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THE ROLE OF CHEMICAL SENSES IN PREDATION, RISK ASSESSMENT, AND SOCIAL BEHAVIOR OF SPINY LOBSTERS

by

SHKELZEN SHABANI

Under the direction of Dr. Charles D. Derby

ABSTRACT

Chemical senses play a critical role in predator-prey and social interactions of many animals. Predators often evoke adaptive escape responses by prey, one of which is the release of chemicals that induce adaptive avoidance behaviors from both predators and conspecifics. I explore the use of chemicals in predator-prey and social interactions, using a crustacean model system, the spiny lobster.

As predators, spiny lobsters are opportunistic, polyphagous feeders, and they rely heavily on their chemical senses during feeding. Some of their potential prey deter attacks through chemical defenses that act through the spiny lobsters' chemical senses. An example of this is sea hares, *Aplysia californica*, which secrete an ink when vigorously attacked by sympatric spiny lobsters, *Panulirus interruptus*. I show that that this ink defends sea hares from spiny lobsters through several mechanisms that include phagomimicry, sensory disruption, and deterrence, and that the ink's efficacy is enhanced by its naturally high acidity.

As prey, spiny lobsters rely heavily on their chemical senses to assess risk from predators. One way to assess risk of predation is through 'alarm cues', which are injury-related chemicals. I show that injured Caribbean spiny lobsters, *Panulirus argus*, release alarm cues in their hemolymph, and that nearby conspecifics detect these cues using olfaction. Hemolymph

from conspecifics induces primarily alarm behavior in the form of retreat, sheltering, and suppression of appetitive responses. In contrast, hemolymph from heterospecifics, depending on phylogenetic relatedness, induces either mixed alarm and appetitive behaviors or primarily appetitive behaviors.

Spiny lobsters also use chemical cues to assess risk during social interactions with conspecific. I show that spiny lobsters use urine-borne chemical signals and agonistic behaviors to communicate social status and that these chemical signals are detected exclusively by the olfactory pathway. Dominant animals increase urine release during social interactions, whereas subordinates do not. Experimental prevention of urine release during interactions causes an increase in agonism, but this increase is abolished when urine of dominants is reintroduced. My findings lay the foundation for neuroethological studies of risk-assessment systems mediated by intraspecific chemical cues.

INDEX WORDS: Agonistic, Alarm cue, Alarm pheromone, Avoidance, *Aplysia californica*, Chemical defenses, Deterrence, Dominant, Olfaction, *Panulirus argus*, *Panulirus interruptus*, Phagomimicry, Risk assessment, Sea hare, Sensory disruption, Signal, Social status, Spiny lobster, Subordinate, Urine.

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SHKELZEN SHABANI

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

In the College of Arts and Sciences

Georgia State University

2008

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BEHAVIOR OF SPINY LOBSTERS

by

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To my caring American dad, Rick Hill

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written proposals, no matter how small they were, were read and returned by Chuck in a timely manner. Indeed, I am proud of his dependable nature. He was also sensitive in giving me the attention I needed, though one carrot at a time. I could go on for some time about Chuck, but just want to say, ‘C, thank you and remember that in my eyes you always rock’.

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TABLE OF CONTENTS

DEDICATION	iv
ACKNOWLEDGMENTS	v
LIST OF TABLES	x
LIST OF FIGURES	xi
LIST OF ABBREVIATIONS	xii
CHAPTERS	
1 GENERAL INTRODUCTION	1
- Chemosensory role in avoiding chemical defenses	3
- Alarm cues in spiny lobsters	8
- Chemical communication of social status	13
2 SEA HARES USE NOVEL ANTIPREDATORY CHEMICAL DEFENSES	19
Introduction	20
Methods and Material	20
Results	24
Discussion	35
3 ACIDITY ENHANCES THE EFFECTIVENESS OF ACTIVE CHEMICAL DEFENSIVE SECRETIONS OF SEA HARES, <i>APLYSIA CALIFORNICA</i> , AGAINST SPINY LOBSTERS, <i>PANULIRUS INTERRUPTUS</i>	38
Introduction	39
Methods and Material	41

Results	49
Discussion	60
4 SPINY LOBSTERS DETECT CONSPECIFIC BLOOD-BORNE ALARM CUES EXCLUSIVELY THROUGH OLFACTORY SENSILLA	64
Introduction	65
Methods and Material	68
Results	77
Discussion	86
5 SPINY LOBSTERS USE URINE-BORNE SIGNALS TO COMMUNICATE SOCIAL STATUS	92
Introduction	93
Methods	97
Results	109
Discussion	123
6 GENERAL DISCUSSION	130
- Discussion	130
- Mechanisms of chemical defenses at the chemosensory level	130
- Mechanism of action of the chemical defenses of sea hares	133
- Avoidance of active predators through intraspecific chemical cues	134
- Risk assessment pathway	139
- Communication of social status	141

- Function of urine-borne signals	144
LITERATURE CITED	147

LIST OF TABLES

Chapter 2

Table 2.1	Concentrations of amino acids, ammonia, and urea in ink, opaline, and hemolymph of <i>Aplysia californica</i> and <i>Aplysia dactylomela</i> .	29
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Chapter 4

Table 4.1	Intensity scale for the responses of <i>P. argus</i> to stimuli	74
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LIST OF FIGURES

2-1	Effect of ink and opaline secretions on survival of sea hare and spiny lobster behavior in attacks by spiny lobsters.	26
2-2	Composition of opaline, ink, and hemolymph of sea hares.	28
2-3	Behavioral responses of spiny lobsters to defensive secretions of sea hares.	31
2-4	Responses of antennular and mouthpart chemoreceptor neurons of spiny lobsters to sea hare defensive secretions.	34
3-1	The percentage of lobsters attracted to acidic stimuli was significantly higher for all stimuli.	51
3-2	In the 2nd maxillipeds, over 50% of chemoreceptor neurons were highly sensitive to pH change alone and to other stimuli.	53
3-3	In the antennules, over 50% of chemoreceptor neurons were highly sensitive to pH change alone and to other stimuli.	57
3-4	The response of a population of olfactory receptor neurons ($n = 39$) to high concentrations of chemicals was enhanced at acidic pH.	58
3-5	Three examples of the diversity of responses of ORNs to stimulation with 0.1% ink at three pH values	59
4-1	Chemosensory organs of spiny lobsters. A1, first antenna or antennule; A2, second antenna.	67
4-2	Hemolymph (HEM) induced alarm responses.	78
4-3	Ablating aesthetasc sensilla eliminated all forms of alarm response to hemolymph.	81
4-4	Ablation of non-aesthetasc sensilla did not affect any form of alarm	83

behavior in response to hemolymph.

4-5	Spiny lobsters were more likely to show alarm responses to conspecific hemolymph compared with either congeneric hemolymph or hemolymph from a brachyuran crab.	85
4-6	Field tests of alarm responses of <i>P. argus</i> to conspecific hemolymph.	86
5-1	Behavior and urine release by a pair of dominant and subordinate spiny lobsters.	112
5-2	Dominants A) engaged primarily in offensive behaviors whereas subordinates engaged in defensive behaviors (N = 17 pairs).	115
5-3	Only dominants increased the frequency of urine release during social interactions.	117
5-4	Urine induced avoidance responses in a significantly greater percentage of lobsters than did sea water.	119
5-5	Effect of urine concentration on behavioral responses of spiny lobsters.	120
5-6	Ablation of aesthetasc (n = 9) but not non-aesthetasc sensilla (n = 10) eliminated all forms of avoidance responses to urine.	122

LIST OF ABBREVIATIONS

HEM	Hemolymph
SW	Sea water
ORNs	Olfactory receptor neurons

CHAPTER 1

GENERAL INTRODUCTION

Introduction

Predator-prey and social interactions in animals can depend heavily on chemical senses. In predator-prey interactions, prey animals use various evasive tactics against attacking and threatening predators. These evasive responses of prey can be triggered either directly by attacking predators or cues released by conspecifics that are attacked by predators. For example, when attacked by predators, prey may release chemicals that either defend the prey from predators by being noxious and/or toxic to predators or defend nearby genetically related conspecifics by evoking escape behaviors (Blum, 1985; 1996; Wyatt, 2003; Derby, 2007; Paul et al., 2007). These responses give prey important advantages in predator-prey interactions (Lima and Dill, 1990; Lima, 1998). In social interactions, animals often compete with their conspecifics for resources such as food, space, and mates. In the process, they often use chemical signals to gain advantages (Rowell, 1974; Drews, 1993; Barroso and Boza, 2000; Petrulis et al., 2004; Burmeister et al., 2005; Gherardi, 2006; Barata et al., 2007; Hovland et al., 2008; Izawa and Watanabe, 2008; Val-Laillet et al., 2008). Chemical signals can induce aggressive and avoidance behaviors, depending on the social status of the animals. My dissertation is an exploration of the involvement of chemicals in predatory-prey and social interactions of spiny lobsters.

An example of this is the use of chemical cues released by prey to either escape from predators and to either deliberately or circumstantially warn nearby conspecifics. This dual function of chemicals is best characterized in eusocial insects (Blum, 1985). Aphids release

cornicle droplets that both glue the mouth pieces of predators and release chemicals that induce avoidance responses in nearby conspecifics (Nault, 1973; Hardie et al., 1999; Wyatt, 2003).

Other tactics of escape involve chemical cues leaked after injury that circumstantially benefit nearby conspecifics (Chivers and Smith, 1998). This is common among aquatic animals (Pfeiffer, 1977; Smith, 1992; Chivers and Smith, 1998). For example, fish injured or killed when attacked by a predator leak chemicals that induce avoidance responses in nearby conspecifics (Pfeiffer, 1977; Wisenden et al., 2008). Some animals, including cephalopods, lizards, and decapod crustaceans, use escape tactics, such as autotomy, which is the release of limbs at specific breakage planes when those limbs are grabbed or damaged during attacks (Fleming et al., 2007). Autotomized limbs and bodily injuries leak chemicals that can benefit conspecifics nearby that detect them. Decapod crustaceans provide examples of this (Juanes and Smith, 1995; Weiss et al., 2006). My dissertation includes an exploration of chemosensory mechanisms underlying the detection of alarm cues in Caribbean spiny lobsters.

Chemical communication is important in social interactions of many species (Rowell, 1974; Drews, 1993; Barroso and Boza, 2000; Petrulis et al., 2004; Burmeister et al., 2005; Gherardi, 2006; Hovland et al., 2008; Izawa and Watanabe, 2008; Val-Laillet et al., 2008). Fights between two individuals, called dyadic fights, may start symmetrical, with the two acting equally aggressively, and then progress with one winning and the other losing. Those that win show aggressive behavior and are referred to as dominants, and those that lose show avoidance or defensive behaviors and are referred to as subordinates (Drews, 1993). Dominants often gain greater access to resources (Wilson, 1975). During dyadic fights, many animals communicate using chemical signals (Wyatt, 2003; Petrulis et al., 2004; Barata et al., 2007). For example, dominant rodents release scent-marks that facilitate and enforce the social status of subordinates.

These scent-marks are detected by the main olfactory and vomeronasal pathways (Mandiyan et al., 2006; Chamero et al., 2007). Similarly, fish communicate using chemical signals during dyadic fights (Barata et al., 2007). Chemical signaling can end the fight sooner or even prevent it, thus minimizing chances of injury or expenditure of energy between opponents (Rutte et al., 2006). Many solitary and gregarious decapod crustaceans chemically communicate during interactions (Berrill, 1975, 1976; Scrivener, 1971; Bruski and Dunham, 1987; Issa et al., 1999; Breithaupt and Atema, 2000; Breithaupt and Eger, 2002). My dissertation includes an analysis of the roles of chemical communication in social interactions, including sensory mechanisms, using Caribbean spiny lobsters.

Chemosensory role in avoiding chemical defenses of prey

Sea hares, which are gastropods with reduced and internalized shells, escape and avoid capture by predators through multifunctional and chemically-rich defensive secretions. Sea hares can secrete defensive compounds both passively and actively (Kinnel et al., 1979; Faulkner, 1992; Nolen et al., 1995; Johnson and Willows, 1999; Cimino and Gavagnin, 2006). Passive chemical defenses are present as deterrent compounds in the skin, some of which are acquired through diet, and do not require neural activation for their release (Thompson, 1960; Ginsburg and Paul 2001). Active chemical defenses are released under nervous system control during physical attacks by predators (Carew and Kandel 1977; Johnson and Willows, 1999). Actively released defensive secretions have various functions, among which two are uniquely used for escape and avoidance from predators. Defensive secretions protect sea hares by inducing aversive responses on predators (DiMatteo, 1981, 1982; Walters et al., 1993; Pennings, 1994; Nolen et al., 1995; Kicklighter et al., 2005; Kicklighter and Derby, 2006). These

defensive secretions are also sensed by nearby conspecifics as an indication of active predation (Kicklighter et al., 2007).

A dramatic example of an active release of defensive compounds is inking by sea hares (*Aplysia*), wherein a purple cloud of pigmented and other substances is formed. This slimy secretion is composed of two glandular products, ink and opaline, that are typically released simultaneously (Tritt and Byrne, 1980; Prince et al., 1998; Nolen and Johnson, 2001). Ink is diffusible and purple, whereas opaline is whitish and highly viscous. Ink contains red-algal-derived pigments, secondary metabolites, proteins, and other chemicals (MacColl, 1990; Pennings and Paul, 1993; Johnson and Willows, 1999; Petzelt et al., 2002). Opaline contains algal secondary metabolites, proteins, and other compounds (Johnson and Willows, 1999; Rogers et al., 2000). Ink and opaline are acidic (~ pH 5: personal observation), and this acidity may contribute to the efficacy as chemical defense. A striking feature of some chemical defenses of sea hares and other opisthobranchs is the use of acids (Gillette et al., 1991; Thompson, 1960, 1983, 1986, 1988).

Sea hares not only generates a cloud that can affect visually oriented predators, but chemicals in it can interact with the chemosensory organs of predators (Derby, 2007). The suggestion that ink of sea hares generates visual distraction on visual predators comes from examples on other mollusks. Observational work on cephalopod mollusks such as octopus and squid has implied that these animals use ink for visually distracting threatening predators (Bush and Robinson, 2008; Wood et al., 2008). A threatened squid will rapidly swim away from the stimulus and release a cloud of ink. The cloud of ink distracts predators long enough for the squid to escape. However, manipulation studies to test the function of inking behavior in cephalopods are lacking (Derby, 2007; Wood et al., 2008). For mollusks such as sea hares,

however, visual distraction is a weaker explanation, since ink and opaline are released only after a sea hare is in the mouth of the predator. Furthermore, the slow escape behavior of sea hares makes it difficult for the sea hare to escape before the ink cloud dissipates. A more likely explanation is that chemicals in ink and opaline affect the chemosensory organs of predators, allowing the sea hares to escape. Some have hypothesized that these chemicals have many functions, including bile excretion, antimicrobial effects, camouflage, aposematism, startle, alarm signaling, sensory irritation, and feeding deterrence (Pennings and Paul, 1993; Carlson and Nolen, 1997; Carefoot et al., 1999; Johnson and Willows, 1999; Petzelt et al., 2002). Most of these hypotheses and particularly those regarding escape remain largely untested.

Most chemical defenses are thought to be toxic or deterrent, and this may be one mode of action of sea hares' chemical defenses. Sea hare ink can be a chemical deterrents against predatory sea anemones, fish, and spiny lobsters (Kicklighter et al., 2005; Kicklighter and Derby, 2006; Kamio et al., 2007). Some of the deterrent molecules are known. Escapin, an L-amino acid oxidase in ink, reacts with L-lysine to generate hydrogen peroxide and an equilibrium mixture of compounds, some of which are anti-microbial and predator deterrents (Yang et al., 2005; Aggio and Derby, 2008). Feeding assays using spiny lobsters and fish in our laboratory show that these molecules suppress appetitive and ingestive responses to food. Ink and opaline however contain many components, the functions of which remain unknown but may vary depending on the predator. For example, ink of *A. californica* induces aversive responses in sea anemones but appetitive responses in crustaceans. Sea hares can be completely engulfed by sea anemones before they release ink and opaline. Upon release of ink, the sea anemone will regurgitate the sea hare and retract its tentacles. Thus, ink in particular has chemicals that induce tentacle retraction (Kicklighter and Derby, 2006). Although opaline alone may contain deterrent

compounds, it is not sufficient to deter the sea anemone from eating a sea hare. In fact, opaline stimulates appetitive responses in sea anemones (Kicklighter and Derby, 2006). However, ink and opaline have opposite effects on portunid crabs and spiny lobsters (Aggio and Derby, 2008; Johnson, 2002). Only opaline contains high enough concentration of deterrent chemicals to induce aversive responses in spiny lobsters (Aggio and Derby, 2008). Ink and opaline can induce appetitive responses in California spiny lobsters *P. interruptus*. The mechanisms whereby the chemicals in the defensive secretion of *Aplysia* manipulate predators' chemosensory systems and provide a defense have only recently begun to be understood. Chapters 2 and 3 examine mechanisms of chemical defense by the sea hare *A. californica* against its sympatric predator *P. interruptus*, taking advantage of the fact that spiny lobsters are model systems in chemosensory neurobiology (Zimmer-Faust, 1987; Derby, 2000; Ache, 2002; Caprio and Derby, 2008).

