-pairs tests, P<0.05). The dominant animal typically acquired food introduced into the aquarium and subsequently released long pulses of urine (see supplementary material Movie 7). In the one pair of the six for which we were unable to determine social status as they showed aggressive and submissive behaviors equally often, both members of the pair released long pulses of urine during all of their interactions, and both ingested food.

**Role of urine in influencing social status**

Dominants always initiated attacks on subordinates and subordinates almost always retreated in response during the 1 h of interaction. Dominance score, D, was 95.1±2.8 (N=17) for dominants and –74.7±9.9 for subordinates.

The behavior of the dominant toward the subordinate was affected by the presence of urine (Fig. 2). Dominants from the catheterized spiny lobsters that could not release urine into the aquarium (Cath) engaged in significantly more attacks than did dominants from catheterized spiny lobsters paired with experimenter-introduced urine (Cath+Urine; Fig. 2A; Mann–Whitney test, P<0.05). Dominants from the control group had a low number of attacks, similar to Cath+Urine animals, but the difference between Control and Cath animals was a strong trend but not statistically significant (P=0.052). Consequently, the total duration of physical interactions after attacks by the dominant animal was significantly longer for the Cath group than for either the Cath+Urine or the Control group (Fig. 2B; Mann–Whitney test, P<0.05). For the Cath+Urine group, the member of the pair whose...
The protocol used to generate these data is shown in supplementary material Fig. S3. The response of laboratory spiny lobsters to urine was dependent on concentration (Fig. 6). Urine at 1% and 0.1% of full strength evoked significantly more avoidance responses than did sea water, expressed as percentage of animals showing avoidance responses (N=53; McNemar test, P<0.05; Fig. 4A), percentage of time spent inside a shelter (Wilcoxon matched-pairs test, P<0.05; Fig. 4B), or percentage of animals with a suppressed appetitive response to food odor (McNemar test, P<0.05; Fig. 4C). Similar results were seen when the urine was from undisturbed spiny lobsters (data in Fig. 4) or from disturbed spiny lobsters (data not shown). Because urine from disturbed and undisturbed spiny lobsters induced the same responses, we tested only urine of undisturbed spiny lobsters in remaining tests.

Ablating the aesthetasc sensilla abolished avoidance responses to urine. Instead, these animals responded to urine with appetitive responses (N=9; Fig. 5A; McNemar test, P<0.05) and similarly spent significantly less time inside the shelter (Fig. 5B; Wilcoxon matched-pairs test, P<0.05). They also tended to show less suppression of responses to food odor after urine, though this difference was not statistically significant (Fig. 5C; McNemar test, P=0.063). On the other hand, ablation of non-aesthetasc sensilla did not affect responses to urine. Ablated animals continued to respond to urine with avoidance behaviors (N=10; Fig. 5A), sheltering (Fig. 5B), and suppression of responses to food odor (Fig. 5C) although at slightly though non-significantly reduced levels compared with pre-ablation levels. Sham-treated animals showed no appreciable changes in behavior after treatment (N=21; data not shown).

The response of laboratory spiny lobsters to urine was dependent on concentration (Fig. 6). Urine at 1% and 0.1% of full strength (when presented in the aquarium, and thus without factoring in any

![Fig. 1. Behavior and urine release by paired VU lobsters. (A) Dominants released long pulses of urine significantly more frequently than subordinates (Wilcoxon matched-pairs tests, *P<0.05; N=5 pairs of lobsters). (B) Total duration of urine release during interactions. Dominants released urine for significantly longer than subordinates (Wilcoxon matched-pairs tests, *P<0.05; N=5 pairs of lobsters). (C) Total duration of urine release for animals (dominant and subordinate combined) when either in physical interaction (phys-int) or within half a body length away and not interacting (in contact) vs when more than half a body length away (distant). Animals released urine for significantly longer when in phys-int or in contact vs when distant (Wilcoxon matched-pairs tests, P<0.05; N=10 animals). Boxes and error bars indicate median and interquartile range. The protocol used to generate these data is shown in supplementary material Fig. S3.](image_url)
Further dilution in the aquarium (N=24, McNemar test, P<0.05). Urine at 0.01% or lower was no more effective in eliciting avoidance than was sea water (N=18, McNemar test, P>0.05).

In the field, wild spiny lobsters (N=56) responded to urine in a similar way to laboratory animals (see supplementary material Movie 8). Urine induced avoidance responses in a significantly greater percentage of spiny lobsters than did sea water (66% vs 7%; McNemar test, P<0.05). Among wild spiny lobsters that showed avoidance behavior, 70% stayed in the same shelter and 30% moved to another shelter.