California spiny lobsters have been anecdotally reported to attack *A. californica* in their natural environment (Johnson, 2002); however, the success rate of such attacks is not known. In general, the diet of spiny lobsters consists mostly of various mollusks and small crustaceans. California spiny lobsters attack and consume *A. californica* in the laboratory (Pennings, 1990). Furthermore, according to preliminary results by Johnson (2002), a significantly greater number of *Aplysia* with ink and opaline glands removed than with ink and opaline glands intact were killed by the California spiny lobsters. In all of these interactions, sea hares with intact ink and opaline glands escaped after release of ink and opaline. Interestingly, in response to these secretions, spiny lobsters engaged in a number of behaviors, including appetitive behaviors. These appetitive responses were not out of context since amino acid analysis of ink and opaline secretions revealed high amounts of amino acids and ammonium. Amino acid concentrations were above threshold levels for sensing by the spiny lobsters. These amino acids induced

appetitive responses from spiny lobsters in the laboratory (Johnson, 2002). However, these experiments did not test the necessity and sufficiency of ink and opaline glands on the survival of sea hares against predatory spiny lobster. Based on these limited results, Johnson (2002) suggested that one mechanism by which sea hares may survive is through ‘phagomimicry’. Phagomimicry is defined as a deception in which process chemical defenses stimulate the chemosensory system of the spiny lobsters to attend to false food stimulus. Phagomimicry functions because ink and opaline have high quantities of amino acids and other food-associated compounds that are highly stimulatory to the chemical senses of spiny lobsters and other predators. Another possible mechanism is sensory disruption, in which the sticky secretion, with its high concentrations of stimulants, adheres to the predators’ sensory organs and functionally disrupts them. Tests of these hypotheses are presented in Chapter 2.

Spiny lobsters rely heavily on their chemosensory organs during foraging and feeding. To forage, they use their antennules for detection and orientation toward distant food odors. To feed, spiny lobsters use legs and mouthparts to taste and handle food (Garm, 2004; Garm et al., 2005; Garm and Høeg, 2006). Functional disruption of chemosensory activity by sea hare secretions may explain the behavior of spiny lobsters toward sea hare secretions, such as extensive grooming of mouthparts and antennules, which allow the sea hare to escape. Therefore, I explored the possibility of this phenomenon through behavior and electrophysiological tests in the chemosensory organs of the California spiny lobster.

Another aspect of ink and opaline ignored previously is its acidity. Acids are widely used by marine gastropods as chemical defenses. Acids such as sulfuric acid are packaged in special glands in the skin (Thompson, 1960, 1983, 1986, 1988). When marine gastropods are attacked by predators, these glands quickly release copious amounts of mucus laden with acid, making the

skin highly acidic. For example, irritation of the skin causes skin mucus of *Pleurobranchaea californica* to reach a pH of ~2 (Gillette et al., 1991) and the mucus of *A. californica* to reach a pH of 3-4 (personal observation). How ink and opaline are made acidic – whether by sulfuric acid or other acids – is unknown. How the acidity in these gastropod skin secretions affects chemical responses of predators is also unknown. Spiny lobsters that grab sea hares will encounter the acidic skin mucus with their legs, mouthparts, and possibly even the antennules. I hypothesize that the acidity of the to ink and opaline enhances their defensive properties in a manner similar to skin secretions.

Therefore, in Chapter 3, I asked whether acidity of sea hare secretions enhances the phagomimetic defense by enhancing the neural and behavioral responses of predators to ink and opaline secretions. To answer this question, I measured behavioral and neural chemosensory responses of spiny lobsters to secretions of sea hares presented at a range of pH values. The spiny lobster is a good model predator for this work, since it has been used already to demonstrate effects of ink and opaline secretions on the function of chemosensory neurons and on chemically evoked behavior (Kicklighter et al., 2005), and since the spiny lobster's chemosensory systems are well studied (e.g. Derby, 2000; Derby et al., 2001). First, I measured behavioral responses that are controlled by either the antennules (attraction) or mouthparts and legs (handling and ingestion). Second, complementary to behavior, I measured electrophysiological responses of chemoreceptor neurons in the mouthparts and antennules.

Alarm cues in spiny lobsters

Many animals have chemically mediated risk-assessment systems that help them avoid or defend against dangerous predators (Pfeiffer, 1977; Blum, 1985; Smith, 1992; Chivers and

Smith, 1998; Wyatt, 2003). Consequentially, the risk-assessment systems have a heavy influence on the behavior of animals. Animals engage in a variety of activities that potentially increase their fitness but that may increase the risk of injury. Some activities are more frequent than others. Foraging for food is frequent, necessary, and risky. These risks are minimized by avoiding predation areas at the expense of food acquisition. Predation areas often are marked by chemical cues that are released either directly by predators or indirectly by injured conspecifics (Chivers and Smith, 1998). In particular, injury-related chemicals released by conspecifics are adaptive for receivers since they indicate risk of active predators. Injury-related chemicals can also mediate learning of active predators on receivers because they linger long after prey die (Brown, 2003; Chivers and Smith, 1998; Weiss et al., 2008). Behavioral responses to these chemicals are often mediated by unique chemosensory pathways (Galizia et al., 1999; Hamdani and Døving, 2007; Yamagata et al., 2006, 2007).

Some animals that are attacked by predators release warning cues or signals for the nearby conspecifics. Some warning signals are of short duration and require conspecific to be nearby to sense them. Examples of short-term warning signals are sound signaling of distress in fish, birds, and mammals (Heyd and Pfeiffer, 2000; Gil-da-Costa et al., 2003; Marler, 2004; Shelley and Blumstein, 2004). Chemical cues however can linger long after prey die and disperse over considerable distances (Chivers and Smith, 1998). For example, octopus can take a long time to consume spiny lobsters, during which chemicals are continuously released from carcass (Weiss et al., 2008). Some chemical cues in consumed prey resist modification in the predator's digestive system and are released as waste products (Chivers and Smith, 1998). Nearby conspecifics can avoid areas that contain these chemical cues and consequentially encounters with predators.

Warning chemical cues or signals can benefit the sender, receiver, or both, and depending on which benefit, are called ‘alarm pheromones’ or ‘alarm cues’ (Smith, 1992). Alarm pheromones are often released through specific glands and benefit genetically related conspecifics (Blum, 1985; Wyatt, 2003). This is common among social insects (Blum, 1985). For example, honeybees release alarm pheromones from a specialized stinging apparatus, which alarms and mobilizes conspecifics from the colony against the intruders (Hunt et al., 2003; Breed et al., 2004). This stinging apparatus autotomizes onto the intruder, making it a target of other attacking honeybees. Other insects also use specialized glands to release alarm pheromones in conjunction with chemical defenses against predators (Blum, 1985). Most eusocial insect colonies consist of genetically related individuals, and consequently these alarm pheromones benefit signalers and receivers. Insects detect these alarm pheromones primarily through their olfactory pathway (Galizia et al., 1999; Yamagata et al., 2006, 2007).

In contrast, alarm cues are released passively by injured or killed prey, and benefit nearby conspecifics that receive the cue but do not always the releaser (Smith, 1992; Seeley, 1995; Viney and Franks, 2004). 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 can persist from long after their release (Smith, 1992). They mediate their affect via a specific sensory and olfactory bulb pathway (Hamdani and Døving, 2000, 2002, 2003; Zippel et al., 2000). In fact, separate anatomical and functional olfactory pathways mediate detection of sex pheromones and food odors (Hamdani and Døving, 2007).

The passive release of alarm cues during predation events is also frequent in aquatic invertebrates. 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 avoid shelters with fluids of injured conspecifics (Parsons and Eggleston, 2005, 2006; Bouwma, 2006; Briones-Fourzán et al., 2006; Briones-Fourzán and Lozano-Álvarez, 2008).

Alarm cues may be indispensable for spiny lobsters. Caribbean spiny lobsters are preyed upon by fish such as groupers, snappers, triggerfish, nurse sharks, and sting rays, mollusks such as common octopus, sea turtles, and other predators. Caribbean spiny lobsters actively defend themselves against predators using their antennae and legs, during which they may lose or have damaged those limbs (Briones-Fourzán et al., 2006; Weiss et al., 2008). They can also autotomize their limbs. During these attacks, whether successful or not, alarm cues may be released over many minutes. These may be detected by neighboring conspecifics, thus indirectly affecting their response to predators. This indicates that spiny lobsters have a chemically mediated risk-assessment system. However, little is known about the mechanism of alarm cues inducing avoidance behaviors in spiny lobsters or any decapod crustaceans. For example, the source of chemical alarm cues from injured conspecifics and the immediate behavioral responses to such cues are poorly characterized. The specificity of alarm cues and the chemosensory pathways mediating the effects are unknown.

Decapod crustaceans have dual chemosensory pathways in their antennules, a major chemosensory organ. This is best described in the Caribbean spiny lobster. One of these pathways is analogous to the olfactory pathway in many taxa, from arthropods to mammals. This olfactory pathway is based on aesthetasc sensilla, which are innervated by the dendrites of olfactory receptor neurons. The axons of these neurons project to the olfactory lobes (Grünert and Ache, 1988; Schmidt and Ache, 1996a, 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, 1996b). The aesthetasc/olfactory lobe pathway may uniquely process 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, 1992; Steullet et al., 2002; Schmidt and Derby, 2005; Johnson and Atema, 2005; Horner et al., 2008a, 2008b).

The olfactory pathway of decapod crustaceans is similar to that of insects and vertebrates. The peripheral olfactory pathway, composed of aesthetasc sensilla (up to 2400 sensilla in *P. argus*), is exclusively and richly innervated by olfactory receptor neurons (ORNs; often in hundreds per sensillum) (Derby et al., 2003; Derby and Caprio, 2008; Hallberg et al., 1992, 1997). These ORNs project ipsilaterally to the first synaptic relay of the olfactory pathway, the olfactory lobes (Schmidt and Ache, 1992). The olfactory lobes are glomerular in organization, common among first synaptic relays in antennal lobes of insects and olfactory bulbs of vertebrates (Dahanukar et al., 2005; Hildebrand and Shepherd, 1997; Kay and Stopfer, 2006; Schachtner et al., 2005). The olfactory lobes contain as few as 200 glomeruli in some species and as many as 1000 or more in others such as *P. argus* (Beltz et al., 2003). Variation in number

of glomeruli is speculated to be a reflection of phylogenetic affinities and habitat (Beltz et al., 2003). Besides sensory input from aesthetascs, the olfactory lobes receive innervation from three central sources: projection interneurons, local interneurons, and centrifugal neurons. Projection interneurons often innervate multiple glomeruli but not equally in all glomeruli. The output from these interneurons is projected to higher processing centers of the lateral protocerebrum in the eyestalks (Mellon et al., 1992a, b; Sullivan and Beltz, 2005; Wachowiak and Ache, 1994). While the local interneurons interconnect glomeruli and synapse with ORNs whereas, the centrifugal neurons synapse on local interneurons (Derby and Caprio, 2008; Schmidt and Ache, 1996; Schmidt, 1997). Local interneurons provide presynaptic inhibition through either GABA or histamine similar to the presynaptic inhibition in the olfactory bulb in vertebrates and the lateral inhibitory pathways in insects (Ache, 2002; Derby and Caprio, 2008; Wachowiak et al., 2002; Wachowiak and Ache, 1997, 1998).

Because of the wealth of knowledge on the chemosensory system of Caribbean spiny lobsters, I chose this species to explore the mechanism of alarm cues and predation-risk-assessment system. Chapter 4 examines 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. Further, I explored if Caribbean spiny lobsters alarm only to conspecific alarm cues or if they respond to alarm cues from other crustaceans of differing phylogenetic relatedness to *P. argus*. Finally, I examined the role of each of the two antennular chemosensory pathways in mediating the behavioral responses to alarm cues.

Chemical communication of social status

Decapod crustaceans often compete for social status to gain better access to food, shelters, and mates (Scrivener, 1971; Berrill, 1975, 1976; Bruski and Dunham, 1987; Issa et al., 1999). Social status is communicated through a combination of physical and chemical signals. Physical signals are tactile and other mechanical cues given during behavioral interactions between opponents, which may start symmetrical and end with one animal showing primary offensive and the other defensive behaviors. Decapods also use urine-borne pheromones to enforce and maintain social status. This communication with urine-borne pheromones minimizes physical aggression (Rutte et al., 2006). The mechanism of this communication in spiny lobsters and other decapods is not fully understood. Chapter 5 explains one mechanism of chemical communication in Caribbean spiny lobsters, and the chemosensory pathway that mediates this urine-based communication.

Most decapod crustaceans fight for resources such as shelter and food, with dominant animals gaining these resources over subordinates. For example, in crayfish, after an initial encounter in which social status is established, subordinates avoid fights with most dominant crayfish (Martin and Moore, 2003). Crayfish are not territorial or gregarious by nature, and thus avoiding dominants may reduce chances of injury. Consequently, subordinates have less access to shelters, food, and mates. American lobsters with subordinate status acquire less food, shelters, and potentially mates (Karnofsky and Price, 1989; Karnofsky et al., 1989). However, unlike crayfish, they avoid fights especially with familiar dominants but not unfamiliar dominants, suggesting that they are capable of individual recognition (Johnson and Atema, 2005). Although American lobsters are not gregarious by nature, they are territorial. The authors suggested that when competing for limited space, American lobsters may gain an

advantage from recognizing previous opponents nearby (Johnson and Atema, 2005). This idea of individual recognition in the American lobster however needs considerable experimentation. Spiny lobsters are also aggressive around shelters (Fielder, 1965; Berrill, 1975, 1976). Most spiny lobster species are gregarious by nature and yet often compete for better access to shelters, food, and mates. This competition starts as early as the first post-larval stage (Berrill, 1976), in which they lunge with their antenna and emit rasps or stridulations toward each other when competing around food. Juvenile Caribbean spiny lobsters also fight for shelters and food (Berrill, 1975; Meyer-Rochow, 1976; Lozano-Álvarez, 1996). Fights are characterized by offensive behaviors such as antenna and leg grabbing and antenna locking, and by defensive behaviors such as retreating and tail-flipping. Juveniles of another spiny lobster, the rock spiny lobster *P. cygnus*, also show similar behaviors around food, where large juveniles prevented small juveniles from entering baited traps (Chittleborough, 1974).

Similar results were observed in adult lobsters, including spiny lobsters, around shelters and baited traps (Fielder, 1965; Jury et al., 2001). Dominant American lobsters prevented other lobsters, which were often smaller, from entering the baited traps. Most lobsters entered the occupied traps only half way and immediately retreated. Close observations revealed that agonistic interactions were the single most important factor for the limited catch of lobsters by the baited traps (Jury et al., 2001). Adult spiny lobsters, *Jasus lalandei*, also fight for shelters and engage in similar offensive and defensive behaviors (Fielder, 1965). For example, subordinates that resist eviction by dominants often retreat inside shelter during which dominants face them and lunge into attacks by grabbing the legs and antenna of subordinates. In response to these attacks, subordinates tail-flip or retreat rapidly away from the shelter. If dominants are challenged in their shelter by an opponent, they often respond with offensive behavior. In

spotted spiny lobsters, *P. guttatus*, dominant lobsters show aggressive behavior toward subordinates and sometimes will evict subordinates from their shelters (Segura-García et al., 2004). The dominant is typically larger in size and commits almost all the attacks on the smaller subordinates. The evicted subordinates will then move to other shelters.

Lobsters also fight during mating season. Male American lobsters defend multiple shelters, which contain females, from other males. Larger males have better chances in mating. Sexually mature male spiny lobsters also defend shelters from other males (Lindberg, 1955). According to anecdotal observations, male spiny lobsters are sometimes found with multiple egg bearing females (Lindberg, 1955). In European lobsters, *Homarus gammarus*, dominant males are more successful in mating than the smaller male subordinates (Debuse et al., 2002).

Although spiny lobsters occasionally fight for social status, most of the time they live in aggregations or forage solitarily for food. During the day, spiny lobsters usually reside in aggregations, from a few to more than a hundred, but they may also live solitarily (Kanciruk, 1980; Herrnkind et al., 1975). The size of the aggregation depends on size and availability of shelters and degree of predation pressure (Eggleston et al., 1990; Eggleston and Lipcius, 1992; Mintz et al., 1994). Furthermore, they show no fidelity to their shelters and there is no evidence that they are territorial. At night, spiny lobsters forage, during which they may move in and out of their shelters several times, then return to shelters before dawn (Herrnkind, 1980; Weiss et al., 2007). Thus agonistic interactions over food are likely rare but around shelters may be common. Spiny lobsters limit the time spent searching for shelters by relying on intraspecific chemical cues (Eggleston and Lipcius, 1992; Childress and Herrnkind, 1997, 2001a, 2001b). Because of predation pressures, some spiny lobsters may engage in aggressive behaviors once they locate occupied shelters to ensure space as fast as possible.

Decapod crustaceans couple their fights with the release of chemical signals to enforce social status. An important source of these signals is urine. The pattern of urine release differs based on the social status of the animal (Breithaupt and Atema, 2000; Breithaupt and Eger, 2002). Social status is established when one opponent, the dominant, engages primarily in offensive behaviors, while the other, the subordinate, engages primarily in defensive or avoidance behaviors (Karavanich and Atema, 1998; Issa et al., 1999). In crayfish, the dominant animal of a pair increases its rate of urine release, whereas the subordinate decreases it (Breithaupt and Eger, 2002). Furthermore, urine is released especially during offensive behaviors and directly in front of the opponent. Subsequent encounters between the opponents show similar rates of urine release even though fighting decreases significantly. Thus dominants release more urine than subordinates during all encounters, suggesting that it plays a role in reduction of fighting behavior. Studies on crayfish by Zulantz Schneider and Moore (2000) and Horner et al. (2008a) complement this view. In social encounters, crayfish prolong their fights significantly when nephropores, from which urine is released, are blocked with glue (Zulantz Schneider and Moore, 2000). Fights are also prolonged if the olfactory sensilla are compromised (Horner et al., 2008a).

Relatively similar results as these for crayfish are also reported for American lobsters. Dominants show offensive behaviors and increase urine release while subordinates show defensive behaviors and suppress urine release. However, unlike crayfish, American lobsters decrease the intensity and duration of fights only when paired with familiar opponents. In addition, lobsters with ablated aesthetasc sensilla fail to recognize previous dominant lobsters (Johnson and Atema, 2005). Consequently these lobsters engage in the same intensity and duration of fights in subsequent encounters.

The aesthetascs mediate detection of urine-borne signals in agonistic interactions and other social contexts (Horner et al., 2008a, 2008b). For example, spiny lobsters are gregarious animals and prefer shelters scented with urine or odors of conspecifics (Zimmer-Faust et al., 1985; Nevitt et al., 2000; Horner et al., 2008b). This preference helps spiny lobsters locate shelters through the guide-post effect (Childress and Herrnkind, 2001), in which they limit their time of searching for shelters. Ablating aesthetasc sensilla eliminates this shelter preference (Horner et al., 2008b). Urine is also important in mating. Female American lobsters prefer shelters occupied by dominant males than subordinate males. However, this preference by females is lost if urine release of males is blocked (Bushman and Atema, 2000). Male blue crabs with ablated aesthetascs lose attraction to female urine (Gleeson, 1982). A similar result is shown in male helmet crabs with ablated distal half of lateral flagellum, which harbors the aesthetasc sensilla (Kamio et al., 2005). This type of parsimonious use of single source of chemicals for signaling in multiple contexts is present in other animals as well (Johnston, 2003). In both American lobsters and crayfish, however, little is known about the direct effect of urine on behavior and whether the olfactory pathway mediates such effects. In addition, little is known about the effect of urine on dominant animals even though dominants initiate almost all fights. Thus, the mechanism of urine-borne signals is not well understood. Furthermore, virtually nothing is known about urine signaling in the Caribbean spiny lobster. In Chapter 5, I explore the role of urine in communicating social status in spiny lobsters. In this work, I developed a new technique of visualizing urine release in spiny lobsters. Our experiments in this chapter complement studies on communication of social status in other decapod crustaceans and lay the foundation for future studies in processing of urine-borne pheromones in spiny lobsters.

CHAPTER 2

SEA HARES USE NOVEL ANTIPREDATORY CHEMICAL DEFENSES.

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Introduction

Numerous studies have demonstrated that chemical defenses protect prey from predation (Eisner and Meinwald, 1966; Tachibana, 1988; Paul, 1992; Pawlik, 1993; Berenbaum, 1995; McClintock and Baker, 2001; Kelly et al., 2003) and have often assumed these defenses function by repelling predators. Surprisingly, few have investigated the mechanisms whereby predators are affected by these defenses (Carefoot et al., 1999; Carlson, 1997). Here, we examine mechanisms of chemical defense of sea hares (*Aplysia californica*), which when attacked by spiny lobsters (*Panulirus interruptus*), release defensive secretions from ink and opaline glands (Johnson and Willows, 1999; Nolen et al., 1995). We show that ink-opaline facilitates escape of sea hares, acting through a combination of novel and conventional mechanisms. Ink-opaline contains millimolar quantities of amino acids that stimulate chemoreceptor neurons in the spiny lobster's nervous system. Ink stimulates appetitive and ingestive behavior, opaline can elicit appetitive behavior but also can inhibit ingestion and evoke escape responses, and both stimulate grooming. These results suggest these secretions function by 'phagomimicry', in which ink-opaline stimulates the feeding pathway to deceive spiny lobsters into attending to a false food stimulus, and by sensory disruption, in which the sticky and potent secretions cause high amplitude, long-lasting chemo-mechanosensory stimulation. In addition, opaline contains a chemical deterrent that opposes appetitive effects. Thus, chemical defenses may act in more complex manners than palatability assays of prey chemistry may suggest.

Materials and methods

Animals and Collection of Ink

California spiny lobsters (*Panulirus interruptus*) and sea hares (*Aplysia californica*) were either collected in the field or provided by the NIH National Resource for *Aplysia*. Unless stated otherwise, results are from field-caught hares. Caribbean spiny lobsters (*Panulirus argus*) and sea hares (*Aplysia dactylomela*) were collected in the Florida Keys or Bermuda. Sea hares were fed red alga, *Gracilaria ferox*. Ink and opaline secretions were collected from dissected ink and opaline glands. Ink glands were gently squeezed, releasing ink. Unless otherwise indicated, opaline glands were centrifuged at 30,000 x g for 1 hr at 4°C to separate opaline secretion from gland tissue. Opaline was also collected by squeezing the glands. Secretions were frozen at -20°C until needed. Although opaline obtained by centrifugation is less sticky than that obtained by squeezing the glands, spiny lobsters find both types of opaline unpalatable.

Assay of the role of ink in success of predation by spiny lobsters on sea hares

Effects of ink and opaline on survival of a total of 55 sea hares in the presence of crustacean predators was analyzed using the sympatric predator-prey pair *P. interruptus* and *A. californica*. Sea hares (40-50 g) were randomly assigned to one of four treatment groups (and were balanced according to sea hare size): both ink and opaline (no glands removed), opaline only (ink gland surgically removed), ink only (opaline gland removed), and neither ink nor opaline (both ink and opaline glands removed). To control for effects of handling and surgery, sea hares in the ‘both’ group were handled as the other groups but underwent a sham surgery in which a portion of the parapodia was instead removed. Glands were removed the day before the experiment, and each sea hare was used only once. Animals in all groups appeared to be in good health on the day of the experiment. Each spiny lobster was used one time and was tested in 80-L aquaria (60 cm L x 30 cm W x 45 cm H) and recorded using a digital video camera. Only

spiny lobsters that are motivated to feed, as determined by them producing a feeding response to sea hare juice (thawed body wall tissue prepared by the same method as squid juice – see below), were included in the data analysis. Interactions were recorded for 5 min after the spiny lobster attacked, and digging, grabbing, antennule grooming, mouthpart grooming, and tailflipping were quantified. Sea hares were scored as ‘eaten’ if the spiny lobster had eaten or was in the process of eating 10 min after attack. Some spiny lobsters dropped partially eaten dead sea hares less than 10 min after attack. These were scored as eaten. If the spiny lobster dropped the (live) sea hare within 10 min of attack, the sea hare was scored as ‘escaped’.

Assay of responses of spiny lobsters to ink, opaline, and other chemicals

We examined feeding responses (grabbing and ingestion) of *P. interruptus* to ink, opaline, and both of *A. californica* in three different assays. To standardize hunger level, spiny lobsters were fed a piece of shrimp 3-5 hr before starting feeding assays. In the first assay, grabbing was examined using a previously described procedure (Derby et al., 1984). Filter paper disks (Fisherbrand P8, 2.5 cm diameter) soaked in stimuli were presented to the legs of spiny lobsters. Grabbing was defined as taking the disk in its legs and transferring it to its mouthparts. Stimuli included: ink, opaline, ink+opaline, artificial ink, and artificial opaline, each at 50% full strength; squid juice (squid soaked in sea water) as a positive control; ASW as a neutral control; and tannic acid as a possible negative (aversive) control. Squid juice was made by homogenizing thawed squid mantle in a volume of water equivalent to the squid and filtering the homogenate through a coffee filter. Squid juice was the fluid that passed through the filter. All spiny lobsters received each type of disk, one disk per trial, randomly presented.

In a second assay, the ability of chemicals to elicit ingestion was examined by presenting to legs and mouthparts of spiny lobsters a 1-ml stimulus using a syringe. Ink+opaline was viscous enough to be presented au naturel (because the opaline obtained by squeezing the opaline glands was very viscous); ink, squid and sea water were mixed in 20 mg/ml carboxymethylcellulose to have viscosity similar to opaline. Ink and opaline were presented at full strength.

In a third assay, the ability of opaline to inhibit ingestion of food was examined by evaluating the effect of adding opaline to freeze-dried shrimp. Spiny lobsters were first offered a palatable control food (freeze-dried shrimp soaked in 500 µl sea water). If this was consumed, spiny lobsters were offered a piece of freeze-dried shrimp soaked in 500 µl full-strength opaline. Spiny lobsters that rejected opaline-soaked shrimp were offered a control shrimp to ensure that rejection was not due to satiation, and spiny lobsters that did not consume either the initial or control shrimp were excluded from analysis. This experiment was performed for opaline from both field-caught and mariculture-raised animals.

Electrophysiological recordings from chemoreceptor neurons

Single-unit extracellular electrophysiological techniques were used to record responses of individual chemoreceptor neurons of spiny lobster's lateral flagellum of the antennule and 2nd maxilliped. We used a perfused preparation in an olfactometer with electronically driven valves for stimulus delivery, and recordings made from CN axons using fine-tipped glass electrodes (Derby, 1995; Garm et al., 2005). CNs from 2nd maxillipeds of *P. interruptus* and from antennules of *P. argus* were tested with glandular secretion of sympatric *Aplysia* species. CNs were identified using 300 mg/L homogenized shrimp or 0.1% full-strength ink+opaline.

Responses were measured to ink, opaline, artificial ink, and artificial opaline (1% for 2nd maxilliped CNs, 0.1% for antennular CNs), to the major single compounds in secretions and their artificial mixtures (taurine, lysine, glutamate, aspartate, histidine, cysteine, ammonium, urea), and tannic acid and adenosine-5' monophosphate (10 μ M for 2nd maxilliped CNs, 100 μ M for antennular CNs), shrimp juice (3000 mg/L for 2nd maxilliped CNs, 300 mg/L for antennular CNs), and ASW (negative control). Higher concentrations were tested for 2nd maxilliped CNs because these neurons have lower sensitivity and higher thresholds than antennular CNs (Garm et al., 2005). For each neuron, response intensity was quantified as the number of spikes in the first 500 msec of response. Two response features were quantified for all stimuli: population response intensity, which represents the relative efficacy of stimuli; and across neuron pattern (or ensemble response patterns), which represents the ability of the population to discriminate between stimuli (see Fig. 4 for description).

Results

To investigate the survival value of the secretions, we presented to spiny lobsters sea hares with and without opaline and/or ink glands (Fig. 1). Sea hares with both opaline and ink glands and sea hares with only opaline glands escaped predation in 60 and 67% respectively of the encounters with spiny lobsters, whereas sea hares with neither opaline nor ink glands and sea hares with only ink glands escaped only 19 and 17% respectively of the encounters (Fig. 1A). This statistically significant protective effect of secretions containing opaline is striking given that these experimental conditions decidedly favored predators. In encounters in which sea hares released secretions and survived, spiny lobsters showed several behaviors suggestive of the

mechanisms of chemical defense. These include digging with the legs into the substrate covered by the secretion ('digging', Fig. 1B) or moving the first two pairs of legs to their mouth ('grabbing', Fig. 1C), behaviors similar to that produced when spiny lobsters are chemically stimulated to search for and sample food items (Derby and Atema, 1982). Other behaviors by spiny lobsters in encounters where sea hares released secretions and survived were grooming of the antennules (Fig. 1D) and grooming of the mouthparts (Fig. 1E), behaviors associated with cleaning their sensory organs following chemical or mechanical fouling (Bauer, 1981). Opaline, either alone or with ink, caused spiny lobsters to tailflip (Fig. 1F), a defensive behavior produced by aversive stimuli (Wine and Krasne, 1972). (Three examples of attacks by spiny lobsters on sea hares, including examples and descriptions of the behaviors described above, are shown in three Supplemental Videos.) These experiments suggest that the secretions can defend sea hares against predatory spiny lobsters by stimulating the predator's chemosensory systems and evoking appetitive or ingestive feeding behavior (ink or opaline), cleaning behavior (ink or opaline), and/or aversive behavior (opaline). These observations led us to perform the following chemical analyses, behavioral, and electrophysiological experiments.

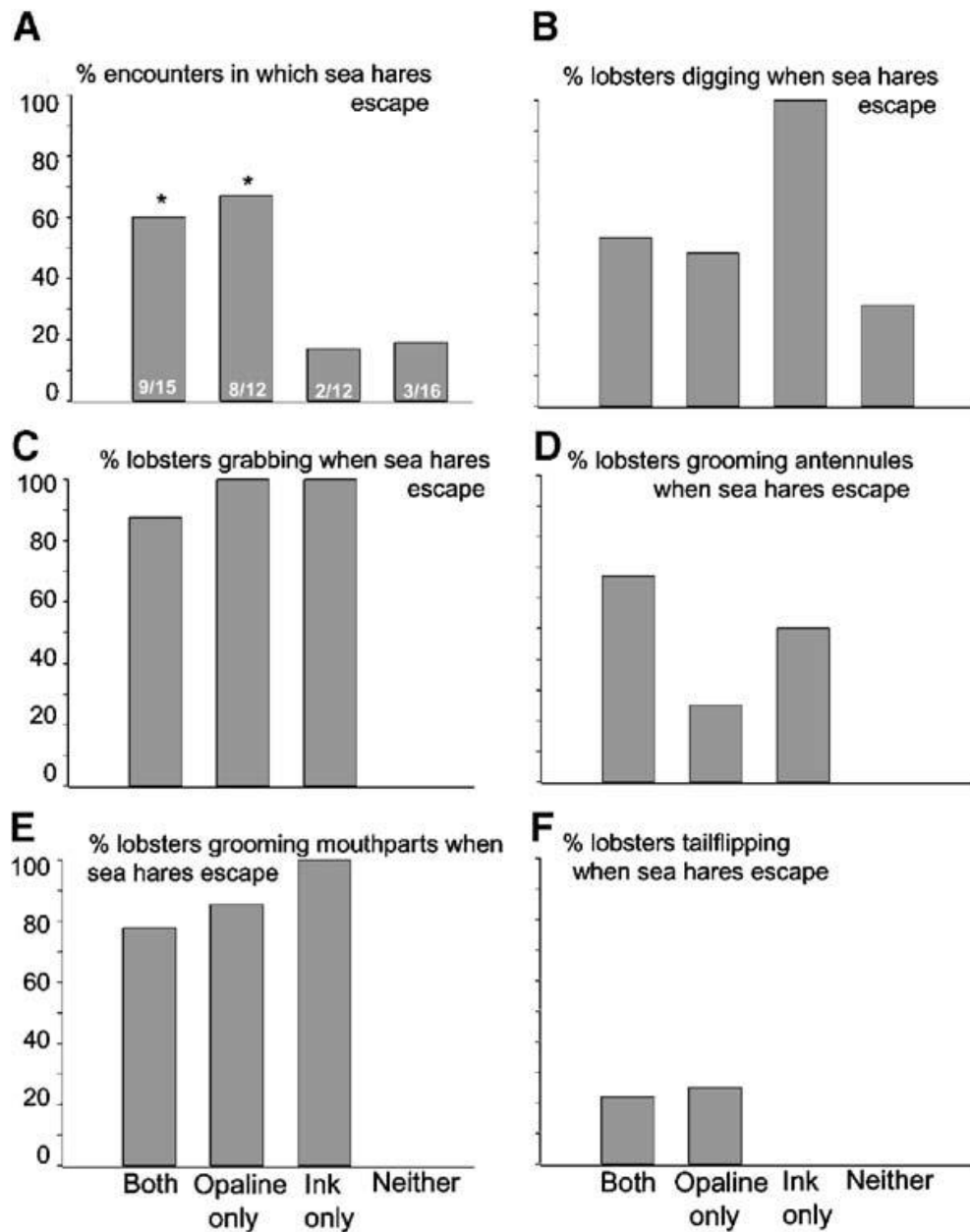


Figure 2-1 Effect of ink and opaline secretions on survival of sea hares and spiny lobster behavior in attacks by spiny lobsters. (A) % of trials in which sea hares escape being eaten by spiny lobsters. Numbers within each bar are the # of escaping sea hares followed by the # of trials. * indicates significant difference from 'Neither' group (Fisher's Exact Test, $p < 0.05$). (B)-(F) For encounters in which sea hares escape, % of spiny lobsters showing (B) digging in substrate, (C) grabbing, (D) antennule grooming, (E) mouthpart grooming, and (F) tailflipping.

To identify possible feeding stimulants in secretions, we analyzed *A. californica* ink and opaline for free amino acids, ammonium, and urea (AA/NH₄/Urea), since these are known to be potent excitants of chemosensory neurons (CNs) and feeding behavior of spiny lobsters and other crustaceans (Derby, 2000; Zimmer-Faust, 1987). Opaline and ink from either field-caught or mariculture-raised sea hares contain enormous concentrations of AA/NH₄/Urea – 319 and 54 mM respectively (Fig. 2; for complete data set, see Table 1). Taurine is the dominant amino acid in opaline – at 231 mM, it constitutes 72% of the total AA/NH₄/Urea. Opaline is also high in lysine (65 mM, 20%), histidine, and ammonium (each at 7 mM, 2%). In ink, ammonium is the dominant component: at 24 mM, it is 44% of the total AA/NH₄/Urea; cysteine (15 mM, 28%) and taurine (8 mM, 15%) are also abundant components. The high levels of taurine in opaline and ink are striking since taurine is one of most potent stimulants of crustacean feeding (Derby, 2000). To test whether these high levels of AA/NH₄/Urea in ink and opaline simply reflect high levels in other body tissues of *A. californica*, we analyzed haemolymph and found only 2 mM AA/NH₄/Urea, of which over 50% was urea (Fig. 2). This total level is only 0.6% and 3.7% of that in opaline and ink respectively, showing that ink and opaline glands secrete very high levels of specific feeding stimulants.