**DISCUSSION**

**Urine-borne signals influence agonistic behavior and social status**

We showed here that the eventual dominant member of a pair of socially interacting Caribbean spiny lobsters uses two types of agonistic behavior – urine-borne chemical signaling and physical aggressive behavior – to influence the behavior and social status of the eventual subordinate animal. Urine-borne signals are primarily released by the dominants. These urine-borne signals have a significant effect on the behavior of both dominants and subordinates, as indicated through experiments in which urine release is controlled. Dominants significantly increase their aggressive behavior when urine is not released during encounters, and this effect is reversed when urine is experimentally introduced into the aquarium. The effect of urine is greatest when animals are in contact or physically interacting with each other. Animals respond to urine-borne signals with avoidance and with suppression of appetitive responses to food odor, food intake and urine release, but only at relatively high concentrations (0.1% full strength or greater), supporting the idea that urine is used in signaling when animals are in close proximity to each other. These effects of urine are mediated mainly by the olfactory pathway and its aesthetasc sensilla. Some of these effects seen in laboratory conditions were validated through observations of wild spiny lobsters in the field.

**Context of urine release**

The release of urine by paired spiny lobsters depends on the animal’s social status and whether animals are in contact with each other.
Dominants released urine more frequently and in greater amounts compared with subordinates (Figs 1 and 3). Spiny lobsters released their urine more often when touching each other (i.e. in physical interaction or in contact with each other) than when distant from and not in contact with each other (Fig. 1C; Fig. 3B). Similarly, dominant American lobsters and crayfish also released urine more frequently than did subordinates, and the urine release occurred most frequently when fighting (Breithaupt et al., 1999; Breithaupt and Attema, 2000; Breithaupt and Eger, 2002). The release of urine by spiny lobsters not only when fighting but also when in contact though not fighting may be related to the fact that spiny lobsters are gregarious animals while American lobsters and crayfish are not. Therefore, spiny lobsters may not need to couple urine release with aggressive physical interactions as interactions around shelters do not always lead to fights and evictions but, rather, sometimes lead to aggregation.

We did not find evidence to support the idea that spiny lobsters use urine to communicate distress or disturbance to nearby conspecifics. Spiny lobsters did not release urine in response to threats (physical disturbance) or to urine or hemolymph from conspecifics. Furthermore, they responded with the same avoidance behaviors to urine from either undisturbed or disturbed conspecifics. Although this result shows that urine induces avoidance responses similar to hemolymph (Shabani et al., 2008), it also shows that neither release nor response is related to disturbance of conspecifics. This finding is unlike some findings reported for crayfish (Hazlett, 1989; Hazlett, 1990; Zulandt Schneider and Moore, 2000). These studies on crayfish concluded that urine from stressed or disturbed crayfish provides disturbance signals to conspecifics. However, some control experiments that would strengthen this argument were not reported. For example, Zulandt Schneider and Moore (Zulandt Schneider and Moore, 2000) did not test urine from undisturbed...
Spiny lobsters, like many decapod crustaceans, interact when competing for food and shelters, and these interactions may be agonistic. Agonistic interactions for space and food are expressed as early as the post-puerulus larval stage in which animals lung with their antenna and emit rasps or stridulations (Berrill, 1976). Juvenile and adult spiny lobsters from several species (P. argus, P. cygnus, J. lalandei) have been reported in field and laboratory studies to show agonistic interactions similar to those described in our study (Fielder, 1965; Chittleborough, 1974; Berrill, 1975; Lozano-Álvarez, 1996). For example, adult J. lalandei subordinates that resisted eviction by dominants often retreated inside the shelter, and dominants responded by facing them and aggressively attacking by grabbing their legs and antenna (Fieldier, 1965). In response to these attacks, subordinates tail flipped or retreated rapidly away from the shelter. If dominants were challenged in their shelter, they often defended with aggressive behaviors. Dominants often evicted the subordinates from their shelter by merely approaching them. Similar behaviors were observed for American lobster around baited traps (Jury et al., 2001). Dominant American lobsters prevented other lobsters, which were often smaller, from entering the baited trap: 89% of lobsters entered the occupied trap only half-way and immediately retreated while only 11% made full entry. These results support the idea that at least one form of agonistic behavior examined in our study of P. argus – physical aggressive acts – also influences the behavior of conspecific lobsters of several species. These other studies did not examine the other agonistic behavior examined in our study – chemical signaling through urine cues – so the use of chemical signals in these cases remains unclear.