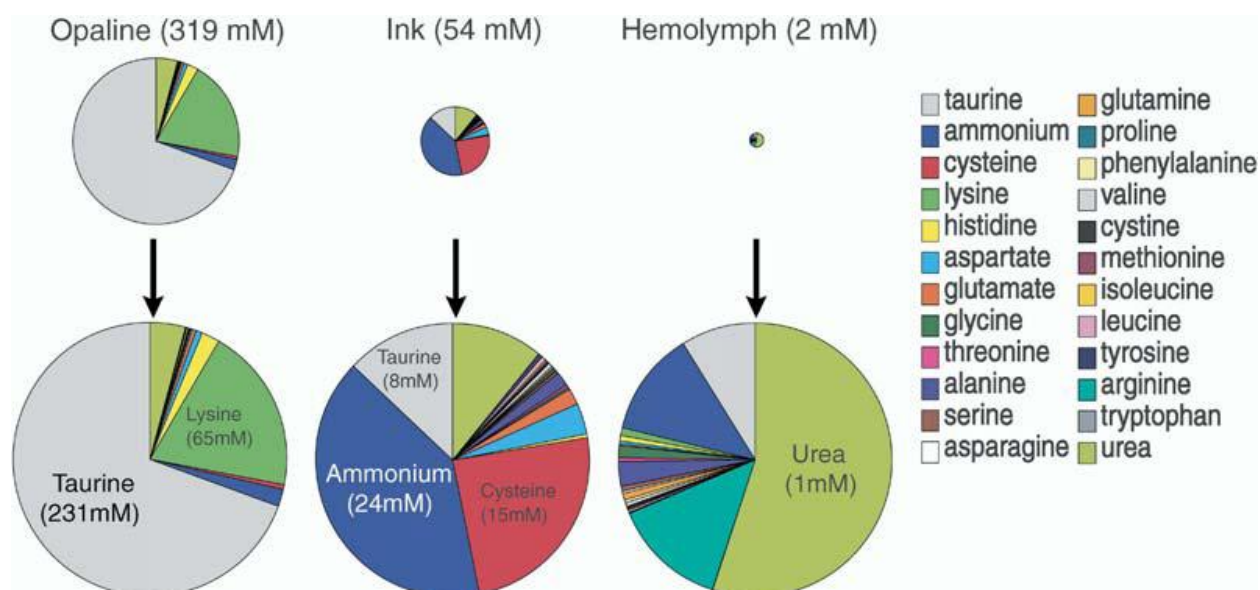


Figure 2-2 Composition of opaline, ink, and haemolymph of sea hares. Samples of ink and opaline were collected from dissected glands of 15 field-collected *A. californica*, and haemolymph was collected from 5 individuals. Concentrations of 22 free amino acids, plus ammonia and urea, were analyzed for pooled samples using an ion exchange, post-column ninhydrin detection system (Beckman Model 6300/7300 Amino Acid Analyzer, The Scientific Research Consortium, Inc.: www.aminoacids.com). The 3 pie charts in the top row represent absolute amounts using the same scale; pie charts in the bottom row show the same results but on a relative scale.

(μM)	<i>Aplysia californica</i>			<i>Aplysia dactylomela</i>	
	ink	opaline	haemolymph	ink	opaline
L-Alanine	1024	339	64	1105	2063
L-Arginine	0	340	296	0	212
L-Asparagine	51	41	6	0	0
L-Aspartic acid	2231	2512	8	4378	21300
L-Cysteine	14830	1844	0	3618	0
L-Cystine	84	16	3	85	14
L-Glutamic acid	1166	1616	4	2023	83800
L-Glutamine	121	9	17	474	0
L-Glycine	181	791	31	239	2554
L-Histidine	255	7185	14	108	1672
L-Isoleucine	135	96	3	145	118
L-Leucine	327	10	6	347	188
L-Lysine	0	65190	20	0	118850
L-Methionine	122	35	4	55	17
L-Phenylalanine	130	648	8	72	75
L-Proline	131	7	0	249	170
L-Serine	214	68	13	185	189
Taurine	7830	231200	194	8770	215350
L-Threonine	193	236	10	227	186
L-Tryptophan	5	0	1	0	496
L-Tyrosine	297	14	3	22	71
L-Valine	301	56	10	607	312
Ammonia	24360	6810	272	9727	36400
Urea	6569	13998	1208	303410	1252000

Table 2.1 Concentrations of amino acids, ammonia, and urea in ink, opaline, and hemolymph of *Aplysia californica* and *Aplysia dactylomela*.

The values for *A. californica* are those used in the pie charts of Figure 2. The concentrations of amino acids are significantly correlated for the same secretion from the two species. For ink, the amino acids concentrations are very similar except for cysteine, which is higher in *A.c.* than in *A.d.* The Pearson correlation coefficient r for ink, using all 22 amino acids, is 0.70 ($P < 0.05$); excluding cysteine from this analysis, $r = 0.98$ ($P < 0.05$).

For opaline, the amino acids concentrations are very similar except for glutamate, which is lower in *A.c.* than in *A.d.* The r value for opaline, using all 22 amino acids, is 0.92 ($P < 0.05$); excluding glutamate from this analysis, $r = 0.97$ ($P < 0.05$). The levels of ammonia and urea are more variable in the two species, and may be more variable within a species; when ammonia and urea are added to the 22 amino acids, the r value for both ink and opaline is 0.18 ($P > 0.05$).

Next, to determine if the secretions induce a feeding response (grabbing and ingestion) by spiny lobsters, behavioral experiments were performed (Fig. 3). We also tested artificial mixtures of ink and opaline, which contained the seven most concentrated identified components in the secretions at their natural concentrations. Ink+opaline, ink, artificial ink, and artificial opaline induced significantly more grabbing than did the negative control (sea water) and as much as natural food (squid juice) (Fig. 3A). Interestingly, artificial opaline elicited significantly more grabbing than did sea water, but natural opaline did not, suggesting the presence of a feeding deterrent in opaline. Tannic acid was included in our experiment as a potential negative (aversive) control, since it is a feeding deterrent to clawed lobsters (Derby et al., 1984); in our study, it elicited grabbing similar to sea water. To examine ingestion, we compared spiny lobsters' ingestion of ink+opaline, ink, and opaline versus that of natural foods (squid juice and freeze-dried shrimp) and sea water. Ink elicited ingestion similar to squid juice and more than sea water (Fig. 3B). When added to ink, opaline inhibited ingestion (Fig. 3C). In fact, ingestion of natural food (freeze-dried shrimp) was inhibited when it was soaked in opaline, either from field-caught or mariculture-raised (on the red alga *Gracilaria ferox*) sea hares (Fig. 3D). These results demonstrate that ink is a feeding excitant that elicits both grabbing and ingestion. Opaline contains feeding excitants, but it also has a feeding inhibitor even present in sea hares fed a limited diet. We are currently investigating the molecular identity of this inhibitor.

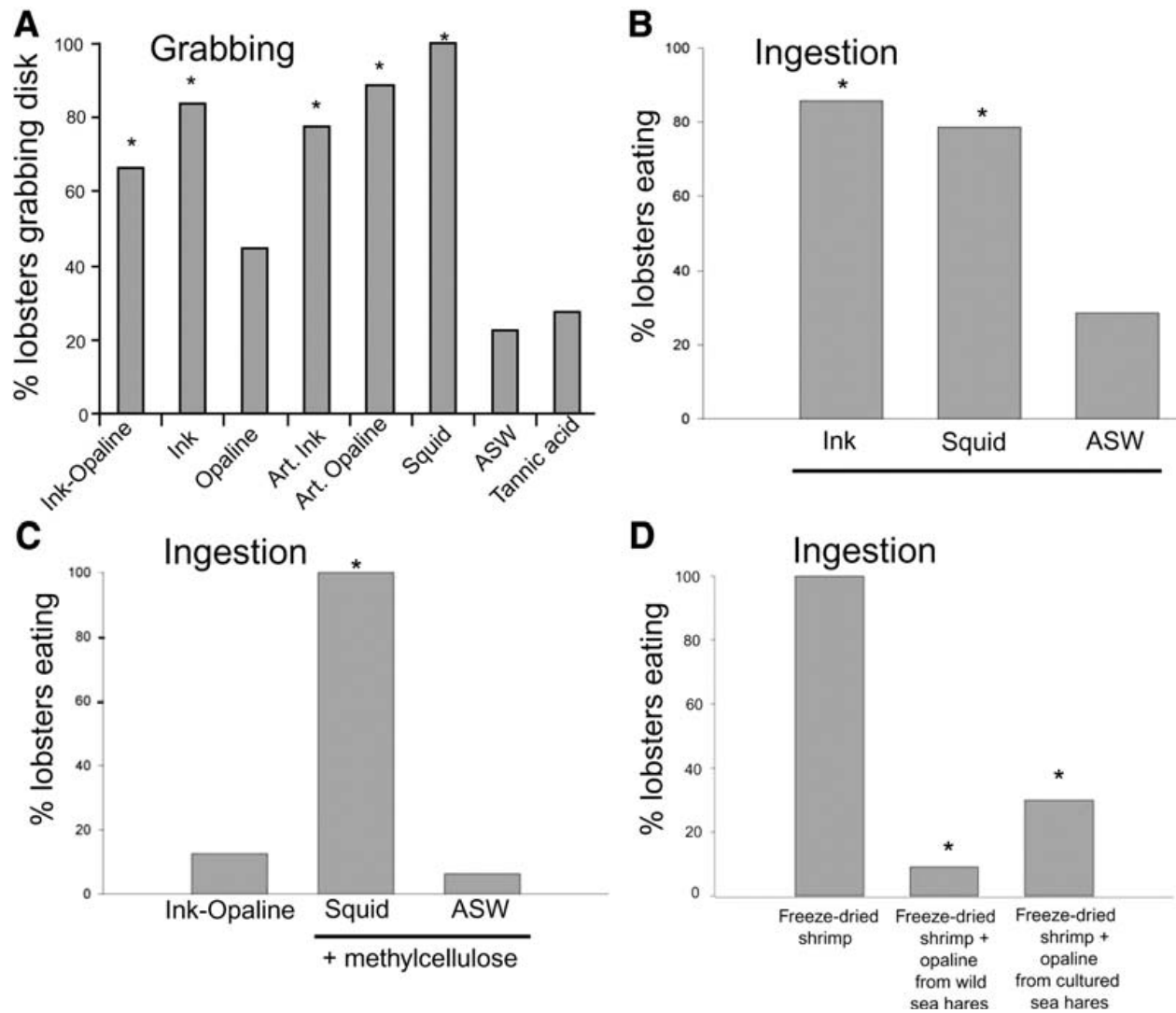


Figure 2-3 Behavioral responses of spiny lobsters to defensive secretions of sea hares. A. Grabbing of ink + opaline, ink, opaline, artificial ink, artificial opaline (all at 50% full strength); squid juice (500 g/L); artificial sea water (ASW: negative control); tannic acid (100 μ M). B-D. Ingestion. In B and C, substances (except ink+opaline) were mixed in 20 mg/ml carboxymethylcellulose. N (# of spiny lobsters tested) = 19 (A), 14 (B), 16 (C), 13 (D). Bars with an asterisk above them are significantly different from ASW (A-C) or freeze-dried shrimp (D) ($P < 0.05$, Cochran Q test for (A), McNemar test for (B)-(D)).

To determine if ink and opaline mimic the activity of food odors in the spiny lobster's neural pathway, we examined the responses of chemosensory neurons (CNs) in spiny lobster's antennules and 2nd maxillipeds, taking advantage of the fact that spiny lobsters are model systems in chemosensory neurobiology (Ache, 2002; Derby et al., 1984; Derby, 2000; Zimmer-Faust, 1987). Because these experiments were conducted at two different locations, we used the local sympatric spiny lobster and sea hare species. Thus, antennule data were from *P. argus* with secretions from *A. dactylomela* (which are similar in amino acid composition to *A. californica*: see Supplemental Table 1), while 2nd maxilliped data were from *P. interruptus* with secretions from *A. californica*. Both antennular and mouthpart CNs were highly excited by 0.1% opaline and 1% ink (Fig. 4A). Even at 0.01% full-strength, secretions elicited significant responses, especially in antennular CNs. Two response properties of these cells were examined: response intensity (Fig. 4B) and across neuron patterns (Fig. 4C), which are the neural codes for stimulus quantity and quality respectively. Ink, opaline, artificial ink, and artificial opaline were more excitatory (i.e. evoked a higher frequency of action potentials, or spiking) than sea water and were similar to shrimp juice and major components of the secretions (such as taurine and ammonium) (Fig. 4B). Analysis of across neuron patterns (ANPs) shows that secretions and artificial mixtures produce ANPs similar to each other and to shrimp, and that components of secretions producing ANPs most similar to the secretions and mixtures are taurine and ammonium. These results are predicted from the compositions of these stimuli and support the conclusion that these defensive secretions may mimic food odors. These results also suggest that the inhibitor in opaline (which deters ingestion) does not function by inhibiting the activity of neurons activated by amino acids and other feeding excitants, which may evoke appetitive spiny lobster behavior, but may function instead by activating a different population of neurons. Thus,

opaline may excite different neuronal populations, sending contradictory messages to spiny lobsters. We identified one candidate neuron – an antennular chemosensory neuron that was excited by opaline but not artificial opaline or any other stimulus. However, conclusive identification of neurons mediating this inhibition requires isolation of deterrent compounds in opaline.

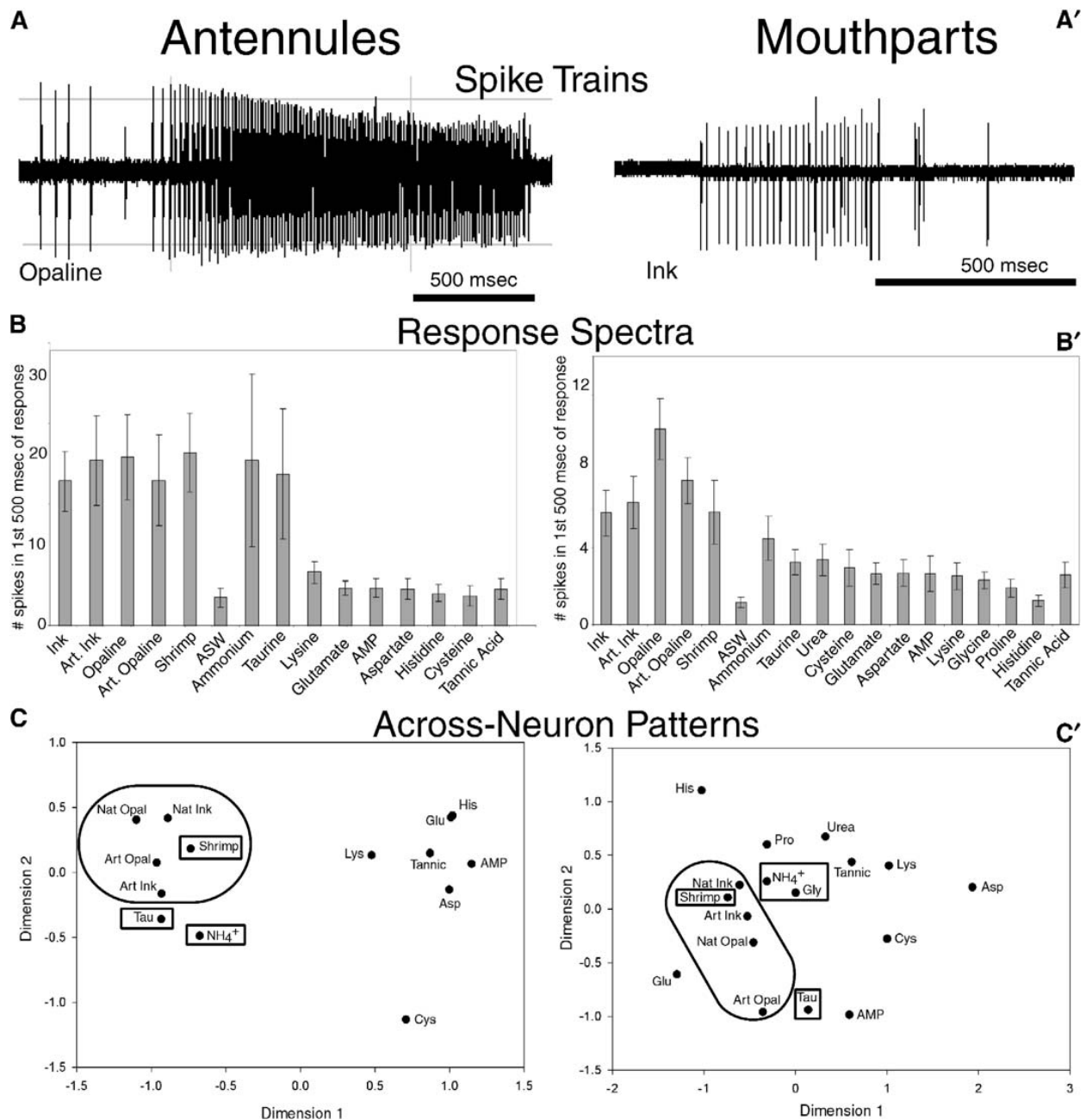


Figure 2-4 Responses of antennular and mouthpart chemoreceptor neurons of spiny lobsters to sea hare defensive secretions. A-1, B-1, C-1: Responses of CNs in the antennular lateral flagellum of *Panulirus argus*. A-2, B-2, C-2: Responses of CNs in the 2nd maxilliped of *Panulirus interruptus*. Examples of single-unit responses of (A-1) antennular CN to 0.1% opaline and (A-2) mouthpart CN to 1% ink. Population response intensity for (B-1) 12

antennular CNs from 10 different preparations from as many animals, following stimulation with 0.1% secretions and the artificial mixtures; single compounds at 10 μ M, and for (B-2) 30 mouthpart neurons from 11 different preparations from as many animals in response to 1% secretions and artificial mixtures; single compounds at 100 μ M. Responses were expressed as mean \pm S.E.M. number of spikes in the first 500 msec of response. Across-neuron patterns (C-1) for the same 12 antennular CNs and same 15 stimuli as in B-1 and (C-2) for the same 30 mouthpart CNs and same 18 stimuli as in B-1. Across-neuron patterns were analyzed using the first 500 msec of response in multidimensional scaling (Statistica, StatSoft Inc.), with similarities determined by Pearson correlation coefficients (8). These two-dimensional solutions account for 94% (C-1) and 84% (C-2) of the variance in the data.

Discussion

Our results show that besides containing unpalatable, aversive chemicals that appear to repel spiny lobsters from feeding on sea hares, sea hares also contain chemicals that protect them by more novel mechanisms. One is a previously undescribed form of chemical defense, ‘phagomimicry’, in which secreted substances mimic the stimulatory properties of food to divert predators, as suggested by the observed digging and grabbing. The enormous concentration difference in free amino acids and ammonia in opaline and ink versus haemolymph suggests that defensive secretions function as a supernormal stimulus – a stimulus that is more effective than the typical stimulus (Tinbergen, 1951). To be effective as a phagomimic, the secretion should be a supernormal stimulus because the prey itself is food to the predator and would release haemolymph when bitten, and a supernormal stimulus is necessary to direct the predator away from the prey. This phagomimic also functions as a sensory trap (Christy, 1995), since the spiny lobster’s chemosensory system is ‘trapped’ to respond in a certain way. Detection of high concentrations of free amino acids typically signals to spiny lobsters the presence of food. Sea

hares exploit this property of their predator's nervous system by releasing secretions that mimic stimulatory properties of food and thereby divert the attention of the attacker. The highly viscous nature of opaline may create a tactile sensation of food, contributing to the mimicry. Sea hares normally release both ink and opaline at about the same time and the ink binds to the sticky opaline, thus keeping the concentrated stimulus near the attacker. This likely explains why sea hares with only ink glands escaped in only 17% of encounters with spiny lobsters, as the less viscous ink rapidly diffused into the water column, away from the spiny lobsters. Anecdotal reports suggest other candidates for phagomimicry (Hoelldobler et al., 1982; LaMunyan and Adams, 1987; Maschwitz et al., 1981). Since using false scents of food has evolved many times for attracting mates, prey, and pollinators (Stowe, 1988), phagomimicry may be a strategy used by many species, serving as an alternative to chemical defenses that harm or deter predators.

Finally, the defensive ink-opaline secretion may also function through sensory disruption or desensitization. This would occur when the sticky ink-opaline coats the spiny lobster's sensory and feeding appendages with concentrated chemical stimuli, resulting in massive and sustained excitation of the chemosensory neurons that may produce confusing sensory messages and inappropriate behaviors, such as extensive grooming, and possibly followed by chemosensory desensitization or adaptation. Although sensory disruption (or related phenomena such as startle or sensory irritation) has been previously suggested as a potential mechanism of antipredatory chemical defense (Carefoot et al., 1999; Carlson, 1997; Johnson and Willows, 1999; Nolen et al., 1995), ours is the first neurophysiological support in sea hares and, to our knowledge, any animal.

In conclusion, sea hares' ink-opaline secretion protects them through a combination of mechanisms, including phagomimicry, sensory disruption, and chemical deterrence. This is one

of the few studies to demonstrate how predators process a prey's chemical defenses at the neural level, illustrating how defenses can have multiple physiological effects on a given predator. This multitude of active chemical defensive mechanisms involving ink-opaline, together with other known passive chemical defenses and other behavioral strategies (Ginsburg and Paul, 2001; Kinnel et al., 1979; MacColl et al., 1990; Nolen et al., 1995; Pennings and Paul, 1993; Yamada and Kigoshi 1997), likely provides highly effective protection against not only spiny lobsters but other predators as well.

CHAPTER 3

ACIDITY ENHANCES THE EFFECTIVENESS OF ACTIVE CHEMICAL DEFENSIVE SECRETIONS OF SEA HARES, *APLYSIA CALIFORNICA*, AGAINST SPINY LOBSTERS, *PANULIRUS INTERRUPTUS*.

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Introduction

Sea hares, like many other opisthobranch gastropods, compensate for the reduction or loss of shell by using an array of defenses (reviewed in Johnson and Willows 1999). Included in this array are passive and active chemical defenses (Kinnel et al. 1979; Faulkner 1992; Nolen et al. 1995; Johnson and Willows 1999; Cimino and Gavagnin 2006). Passive chemical defenses are not released under nervous system control during predatory attacks, but are present as deterrent compounds in the skin, some of which are acquired through diet (Thompson 1960; Ginsburg and Paul 2001). Active chemical defenses however are released under nervous system control only during physical attacks by predators (Carew and Kandel 1977; Johnson and Willows 1999). A dramatic example of an active chemical defense is inking by sea hares (*Aplysia*), wherein a purple cloud composed of pigmented and other substances is formed. This slimy secretion is composed of two different glandular products, ink and opaline, that are typically released simultaneously (Tritt and Byrne 1980; Prince et al. 1998; Nolen and Johnson 2001). Ink is diffusible and purple, whereas opaline is whitish and highly viscous.

The secretion of ink and opaline not only generates a visual cloud that can affect visually oriented predators, but chemicals in it can interact with the chemosensory organs of predators. The mechanisms whereby the chemicals in the defensive secretion of *Aplysia* manipulate predators' chemosensory systems and provide a defense have only recently begun to be understood.

One mechanism for *Aplysia* chemical defenses is through deterrent chemicals that inhibit ingestive behaviors. Examples of this include predatory sea anemones, fish, and spiny lobsters,

though the molecular identities of the deterrents are largely unknown (Kicklighter et al. 2005; Kicklighter and Derby 2006; but see Kamio et al. 2007).

A second mechanism for *Aplysia* chemical defenses is phagomimicry, which involves predators such as spiny lobsters being diverted toward the secretion and away from the sea hare (Kicklighter et al. 2005). This mechanism operates because ink and opaline have high quantities of amino acids and other food-associated compounds that are highly stimulatory to the chemical senses of predators such as spiny lobsters. For example, ink and opaline, as well as artificial mixtures mimicking them based on chemical analysis of the secretions, are attractive, and can evoke feeding behavior from the California spiny lobster, *Panulirus interruptus* (Kicklighter et al. 2005).

A third mechanism is sensory disruption, which is based on the fact that the secretions not only contain very high concentrations of stimulants but are also very sticky and can adhere to the predators' sensory organs. For example, the highly stimulating secretions stick to the chemosensory organs of spiny lobsters – the antennules, legs, and mouthparts (Kicklighter et al. 2005). This secretion stickiness may functionally disrupt the spiny lobsters' chemosensory organs. Spiny lobsters rely heavily on their chemosensory organs during foraging and feeding. To forage, they use their antennules for detection and orientation toward distant food odors. To feed, spiny lobsters use legs and mouthparts to taste and handle food (Garm 2004; Garm et al. 2005; Garm and Høeg 2006). Functional disruption of chemosensory activity by sea hare secretions may explain spiny lobster behaviors to sea hare secretions, such as extensive grooming of mouthparts and antennules, which allow the sea hare to escape (Kicklighter et al. 2005).

A striking feature of some chemical defenses of sea hares and other opisthobranchs is the use of acids. Acid glands of many opisthobranchs release their contents following disturbance of the skin (Thompson 1960, 1983, 1986, 1988). For example, notaspids release copious amounts of sulfuric acid from their skin when irritated, creating mucus with a pH of ~ 2 (Gillette et al. 1991). Likewise, the mucus of sea hares (*Aplysia californica*) becomes acidic (pH ~ 4.0) immediately after physical aggravation of the skin (unpublished data).

Acidity is not limited to the skin. Ink and opaline secretions also are acidic. Our preliminary studies showed that ink and opaline are highly acidic. The acidity of the ink secretions may contribute to their efficacy as chemical defenses. Therefore, we asked whether acidity of sea hare secretions enhances the phagomimetic defense by enhancing the neural and behavioral responses of predators to ink and opaline secretions. To answer this question, we measured behavioral and neural chemosensory responses of spiny lobsters to secretions of sea hares presented at a range of pH values. The spiny lobster is a good model predator for this work, since it has been used already to demonstrate effects of ink and opaline secretions on the function of chemosensory neurons and on chemically evoked behavior (Kicklighter et al. 2005), and since the spiny lobster's chemosensory systems are well studied (e.g. Derby 2000; Derby et al. 2001). First, we measured behavioral responses that are controlled by either the antennules (attraction) or mouthparts and legs (handling and ingestion). Second, complementary to behavior we measured the electrophysiological responses of chemoreceptor neurons in the mouthparts and antennules.

Materials and methods

Animals

California spiny lobsters (*Panulirus interruptus*) with carapace lengths of 60-90 mm were collected in the waters near San Diego, CA. Sea hares (*Aplysia californica*) were supplied by the National Institutes of Health National Resource for *Aplysia*, in Florida. Both were shipped to our laboratory and maintained in aquaria containing filtered and re-circulated artificial seawater (Instant Ocean®, Aquarium Systems, Mentor, OH, USA) at room temperature 20-25 °C and a 12:12 hr light: dark cycle. Spiny lobsters for behavioral experiments were housed individually in 80-l aquaria, and spiny lobsters for electrophysiological assays were held in groups of ~15 in 800-l aquaria. Spiny lobsters were fed shrimp and squid three times per week. Spiny lobsters that were pre-molt or that were not attracted to shrimp or squid juice were not used in our assays.

Determination of the pH of natural ink and opaline

The pH values of ink and opaline at full strength were measured from 7 individual sea hares. Ink and opaline were collected from ink and opaline glands according to Kicklighter et al. (2005) and their pH values were tested immediately after collection. pH values were measured using an Accumet AB 15 pH meter and a combination pH electrode with calomel reference (Fisher Scientific, Pittsburgh, PA, USA).

Stimuli and solutions

Ink and opaline were collected from ink and opaline glands, respectively, of 12 sea hares according to Kicklighter et al. (2005) and stored at -20 °C until used. Full strength squid juice was prepared by homogenizing 20 g of squid mantle in 100 ml of artificial sea water (ASW) (Derby 1995) and then filtering through a Whatman 3 filter paper. Full strength shrimp juice

was prepared as was squid juice except using frozen shrimp (*Penaeus* sp.). Ink, opaline, and squid juice were diluted with ASW for the behavioral and electrophysiological assays, except for the loose-patch electrophysiological recordings for which dilutions were made with *Panulirus* saline (PS) (Derby 1995). The pH of all stimuli in all of our assays was adjusted using sodium hydroxide or hydrochloric acid, which affected osmolarity by less than 0.1%. The pH of solutions remained stable over the course of experiments, since they contained buffers. Sea water contained sodium bicarbonate and saline contained HEPES. We measured the pH of solutions at the beginning, middle, and end of each experiment, and they remained within ± 0.2 pH units. For the behavioral assay and electrophysiological recordings from chemoreceptor axons, we made fresh dilutions and pH adjustments every day. For the electrophysiological recordings from olfactory receptor neuronal somata, dilutions and pH adjustments were made once and solutions were then stored at -20°C . After thawing the stimuli diluted in saline, we adjusted the pH, if necessary, and checked it before, during, and after experiments. The solutions were adjusted to pH values of 7.7 (the pH of sea water), 4.9 (the pH of ink when released from a sea hare), and 6.3 (midway between the pH of sea water and ink). The pH of natural sea water (~ 8.1) is slightly higher than the pH of sea water in our aquaria (7.7). Since lobsters behaved normally in our aquaria, and we needed to test the same pH values in all experiments, we used 7.7 as the ‘natural’ pH of sea water in both behavioral and electrophysiological experiments.

Behavioral assays

In our behavioral assays, we examined whether the acidity of sea hare secretions had a significant effect on the behavioral responses of spiny lobsters. Assays were conducted during the light phase of the 12:12 L:D photoperiod. Stimuli were delivered to each spiny lobster using

a hand-held 1-ml pipette, which allowed introduction of 1 ml of stimulus approximately 8 cm from the antennules of the animal. The assays had two phases: conditioning phase and testing phase.

Conditioning phase. Spiny lobsters normally respond to a food odor by moving forward and flicking their antennules in the direction of odor. Animals normally do not respond to ASW. However, at the beginning of our experiments, animals sometimes responded to introduction of the delivery pipette or delivery of ASW. Thus, we conditioned the animals to respond to delivery of food odor (shrimp juice) but not ASW by acclimating them to the delivery procedure. We conditioned them daily, beginning one week prior to the testing phase of the experiment, by presenting to each animal 1 ml of shrimp juice and 1 ml of ASW three times per day at 1 h intervals. This procedure eventually led to animals consistently responding to shrimp odor by moving forward at least a half body length toward the point of stimulus delivery, and by not moving toward ASW.

Testing phase. Once the conditioning phase was complete, we proceeded with the testing phase. We began by determining the minimum concentration that induced at least 20% of the spiny lobsters to respond to ink, opaline, and squid juice. We tested stimuli, all at pH 7.7, ranging down to 0.0001% full-strength. We identified the minimal effective concentrations to be 0.001% full-strength ink, 0.0005% full-strength opaline, and 0.001% squid juice, which we then used to determine the effect of pH on behavioral responses of spiny lobsters.

The independent variables in our experiment were stimulus type and stimulus pH. We tested 4 stimulus types (ink, opaline, squid juice, ASW), each at 3 pH levels (7.7, 6.3, 4.9) in separate behavioral assays. In each behavioral assay, spiny lobsters were tested with a positive control (0.01% squid juice), negative control (ASW), and one stimulus type at three pH values,

for a total of five trials. We waited approximately 30 min between each stimulus delivery. A response was measured through dichotomous scoring: positive response vs. no response. Significance was established using a non-parametric statistical test, the McNemar pair test. A positive response was defined as forward movement of the spiny lobster toward the stimulus by at least a half body length. A no-response was defined as no forward movement within 20 sec of stimulus delivery. Spiny lobsters that failed to respond to the positive control stimulus or that responded to negative control stimulus were not included in our analysis.

Electrophysiological assays

Antennules of spiny lobsters have ten types of sensilla (Cate and Derby 2001). One of these, the aesthetasc sensilla, contains only chemoreceptor neurons, whereas the other types, collectively called non-aesthetasc sensilla, contain both mechanoreceptor neurons and chemoreceptor neurons. Either aesthetascs or non-aesthetascs are sufficient for allowing spiny lobsters to detect and locate food (Steullet et al. 2001). Unlike antennules, mouthparts of spiny lobsters mainly have bimodal sensilla composed of both mechano- and chemoreceptor neurons (Derby 1989; Corotto et al. 1992; Garm et al. 2005; Garm and Høeg 2006). Chemoreceptor neurons in the mouthparts of *Panulirus* detect the same chemicals as antennules but have broader tuning properties (Garm et al. 2005). Thus, mouthparts complement antennules in assessing chemical compounds before items are ingested. For example, spiny lobsters are attracted to either ink or opaline secretions of *Aplysia*, but they ingest only ink because of feeding deterrent compounds in opaline (Kicklighter et al. 2005).

Two types of preparations were used to examine the effect of pH on the responses of spiny lobster chemoreceptor neurons to defensive secretions. One preparation was used to

record responses from the axons of chemoreceptor neurons in the mouthparts and antennular lateral flagella. This preparation did not allow us to identify the sensillum type innervated by the recorded chemoreceptor neurons. A second preparation was used to record responses from the somata of identified receptor neurons – olfactory receptor neurons in the aesthetascs.

Single-unit extracellular electrophysiological recordings from axons

The effect of pH on response of chemoreceptor neurons (CRNs) in the antennular lateral flagellum and second maxilliped (one of the six pairs of mouthparts) was examined as follows. Axonal recordings were performed via single-unit extracellular electrophysiology (Derby 1995; Garm et al. 2005). We recorded from 14 mouthparts and 18 antennular lateral flagella, often isolating more than one neuron per preparation.

Stimuli were presented to the sensilla via an olfactometer with electronically driven valves. Responsive CRNs were identified using 1% ink or opaline, with ASW as the negative control. These stimuli were also presented periodically over the course of an experiment to ascertain that the responsiveness of the neuron did not change. Our independent variables were stimulus type (ASW and 1% of ink, opaline, and squid juice) and stimulus pH (7.7, 6.3, and 4.9). These stimuli were presented in a random order. Each stimulation was immediately followed with three rinses of ASW and a rest period of 90 sec before the next stimulation.

We analyzed the activity of single CRNs using spike sorting (Spike 2, v. 5.05, CED, Cambridge, UK). In each recording, we collected data for total of 16 sec, divided into 3 time periods: 2 sec prior to chemical stimulation, 2 sec during chemical stimulation, and 12 sec after stimulation. Our dependent variable was spike frequency in the first 500 ms of response to a chemical stimulus. We analyzed these data for statistical differences through both parametric GLM repeated measures ANOVA and non-parametric Wilcoxon two-related-sample tests using

SPSS software (SPSS 12.0). We performed the same type of analysis for all the electrophysiological data. Data were transformed to square root to meet sphericity and normal distribution criteria as needed. After we determined significance for each stimulus, we performed three post-hoc comparisons between pH values: 7.7 vs. 6.3, 7.7 vs. 4.9, and 6.3 vs. 4.9. The appropriate significance levels ($p < 0.05$) were determined after Bonferroni corrections for either parametric or non-parametric tests. Single-unit activity from recordings of axons of antennular CRNs most likely was from non-aesthetasc sensilla rather than aesthetasc olfactory receptor neurons (Steullet et al. 2002).

Extracellular recordings from somata of olfactory receptor neurons

Our antennular preparation and method of recording olfactory receptor neurons (ORNs) were modified from Bobkov and Ache (2003, 2007). The middle section of the aesthetasc region of the lateral flagellum was cut from intermolt animals and bathed in a dish filled with PS. From this section, ~12 individual annuli were cut. The dorsal half of each annulus was removed to expose the ORN clusters. To remove sheath tissue around ORN clusters, annuli were placed in 1 mg trypsin/ml PS for 1 min, after which they were rinsed in PS for 20 min. The distal-most row of ORN clusters in the annulus was then removed to visualize and access the remaining ORN somata. An annulus was then transferred to a 60 x15 mm dish and filled with PS, and the annulus was secured to the bottom using a hair that was secured by dental wax. This preparation was placed on the stage of an inverted microscope (Olympus CK2) and perfused continuously with fresh PS (0.6 ml/min). Stimuli were delivered directly to aesthetascs via electronically controlled rapid-solution changer (RSC-160, Bio-Logic, Claix, France). The stimulus was

released approximately 1 mm from the aesthetascs. In each recording, we collected 2 sec of data before and 13 sec after a 4-sec stimulus recording.