Urine signals that communicate social status in P. argus are probably functioning only at a close distance. We found responses to 0.1% urine but not to lower concentrations (Fig. 6). Measurements on coral reefs near Key Largo suggest that chemical signals are diluted to 0.1% of their original concentration within 1 m of release (R. K. Zimmer, personal communication). Thus, urine released from spiny lobsters in their natural environment is probably functioning at close distances. This is consistent with our finding that animals release and respond to urine when they are close to or even in contact with conspecifics (Figs 1–3). Diluted urine or urine released from a long distance, however, may have a different function. Urine released from a long distance may signal shelters occupied with conspecifics, which would be adaptive in limiting the time spent outside shelters and thus reduce exposure to predators (Childress and Herrnkind, 2001).

Previous studies indicated that when conspecific urine is presented 2 m upstream, spiny lobsters prefer shelters with conspecific urine (Horner et al., 2006; Horner et al., 2008b). Horner and colleagues observed over the course of 30 min the behavior of lobsters that were introduced into one end of a long flume that had shelters at the end. These animals were handled 30 min prior to testing, unlike...
our study in which animals were not handled for at least a week prior to testing avoidance responses. Handling of spiny lobsters and the larger, more open space may have triggered their sheltering behavior. Thus, depending on the context of urine release, spiny lobsters may have very different adaptive responses to urine. It is not known what chemical signals from urine mediate these contrasting responses in different contexts.

**Conspecific cues and their sensory pathways**

The avoidance responses to urine are very similar to avoidance behaviors that spiny lobsters show to hemolymph-borne alarm cues. Avoidance behaviors to hemolymph alarm cues include those examined in this study: retreat, increased shelter time, and suppression of appetitive responses to food odor (Shabani et al., 2008). Risk assessment is especially critical when spiny lobsters return from foraging to occupied shelters. Some shelters may contain conspecific chemical cues of aggregation but also alarm cues from injured conspecífics. Spiny lobsters are known to avoid shelters scented with fluids of injured or diseased conspecifics, or scents of predators (Berger and Butler, 2001; Parsons and Eggleston, 2005; Parsons and Eggleston, 2006; Behringer et al., 2006; Bouwma, 2006; Briones-Fourzán et al., 2008; Horner et al., 2008b).

Urine-borne signals that communicate social status to spiny lobsters (our study) and hemolymph cues that induce alarm responses (Shabani et al., 2008) are both largely or exclusively detected through the olfactory (aesthetasc) pathway (Fig. 5). Spiny lobsters with ablated aesthetasc sensilla showed no avoidance responses to urine, indicating the role of this olfactory pathway in detecting urine signals. Animals with non-aesthetasc antennular chemoreceptors ablated continued to respond to urine, though at a somewhat (statistically non-significant) lower level. This reduction may indicate that non-aesthetasc sensors play a minor role in mediating responses to urine, much less than aesthetasc.

Alternatively, the reduced response in non-aesthetasc ablated animals may be due to collateral damage to aesthetasc that resulted during the surgical elimination of the non-aesthetasc sensilla, wherein ca. 50% of the aesthetasc were damaged. In either case, it is clear that aesthetasc principally drive the response to conspecific urine signals.

Our findings complement suggestions from previous studies about the role of the olfactory pathway in social behavior of American lobsters (Johnson and Atema, 2005) and crayfish (Horner et al., 2008a). American lobsters reduce the duration of fights in subsequent encounters with familiar opponents but fail to reduce it if aesthetasc sensilla are ablated. Crayfish with ablated aesthetasc also fail to reduce fighting in subsequent encounters. Interestingly, in both studies, social status of dominants is not reversed because of aesthetasc ablation. These results suggest that a lack of communication through one mechanism, namely chemical, may be compensated by another mechanism, physical aggression. We hypothesize that the increase in aggression is partly because dominants lack feedback from their own urine signals.

Our results also support the view that urine-borne signals have multiple functions and that these functions are likely to be mediated through the olfactory pathway. For example, spiny lobsters are gregarious animals and prefer shelters scented with odors of conspecifics (Zimmer-Faust et al., 1985; Ratchford and Eggleston, 1998; Nevitt et al., 2000; Homer et al., 2006; Horner et al., 2008b; Childress, 2007; Briones-Fourzán and Lozano-Alvarez, 2008; Briones-Fourzán et al., 2008). This preference may aid spiny lobsters to locate shelters through a guide-post effect, in which they limit their time of searching for shelters (Childress and Herrkind, 2001). Ablating aesthetasc sensilla eliminates this preference for shelters (Horner et al., 2008b). Urine is also important in mating. Female American lobsters show a greater preference for shelters occupied by dominant males than subordinate males; however, this preference by females is lost if urine release by males is blocked (Bushmann and Atema, 2000). Male blue crabs with ablated aesthetasc no longer perform courtship display behavior in response to the pheromone of reproductive females (Gleeson, 1982), and male helmet crabs with an ablated distal half of the lateral flagellum, which harbors the aesthetasc sensilla, do not respond to female signals (Kamio et al., 2005). The use of a single source of chemicals for signaling in multiple contexts occurs in other animals as well. For example, golden hamsters use flank-glands for both individual recognition and identification of sex and reproductive state (Johnston, 2003).