Extracellular recordings were taken from ORN somata of aesthetasc sensilla using loose-patch techniques (Bobkov and Ache 2003, 2007). Borosilicate patch pipettes (GMBH GB150-8P, *Science* Products, Germany) were pulled using a Narishige PP830 pipette puller (Japan) to produce electrodes with a tip diameter of ca. 1 μm . These electrodes were filled with PS and had a resistance of 2-3 $\text{M}\Omega$ in the PS bath. The electrodes usually formed seals with resistance of $\sim 30 \text{ M}\Omega$. The electrode tip contacted the soma of a single ORN innervating a single aesthetasc sensillum. Data acquisition was performed using an amplifier (Axopatch-1D), digitizer (Digidata-1320A), and software (pClamp 8.2) (Axon instruments, Inc). Spike sorting was accomplished with Spike 2 v.5.05 software. The dependent variable was spike frequency, measured during the first 3 sec of response to chemical stimulation. We measured the spike rate at 3 sec instead of 0.5 sec since the spike rate of ORN responses increased at a much slower rate than single unit recordings (see above), and thus a 3-sec measurement is a more reliable indicator of spiking rate. For example, the CRNs in single unit recordings reached their maximum spike rate during their first 0.5 sec, where as the ORNs in single soma recordings reached their maximum spike rate in 2-3 sec.

This preparation was used to examine the effect of pH on the response of ORNs at various concentrations of ink. Our positive control was the fish food TetraMarine (TM) (Tetra, USA) dissolved with PS and filtered (pore diameter 0.22 μm , Fisherbrand) to 0.125 g/ml (full strength). TM was further diluted with PS to 1% when used as a positive control. ORNs that responded to 1% TM (pH 7.7) or in some cases to 0.1% ink (pH 7.7) but not PS alone (pH 7.7) were considered as odor-responsive ORNs and used in our assay. ORNs that showed intrinsic

bursting activity, as reported by Bobkov and Ache (2007), were avoided since stimulation with ink or TM did not appreciably change their spiking activity. Each 15-sec recording consisted of measuring spontaneous activity for 2 sec before stimulus delivery, then the response to the chemical stimulus during 4 sec of its delivery, followed by 9 sec of post-stimulation recordings. The interval between stimulations was 90 sec. Our experimental stimuli included ink at dilutions of 0.1%, 0.01%, and 0.001% full strength, each at three pH values – 7.7, 6.3 and 4.9. Stimuli were delivered in a random order from one preparation to another. However, within a single preparation, a block of stimulations consisted of testing a single concentration of ink at all three pH values, presented in random order, followed by other blocks at different ink concentrations. Within a block, the first ink stimulus was re-tested as a positive control at the end of that block. At the end of all blocks, TM and PS were tested, followed by a measurement of spontaneous activity.

Results

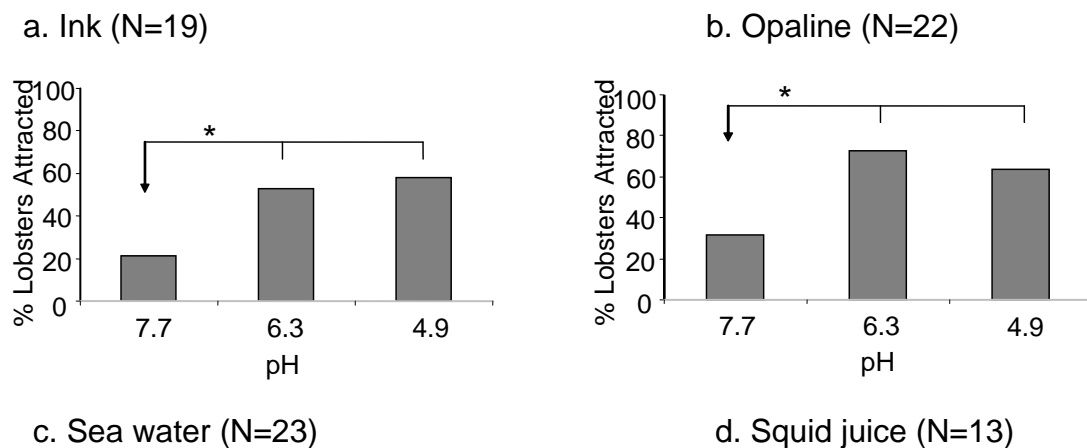
The defensive secretions of sea hares are acidic

Full-strength ink had a pH of 4.9 ± 0.07 (mean \pm S.E.M., $n=7$), and full-strength opaline had a pH of 5.9 ± 0.08 (mean \pm S.E.M., $n=7$). Both had lower pH values than sea water: the pH of sea water in our aquaria was ~ 7.7 .

Phagomimetic behavioral response of spiny lobster to ink and opaline is enhanced at acidic pH

Our previous study showed that spiny lobsters were attracted to the defensive secretions of sea hares – ink and opaline – even if they did not consume one of the secretions, a process

called phagomimicry (Kicklighter et al. 2005). In those studies, both secretions were tested at their natural pH values. To determine whether the pH values of secretions increase phagomimetic attraction, we tested both secretions at near-threshold concentrations, and at pH values ranging from that of ink at full strength (pH 4.9) to that of sea water (pH 7.7). We recorded the number of lobsters that showed attractive responses to ink, opaline, squid juice (SJ, a positive control), and sea water (SW, a negative control), at pH values of 7.7, 6.3, and 4.9, and from these data we calculated the percentage of responding lobsters out of all tested lobsters. The percentage of lobsters that showed attractive responses to stimuli of low pH was significantly higher than that responding to stimuli of high pH for all four stimuli (Fig. 1 a-d). For instance, the percentage of lobsters showing attractive responses to ink, opaline, and sea water of low pH was ~30-40 percentage points higher than the percentage of animals responding to the same stimuli at pH 7.7. Similarly, the percentage of lobsters showing attractive responses to squid juice at low pH was ~60 percentage points higher than the percentage responding to squid juice at pH 7.7.



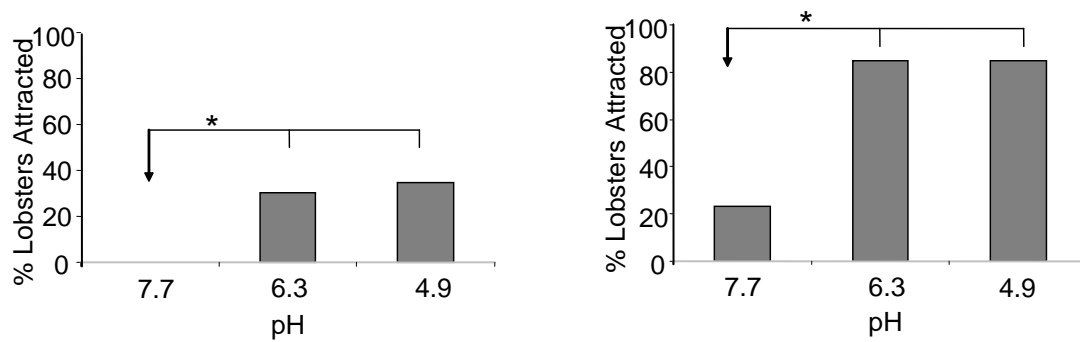


Figure 3-1 The percentage of lobsters attracted to acidic stimuli was significantly higher for all stimuli. (a) 0.001% ink, (b) 0.0005% opaline, (c) sea water, and 0.001% squid juice. N= # of spiny lobsters tested. Asterisks indicate responses at pH 4.9 or 6.3 were significantly different than the response to the stimulus at pH 7.7 sea water (McNemar pair test, $\alpha=0.05$).

Responses of mouthpart and antennular chemoreceptor neurons to ink and opaline are higher at acidic pH

Axonal recordings of second maxilliped chemoreceptor neurons. CRNs from mouthparts clearly fell into two groups: one group was sensitive to changes in pH alone (i.e. sea water at low pH), and another group was insensitive to pH changes. These two groups differed in another way: those sensitive to pH changes alone were also sensitive to a pH change in other stimuli, while those insensitive to pH change alone were generally insensitive to pH changes in these other stimuli. The group of pH-sensitive CRNs responded on average with higher spike frequency to sea water, ink, opaline, and squid juice at low pH than at the pH of sea water (7.7) (Fig. 2a), with responses to sea water and ink being significantly higher at pH 4.9 than at pH 7.7 (Fig. 2a). Note that CRN responses to sea water at any pH were much lower than responses to ink and opaline, and thus the increase in response at pH 6.3 and 4.9 compared to 7.7 was high on a percentage scale but not on an absolute scale. In other words, the increase in absolute responses at lower pH values was in the same range for sea water, ink, and opaline. In fact, subtracting sea water

responses at each pH from the corresponding stimulus of the same pH removed the pH effect for each stimulus. Thus, pH change alone accounted for most of the enhanced chemosensory responses toward either defensive secretions or a food odor. The pH-insensitive CRNs responded with similar intensity across the pH values of each stimulus (Fig. 2b). The pH-insensitive CRNs showed little or no response to sea water at any pH, although they showed high responses to other stimuli such as ink and opaline.

Mouthpart CRNs

a. pH-sensitive
(N=11)

b. pH-insensitive
(N=10)

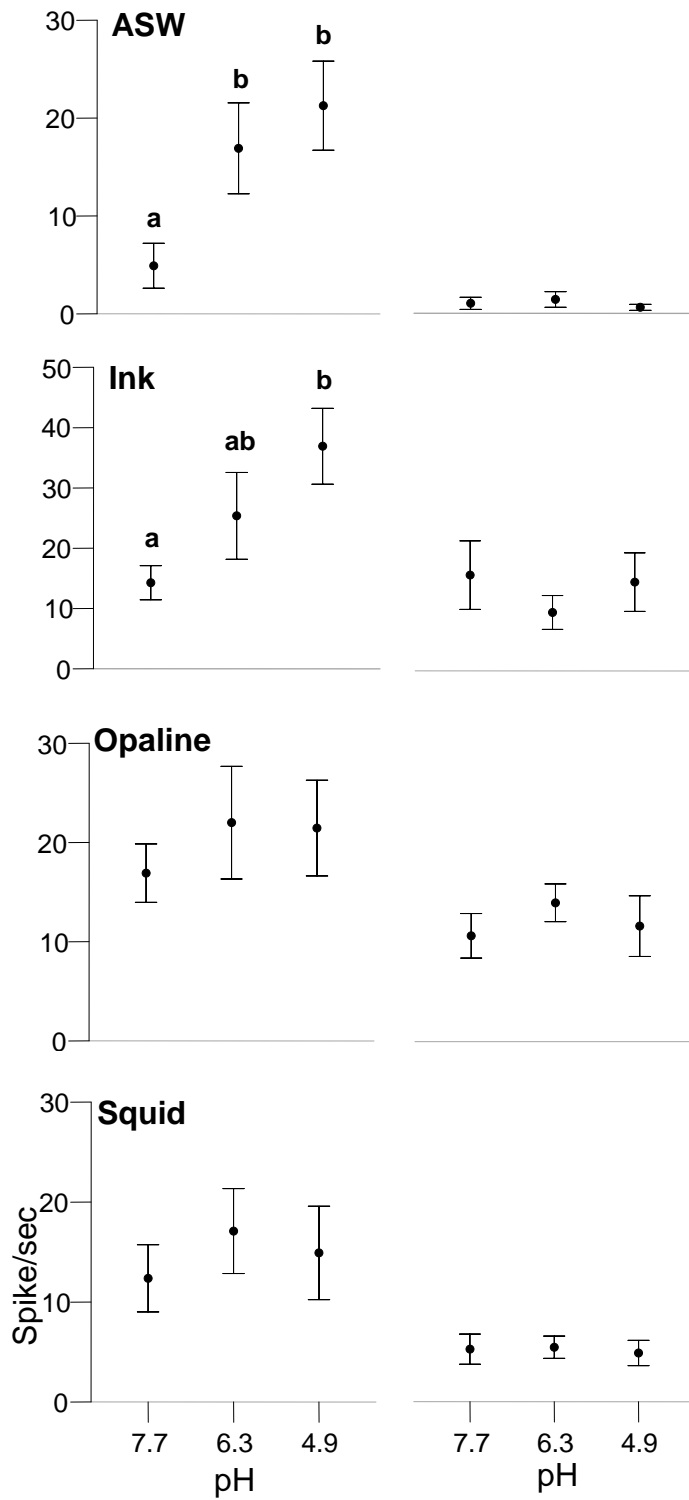
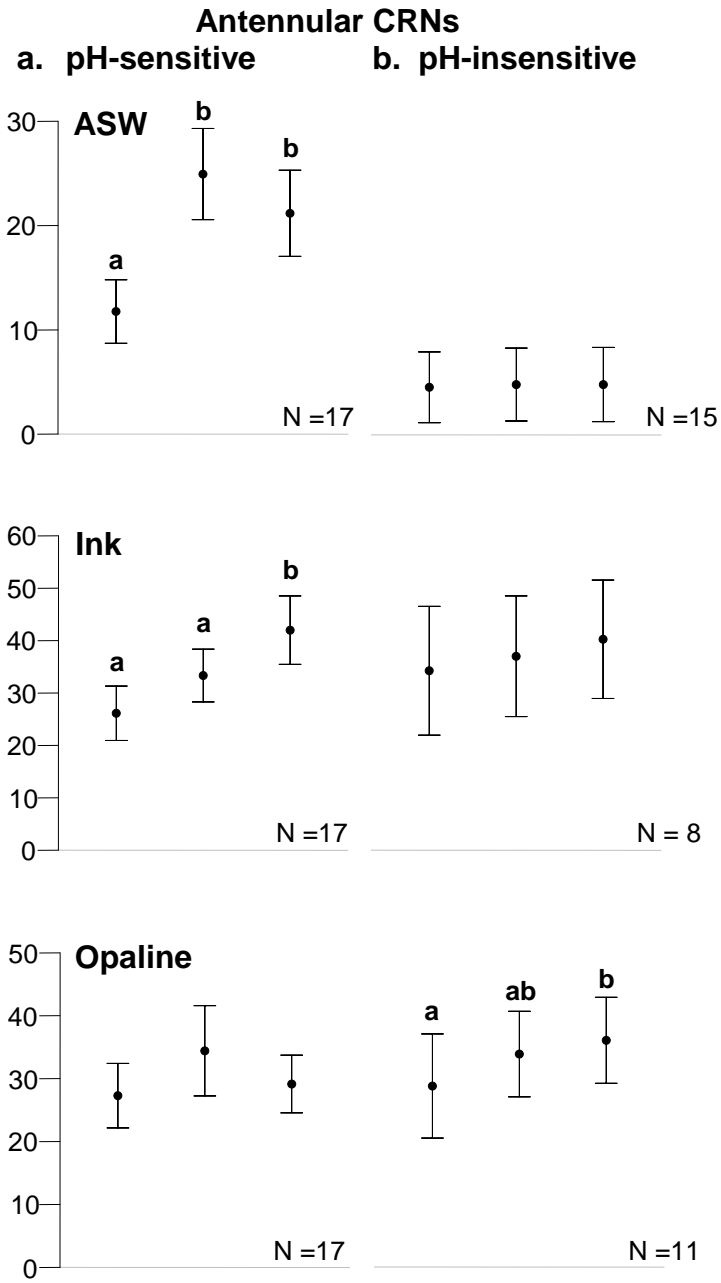


Figure 3-2 In the 2nd maxillipeds, over 50% of chemoreceptor neurons were highly sensitive to pH change alone and to other stimuli (a), the rest were insensitive to pH change across stimuli (b). Activity is expressed as mean \pm S.E.M. of the frequency of action potentials (# of spikes/sec) to ASW, 1% ink, 1% opaline, and (d) 1% squid juice. The letters represent statistical differences between responses to the pH levels (ANOVA or Wilcoxon followed by post-hoc tests after Bonferroni corrections, $\alpha=0.05$).

Axonal recordings from antennular chemoreceptor neurons. CRNs from antennules also partitioned into two groups: one group that was sensitive and another that was insensitive to changes in pH alone. The CRNs sensitive to pH change alone were also sensitive to pH change in other stimuli, and the CRNs insensitive to pH change alone were minimally or not at all sensitive to pH change in other stimuli. The group of pH-sensitive CRNs from antennules responded on average with higher spike frequency to sea water, ink, opaline, and squid juice at low pH than at 7.7 (Fig. 3a), with significantly greater responses to pH 4.9 than 7.7 for both sea water and ink (Fig. 3a). Furthermore, sea water induced much lower responses than other stimuli, so that the change in absolute response with increasing pH was similar for sea water, ink, and opaline. Subtracting the sea water responses from each stimulus of corresponding pH removed the pH effect for each stimulus. Thus, pH change alone accounted for the enhanced responses of antennular CRNs to either defensive secretion or food odors. The pH-insensitive CRNs from antennules, similar to those from mouthparts, responded with the same intensity to sea water across the pH values, and unlike those from mouthparts, responded greater to ink and opaline at low pH than at high pH (Fig. 3b). In fact, these CRNs responded significantly greater to opaline at pH 4.9 than at pH 7.7. Furthermore, when pH-sensitive and insensitive CRNs were grouped together, they responded to stimuli (ink, opaline, squid, and sea water) with significantly greater intensity to low pH (4.9) than high pH (7.7) ($p < 0.05$ ANOVA). However, the pH-

insensitive CRNs from antennules, like those from mouthparts, showed little or no response to sea water at any pH, although they responded well to other stimuli such as ink and opaline.



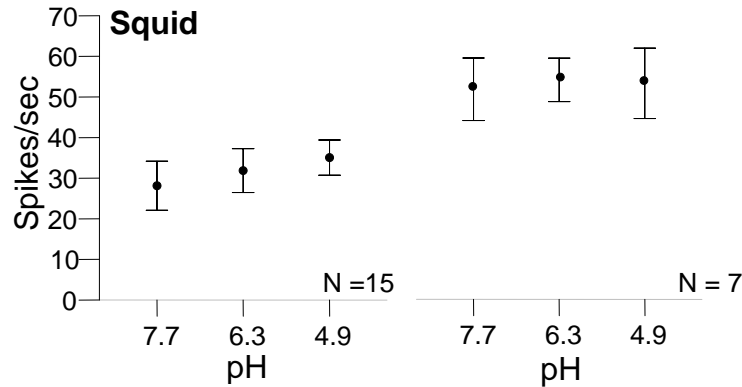


Figure 3-3 In the antennules, over 50% of chemoreceptor neurons were highly sensitive to pH change alone and to other stimuli (a), while the rest were insensitive to pH change alone but sensitive to specific stimuli (b). Activity is expressed as mean \pm S.E.M. of the frequency of action potentials (# of spikes/sec) to ASW, 1% ink, 1% opaline, and 1% squid juice. The letters represent statistical differences between responses to the pH levels (ANOVA or Wilcoxon followed by post-hoc tests after Bonferroni corrections, $\alpha=0.05$).

Soma recordings from aesthetasc olfactory receptor neurons (ORNs). Taken in its entirety, the population of 35 aesthetasc ORNs responded to ink in a manner dependent on both stimulus concentration and stimulus pH (Fig. 4). In statistical terms, concentration explained a significant proportion of response variance (partial $\eta^2 = 0.55$ or $F = 46.4$, $p < 0.0001$), with the highest concentration of ink (0.1%) but not the lower concentrations (0.01% and 0.001%) producing significant responses. pH also explained a significant portion of response variance (partial $\eta^2 = 0.12$ or $F = 5.1$, $p < 0.008$). This pH effect was apparent only at the highest concentration of ink, since this was the only concentration that produced significant responses from the overall ORN population. For 0.1% ink, the response to ink was significantly greater at the lowest pH (4.9) than at 6.3 or 7.7.

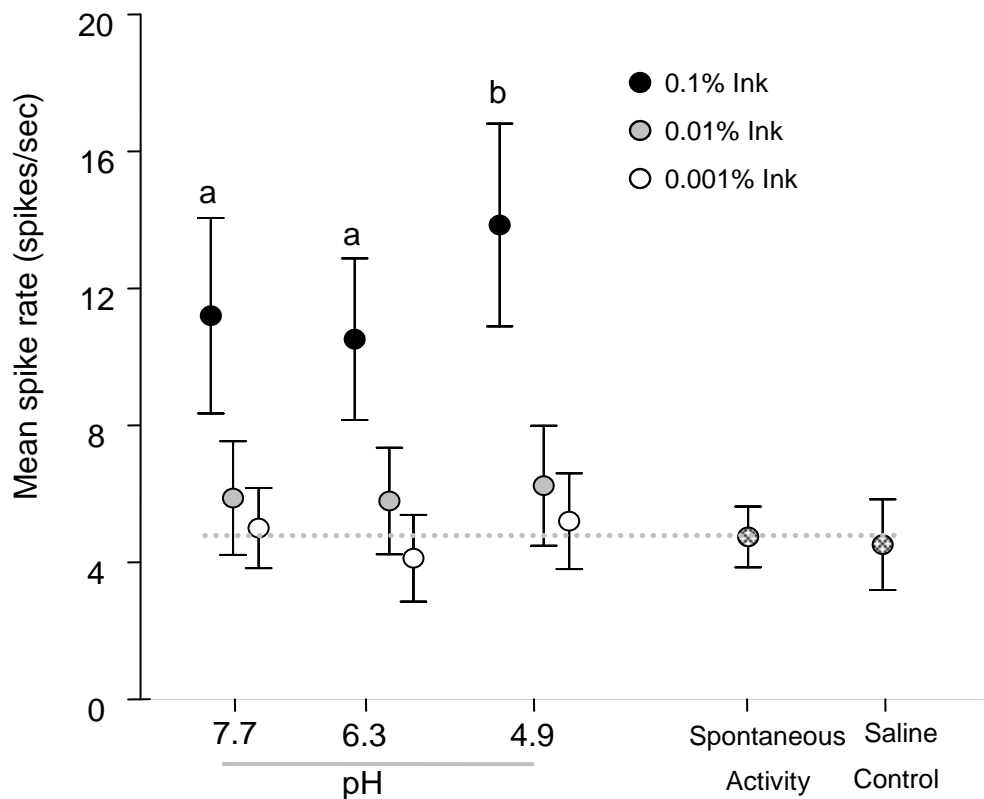


Figure 3-4 The response of a population of olfactory receptor neurons (n=39) to high concentrations of chemicals was enhanced at acidic pH. At high concentration of ink (0.1%), the ORN population responded with significantly higher spike frequencies at low pH (4.9) compared to 6.3 and 7.7. The circle and bars represent mean \pm S.E.M. A dashed line indicates mean spontaneous spiking rate. The letters represent statistical differences between responses to the pH levels at that concentration (ANOVA followed by post-hoc tests, $\alpha=0.05$). This effect of pH was only seen at the highest concentration of ink (0.1%) and not at the lower concentrations (0.01 and 0.001%).

While the above description is for the entire population of ORNs, the response spectrum of individual members of this population of ORNs varied from each other. This can be seen from qualitative observations. Three examples of ORN responses to 0.1% ink are shown in Figures 5a-d. The first example is an ORN that responded more to lower pH ink than to higher

pH ink (Fig. 5a). Over 60% of recorded ORNs responded similar to this ORN. A second example of the diversity of response spectra of the aesthetasc ORNs, as shown in Fig. 5b, responded more to higher pH ink. A third example is an ORN that responded most to either low or high pH ink but little to pH 6.3 ink (Fig. 5c).

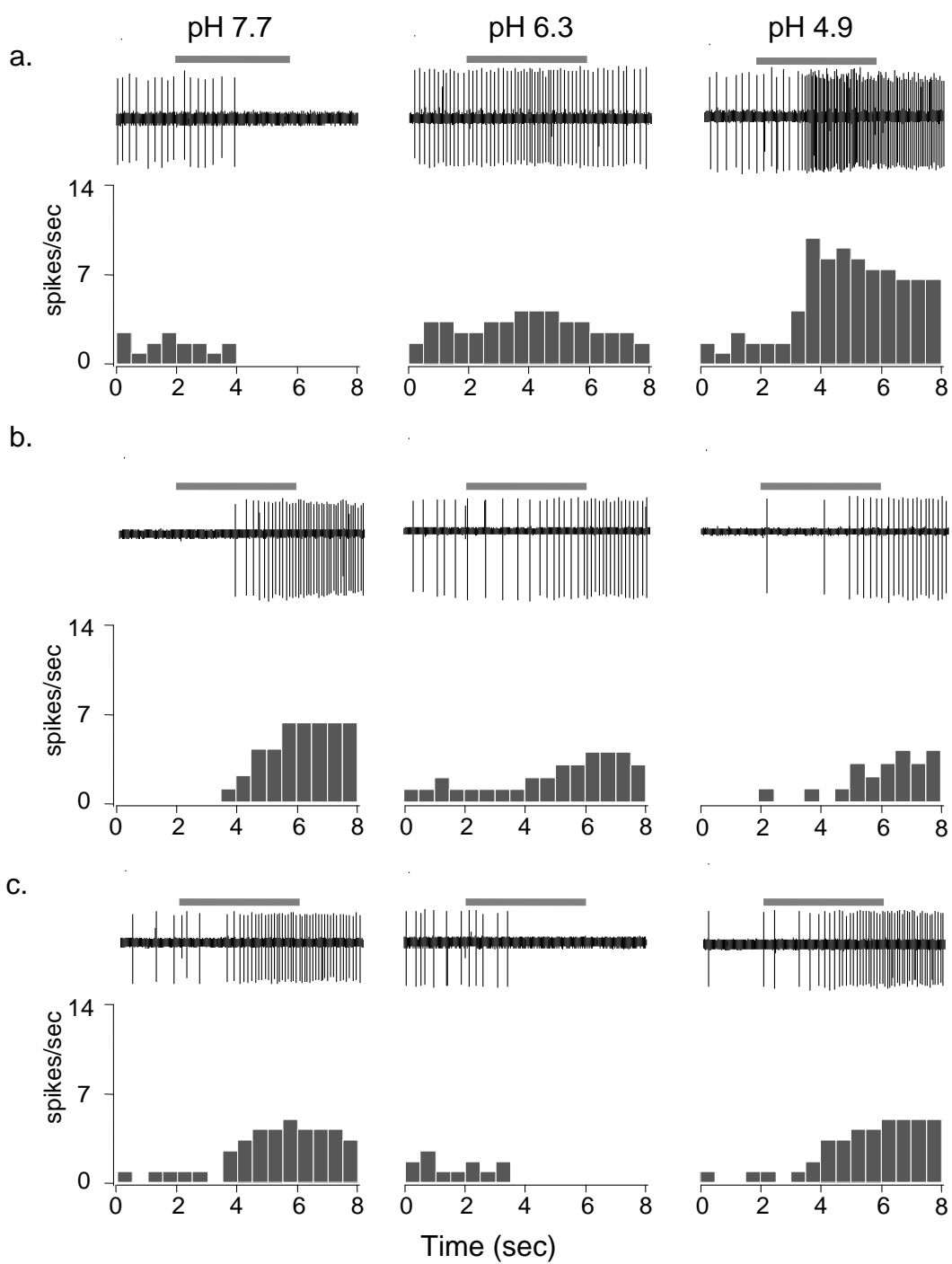


Figure 3-5 Three examples of the diversity of responses of ORNs to stimulation with 0.1% ink at three pH values – 7.7, 6.3, and 4.9 (gray line). (a) This ORN responded greatest to acidic ink. (b) This ORN responded greatest to high pH ink. (c) This ORN responded greatest to high and low pH ink but not to mid pH ink.

Discussion

Our behavioral and electrophysiological study showed that the acidity of *Aplysia*'s active defensive secretions enhanced its phagomimetic properties against the predatory spiny lobster *Panulirus interruptus*. The acidity significantly increased the behavioral attraction of spiny lobsters to the secretions, and the effect of pH alone accounted for most of this increase. These behavioral results correspond closely to changes in responses of specific chemoreceptor neurons in the spiny lobster's antennules and mouthparts. Low pH significantly and consistently increased the pH-sensitive neurons' responses to defensive secretions, and low pH alone accounted for most of the increased excitability.

Acidity enhanced CRNs responses to secretions, depending on the type of CRNs, primarily by direct activation and perhaps secondarily by interacting with secretions. In half of the recorded antennular and mouthpart CRNs, acidity directly activated them and in so doing strongly enhanced the response to the secretions. In the other half of these mouthpart CRNs, acidity did not directly activate them and it did not at all enhance the response to the secretions.

Surprisingly however, the antennular pH-insensitive CRNs, though not sensitive to acid alone, showed moderately enhanced responses to acidic secretions. This suggests that for some cells, pH modulates the response to secretions. Overall for the population of antennular or mouthpart CRNs, acid strongly enhances their responses to the secretions.

Similarly, acid enhanced the responses of olfactory receptor neurons in the aesthetasc sensilla (ORNs) to ink, although these ORNs often showed complex response profiles to ink at different concentration and pH values. For example, acid increased the average response of ORNs to ink only at high ink concentration. This may be due to low, and sometimes high, concentration of ink evoking inhibitory rather than excitatory responses at some pH values (Fig. 5a, c). Such responses were not seen in recordings from antennular and mouthpart CRNs. Because of these complexities in ORNs responses, the overall population of ORNs did not show enhanced excitatory responses to low concentrations of ink.

Acid may affect cellular responses in several ways. One possibility is that the defensive compounds in ink and protons bind to similar sites on the same receptor molecules, i.e. they are competitive agonists. For example, protons enhance nociceptor sensitivity to capsaicin molecule because they bind to and activate similar sites on capsaicin receptors (Jordt et al. 2000; Tominaga and Julius 2000). Similarly, acids interact with bitter, sweet, and amino acid tastants on similar sites of the taste receptor TRPM5 (Liu et al. 2005). A second possibility is that protons affect post-receptor components of the transduction pathway separate from that by other components of ink, i.e. a non-competitive effect. A third possibility is that protons affect the molecular structure of the defensive compounds themselves. For example, ink changes from purple to reddish when pH is increased, indicating that at least some chemical properties are changed. In addition, previous studies have suggested that protons change amino acid charge

and ultimately amino acid effectiveness in inducing chemosensory responses (Tierney and Atema 1988). Because low pH sea water can stimulate both behavioral and physiological responses, we do not believe that this third possibility is responsible for our reported effects.

Our results suggest that the acidity of ink and opaline, by enhancing the response of chemosensory neurons to these secretion, may enhance the phagomimetic and/or sensory disruptive defensive properties of these secretions. The secretions are effective in diverting the attack of spiny lobsters toward the defensive secretions, causing grabbing and digging behaviors, and away from the sea hare itself and thus allowing it to escape (Kicklighter et al. 2005). We show here that the acidity of ink and opaline enhances their effectiveness. The acid's stimulation of chemosensory neurons should likewise enhance the sensory disruptive defensive properties of these secretions, although this was not studied in our behavioral tests.

The use of acids as chemical defenses is widespread among marine gastropods. Such acids, including sulfuric acid, are packaged in special glands in the skin (Thompson 1960, 1983, 1986, 1988). When attacked by predators, these glands quickly release copious amounts of mucus laden with sulfuric acid, making the skin highly acidic. For example, irritation of the skin causes skin mucus of *Pleurobranchaea californica* to reach a pH of ~2 (Gillette et al. 1991) and the mucus of *A. californica* to reach a pH of 3-4 (personal observation). How ink and opaline are made acidic – whether by sulfuric acid or other acids – is unknown. How the acidity in these gastropod skin secretions affects chemical responses of predators is also unknown. Spiny lobsters that grab sea hares will encounter the acidic skin mucus with their legs, mouthparts, and possibly even the antennules. We hypothesize that the acidity of the skin secretions enhances their defensive properties in a manner similar to that which we showed here for ink and opaline.

In summary, our results demonstrate another weapon in the arsenal of active chemical defenses by sea hares – the acids. These acids modify the behavior of predators either by directly stimulating or by indirectly enhancing the chemosensory responses of predators such as spiny lobsters. Anecdotal reports have previously indicated that acids alone or a change in the pH of amino acid stimuli can affect the activity of crustacean chemoreceptor neurons, but without suggesting an ecological context for this effect (Case 1964; van Weel and Christofferson 1966; van Weel and Correa 1967; Johnson and Ache 1978). Therefore, our report provides the first demonstration within a neuroethological and functional context that acids enhance the chemosensory responses of crustaceans.

CHAPTER 4

SPINY LOBSTERS DETECT CONSPECIFIC BLOOD-BORNE ALARM CUES EXCLUSIVELY THROUGH OLFACTORY SENSILLA.

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Introduction

Many animal taxa when exposed to hazardous situations such as predatory attacks 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 behaviour 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 behaviour 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 behaviour by conspecifics (Blum, 1985). These alarm pheromones mediate these behavioural responses primarily through the insect's olfactory pathway (Galizia et al., 1999; Yamagata et al., 2006, 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 odours (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 behaviour 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, 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 behavioural 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, 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, 1996b). The aesthetasc/olfactory lobe pathway may uniquely process detection of pheromones and other conspecific odours, whereas the non-aesthetasc/lateral antennular neuropil pathway and the aesthetasc pathway both detect general odours including food chemicals (Gleeson, 1980, 1992; Steullet et al., 2002; Schmidt and Derby, 2005; Johnson and Atema, 2005; Horner et al., 2008a, 2008b).

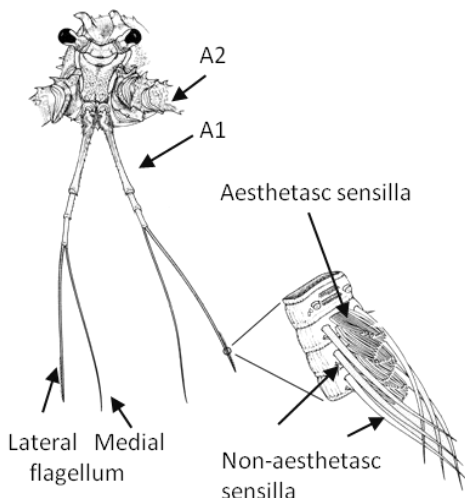


Figure 4-1 Chemosensory organs of spiny lobsters. A1, 1st antenna or antennule; A2, 2nd antenna. A1 bifurcates after the basal segments into the lateral and medial flagella, which share many of the same non-

aesthetasc sensilla. However, only the lateral flagellum contains rows of the aesthetasc sensilla. Diagram modified from Schmidt et al. (2006).

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 behaviour induced by such alarm cues, under both field and laboratory conditions. To answer the 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 if Caribbean spiny lobsters respond with alarm only to conspecific hemolymph or if 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 behavioural responses to hemolymph, by examining behavioural responses to hemolymph before and after surgical manipulations of different types of sensilla.

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 \pm 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 12h:12h light/dark phases. Lobsters were individually held in 80-liter aquaria (60 cm L x 30 cm W x 45 cm H) containing filtered sea water (Instant Ocean®, Aquarium Systems, Mentor, OH) at 25 °C. Each aquarium contained a hand-made plastic shelter at one end. The shelter was 25.4 cm x 24 cm x 24 cm (height x width x 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 behaviour 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 Dickenson, Franklin Lakes, NJ) inserted at the base of the fourth or fifth leg. We used fresh hemolymph diluted 100 times with sea water for each experimental day for most behavioural tests. Hemolymph diluted at 300 times was used only for the behavioural assay on stimulus specificity since it was as potent as hemolymph at 100 times dilution. Sea water (SW) was used as a negative control stimulus in all experiments. Shrimp odour was used as a feeding stimulus. It was prepared by blending shrimp tissue in sea water at a concentration of 2 mg/ml, then filtering.

Ablation of sensilla

To determine if the aesthetasc pathway is necessary or sufficient to mediate alarm behaviour, we performed behavioural experiments before and after ablation of aesthetasc or non-aesthetasc sensilla. To test for necessity of aesthetascs, we performed behavioural tests during February and March of 2005 on 20 lobsters, first on all animals before treatment ('intact') and then after either ablation of aesthetasc pathway (9 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 Sterilization Pan; Lima, OH, USA) filled with sea water ~ 10 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 one week before we performed the same behavioural experiments as before treatment.