**Conclusions**

Our studies on Caribbean spiny lobsters and the studies of others on other decapod crustaceans indicate that urine-borne signals have an important role in communicating social status. These collective studies support the idea that urine-borne signals released primarily by a dominant animal affect the length of an interaction not just because they induce avoidance behavior from the subordinate but also because they provide feedback to the dominant. Spiny lobsters control their release of urine according to whether they are socially interacting – that is, they are more likely to release urine when in contact or physically interacting than when they are more distant from each other. Furthermore, we show that the urine-borne signals communicating social status are detected by the olfactory (aesthetasc) sensilla, which are the same sensors that detect other conspecific cues – urine-borne aggregation cues and hemolymph-borne conspecific alarm cues. Therefore, our study provides new information about the mechanism of social competition in spiny lobsters. Furthermore, because spiny lobsters are gregarious animals, they are an excellent model in which to contrast mechanisms of urine-borne communication during competition and aggregation, as well as with competition among other decapods that are solitary.

We thank Thomas Matthews and Kerry Maxwell (Florida Fish and Wildlife Conservation Commission in Marathon, Florida) for providing space, animals and other support during the field studies, Fatbardhe Krasniqi for invaluable assistance with the field work, and Mark Hay, Michael Schmidt, Paul Katz and Ingrid Grober for discussion and review of the manuscript. Funding was provided by NSF grants IBN-0077474, IBN-0324435 and IBN-0614685, and a graduate fellowship from the Center for Behavioral Neuroscience through the STC Program of NSF under Agreement No. IBN-9876754.

**REFERENCES**


Brock, F. (1930). Das Verhalten von Einzelkrebsen Pagurus angustus aus Herbst. S. Shabani, M. Kamio and C. D. Derby


A

One-way valve

3-way connector

Tube C

Silica bead

Spiny lobster nephropores

Tube A

Tube B

B

Tube C

Spiny lobster nephropores

Tube A

Tube B

Fluorescein cartridge
Behavioral Assay of Stimuli Influencing Urine Release

Sea Water  

**Stimulus**

Food offered

Measurements:  

<table>
<thead>
<tr>
<th></th>
<th>UR</th>
<th>UR</th>
<th>Ingestion &amp; UR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min):</td>
<td>5</td>
<td>5</td>
<td>30-60</td>
</tr>
</tbody>
</table>

3 days

Physical disturbance  →  UR measured

5 days

Pair VU lobster with another conspecific

2 days

Repeat procedure

*Urine Release (UR) measured as number and duration of pulses

**Stimulus = conspecific urine, conspecific hemolymph, shrimp juice, or physical disturbance
Behavioral Assay of Urine Release During Social Interactions

Measurements: | Behavior & UR* | Ingestion & UR
---|---|---
Time: | 1 week | 1st hr video-recorded
Animals | Animals | Food
Isolated | Paired | Offered

*Behaviors:

**Agonistic**: Antennae-locking, Antenna-grabbing, Leg-grabbing, Abdomen-poking

**Avoidance**: Retreat, Tail-flipping, Stridulating

*Urine Release (UR)*: measured as # of pulses and duration of each pulse (sec)
Behavioral Assay of the Role of Urine Release in Communicating Social Status

Measurements: UR | Behavior & UR* | Ingestion & UR | Ingestion & UR
Time: 1 week | 1st hr video-recorded | 24 h | 24 h
Animals Isolated | Animals Paired | Food offered | Food offered

*Behaviors:
Agonistic: Antennae-locking, Antenna-grabbing, Leg-grabbing, Abdomen-poking
Avoidance: Retreat, Tail-flipping, Stridulating

*UR - Urine Release measured as volume per mL
Behavioral Assay of Responses to Urine

% of time inside shelter

Alarm and Appetitive Responses

45 sec
1-10 ml Shrimp juice

120 sec
10 ml Stimulus*

30 sec
1-10 ml Shrimp juice

* Stimulus: Urine from undisturbed and disturbed lobsters, seawater

** Appetitive: Appetitive (approach) responses before and after stimulus delivery