To test for the sufficiency of aesthetascs to mediate alarm responses, we performed behavioural 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 we did 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, California, 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 one week before we resumed behavioural testing. In these experiments, concrete rectangular blocks (23 x 23 cm) were used as shelters.

To determine the effectiveness of our sensillar ablations, we collected the lobsters' antennular flagella after completing the behavioural assays according to 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 KH₂PO₄ and 21.3 g Na₂HPO₄ in 1 liter deionized H₂O, adjusted 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 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% (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.

Behavioural tests

The behavioural 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 behavioural testing paradigm. This included feeding lobsters a piece of shrimp using tongs and delivering sea water 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 shrimp odour, but not to negative controls (tongs without shrimp or pipettes releasing 10 ml sea water).

Following this acclimation phase, the test phase began, in which we measured appetitive and alarm behavioural responses to chemical stimuli, as defined below. In the test phase, we delivered 1-10 ml of 2 mg ml⁻¹ shrimp odour, observed for 45 sec, delivered 10 ml of an experimental stimulus (hemolymph) or control stimulus (SW), observed for 120 sec, and then again delivered 1-10 ml of shrimp odour and observed for 30 sec. All experimental events were videorecorded (Sony DCR PC110) under low-intensity red light during the dark phase, and analysed later.

We used three dependent measures to assess alarm response to hemolymph. The first measurement was a quantification of the occurrence and intensity of alarm response of each animal during the 120-sec 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 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 behaviour was the most stereotypical and consistent alarm behaviour, we used it as one dependent measure of alarm response. The intensity of alarm response according to our first dependent measure was assessed using an ordinal scale, from -3 (most alarming) to 0 (no alarm; Table 1) (Table 1). The difference in intensity of 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 the nominal scale, a response intensity of “-1” or lower (Table 1) was rated as a ‘yes’ for alarm response. Differences between nominal measures were tested for significance using McNemar tests.

A second dependent measure of alarm was time spent in the shelter, expressed as percentage of the 150-sec 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 % time in shelter indicated that lobsters are alarmed by the stimulus.

The third dependent measure was the suppression of appetitive response to a food odour by hemolymph. An appetitive response is defined as the animal moving forward towards the source of the chemical. The intensity of appetitive response to shrimp odour is measured on an ordinal scale from +3 (most attractive) to 0 (not attractive) (Table 1). The intensity of the suppression of 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 measure to the first presentation of shrimp odour was significantly greater than the

response to the second presentation of shrimp odour (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 measure 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 odour, after a response of “+1” or greater to the first presentation of shrimp odour, was rated as suppression of appetitive response by hemolymph. Differences between nominal measures were tested for significance using McNemar tests.

Table 4.1. Intensity scale for the responses of *P. argus* to stimuli.

Score	Behaviour toward stimuli
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 2 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 body length and lowers against the substrate
-2	Lobster moves backward at least 1 body length or inside shelter from outside
-3	Lobster moves backward inside the shelter and moves up the ramp of the shelter

Our data were collected in the course of three experiments. Our first experiment tested whether Caribbean spiny lobsters respond with alarm behaviour when exposed to conspecific hemolymph before and after ablations of the aesthetasc sensilla. The control stimulus was SW.

These two stimuli were delivered ‘blindfolded’ randomly over two days, with a maximum of two stimulus presentations per day per lobster. All three dependent measures were used in this analysis.

Our second experiment tested whether spiny lobsters respond with alarm behaviour 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 behavioural responses to hemolymph from *P. argus*, *P. interruptus*, and *C. sapidus* as experimental stimuli and sea water 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 odour. Thus in this experiment, we only used the first dependent measure of alarm response to hemolymph, and did not use suppression of appetitive response by hemolymph. Statistical significance was investigated using Cochran-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 square test for independent measures.

Field experiments

A field study was performed to determine whether wild Caribbean spiny lobsters show the same alarm responses to hemolymph of conspecifics as do 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 videorecorded using an underwater micro videocamera (Micro Video TM; Bobcaygeon, Ontario, Canada) mounted in a PVC pipe and positioned approximately 1 m from the lobster. Recordings started at dusk, around 19:30, and usually ended before 21:30. 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 required artificial illumination through two IR illuminators (IR-200 ProVideo; Surveillance-Video.com; Manhattan, New York, USA). 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 behavioural 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 of the water. One of those plastic tubes delivered the experimental stimulus (hemolymph, diluted 100 times with filtered sea water) and the other delivered the control stimulus (filtered sea water). 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 two stimuli. Sea water, 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 sec. 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 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 negative control first.

All behavioural responses were recorded, with an emphasis on 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 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 behaviour

Hemolymph induced several types of responses, all indicative of alarm behaviour, including retreat, increased sheltering, and reduced appetitive responses to food (supplementary material Movies 1 and 2; Table1). Hemolymph induced an alarm response in a significantly greater percentage of lobsters than did sea water (Fig. 2A). Hemolymph also induced significantly more sheltering than control stimuli (Fig. 2B). In addition, hemolymph eliminated appetitive responses to shrimp odour in a significant percentage of lobsters (Fig. 2C).

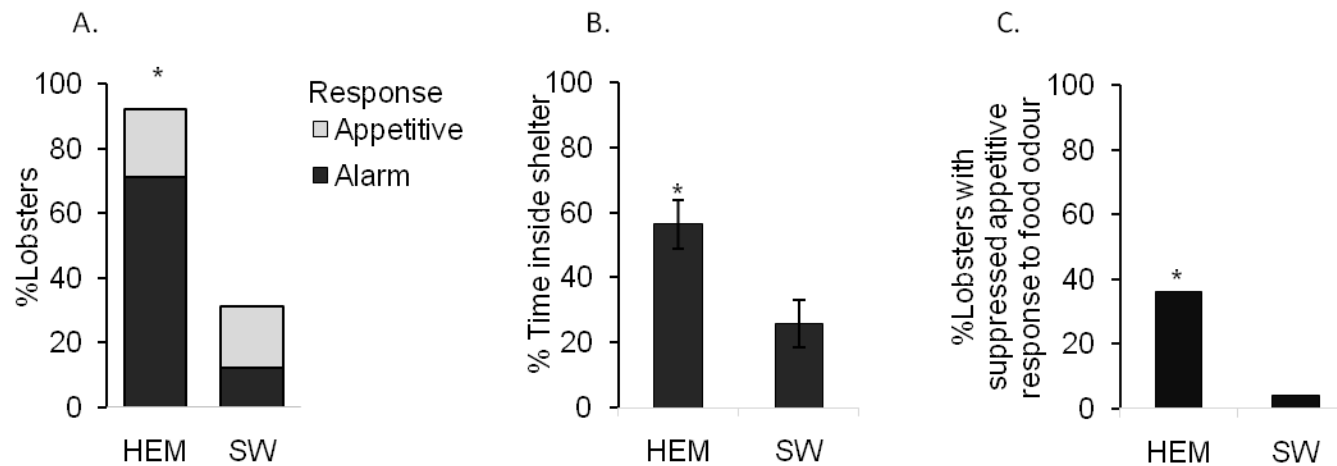


Figure 4-2 (A) Hemolymph (HEM) induced alarm responses in significantly more spiny lobsters than did sea water (SW). In contrast, HEM and SW induced a similarly low frequency of appetitive responses. (B) Hemolymph caused spiny lobsters to spend significantly more time inside shelter than did SW. (C) Hemolymph suppressed the appetitive response to shrimp odour in significantly more lobsters than did SW. Results are based on 53 lobsters. * denotes a significant difference between HEM and SW using nominal data and McNemar tests ($P < 0.05$) as described in the Methods. Evaluation of the results based on ordinal data using Wilcoxon matched-pairs test showed similar results.

Aesthetasc ablation eliminates alarm behaviour

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 ablations was significantly higher than after ablations of the aesthetasc sensilla. However, the percentage of control lobsters showing alarm responses to hemolymph was similar either before or after sham treatments (Fig. 3A).

Lobsters with ablated aesthetascs reversed their behaviour 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 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 odour was significantly higher when shrimp odour was presented

after exposure to sea water than after exposure to hemolymph. However, this suppression of response to shrimp odour by hemolymph was eliminated following aesthetasc ablation. In contrast to ablated lobsters, sham-treated lobsters showed the suppression by hemolymph. However, this suppression was not significantly different than suppression by sea water (McNemar tests, $P > 0.05$).

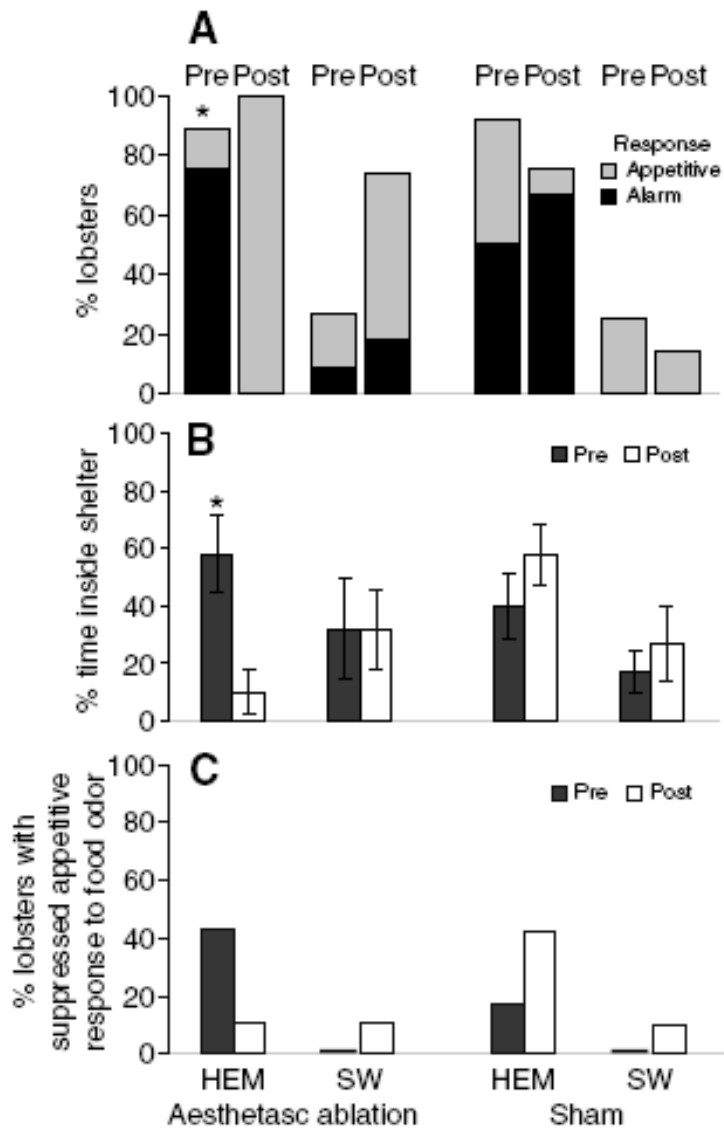


Figure 4-3 Ablating aesthetasc sensilla eliminated all forms of alarm responses to hemolymph (HEM). (A) Before (Pre) ablation of aesthetasc sensilla, a significantly (indicated by the asterisk) higher percentage of experimental lobsters (left graph, $n = 9$) showed alarm responses to hemolymph than after (Post) ablations (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 (indicated by asterisk, 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 treatments. (B) Experimental lobsters spent significantly

more time inside shelter in response to hemolymph before than after ablation (Wilcoxon matched-pairs test, $*P < 0.05$). Control lobsters spent similar amount of time inside 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 odour 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 test.

Non-aesthetasc ablation does not affect the alarm response

None of the four measures 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).

Third, the amount of time that lobsters spent inside 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 shelter following presentation of hemolymph (Fig. 4B).

Fourth, suppression of appetitive responses to shrimp odour 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 odour (Fig. 4C).

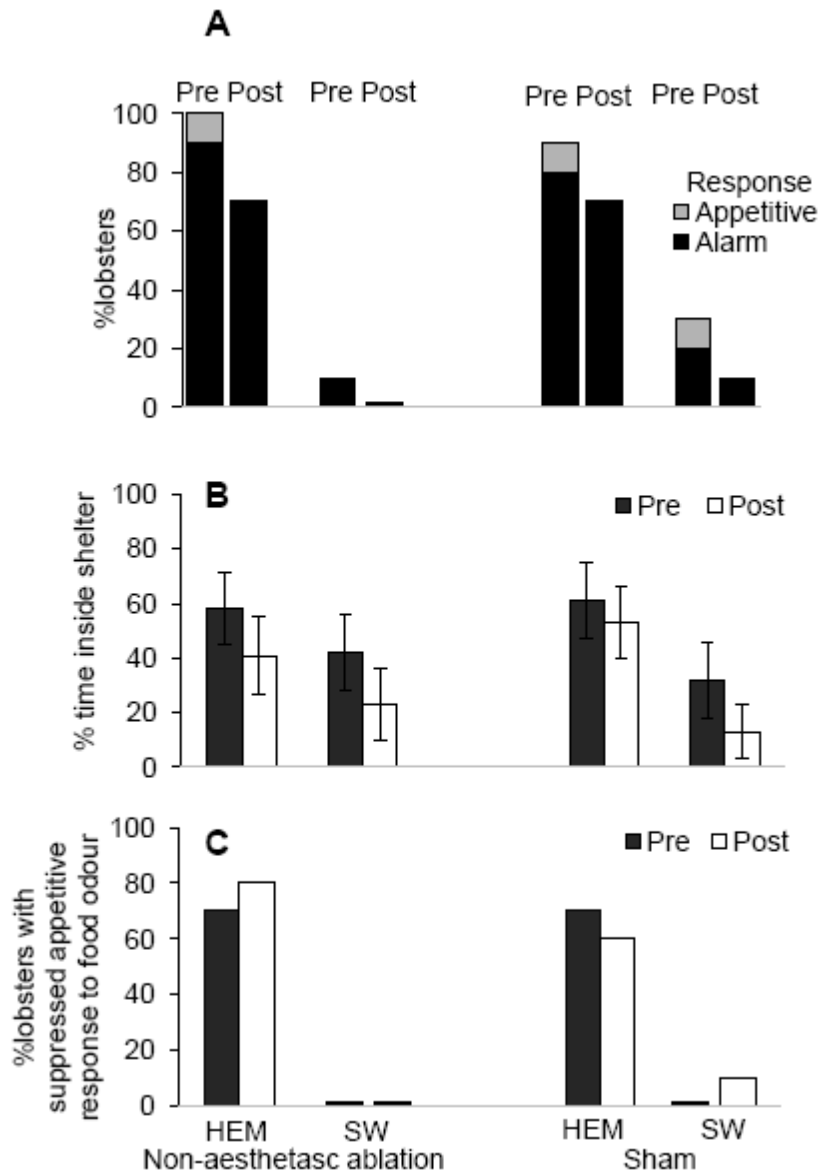


Figure 4-4 Ablation of non-aesthetasc sensilla did not affect any form of alarm behaviour to hemolymph (HEM). The percentage of either experimental (left graph, $n = 10$) or control (right graph, $n = 10$) lobsters that showed (A) alarm or appetitive responses to hemolymph remained the same after (Post) ablation of non-aesthetascs or sham treatment. (B) Likewise, both experimental and control lobsters spent similar amount of time inside shelter in response to hemolymph before and after either treatment. (C) Both experimental and control lobsters before or after treatments showed similar suppression of appetitive responses to shrimp odour when shrimp odour was presented after hemolymph. Nominal data were analysed using McNemar

tests ($P < 0.05$) as described in the Methods. Evaluation of the results based on ordinal data using Wilcoxon matched-pairs test showed similar results.

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 to hemolymph from conspecifics than to 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 sea water, 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.

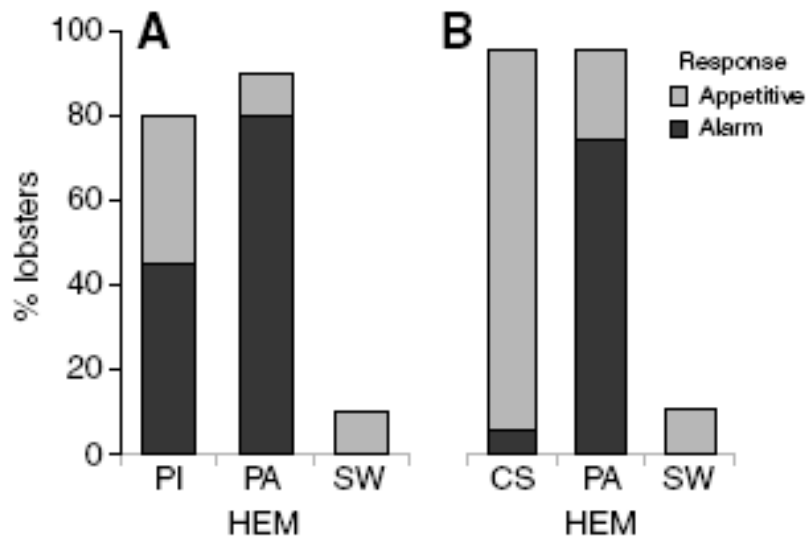


Figure 4-5 Spiny lobsters were more likely to show alarm responses to conspecific hemolymph compared to either congeneric hemolymph or a hemolymph from a brachyuran crab. Responses of two groups of *P. argus* lobsters are shown in A and B. *P. argus* hemolymph (PA Hem) induced alarm responses in a significantly greater percentage of *P. argus* lobsters than did hemolymph of *Panulirus interruptus* (PI Hem) or the sea water control, and PI Hem induced alarm responses in a significantly greater percentage of lobsters than did sea water (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 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 test showed similar results.

Hemolymph induces alarm behaviour in wild lobsters

Lobsters in the field showed similar alarm behaviour to hemolymph as did animals in our laboratory studies. These behavioural 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 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.

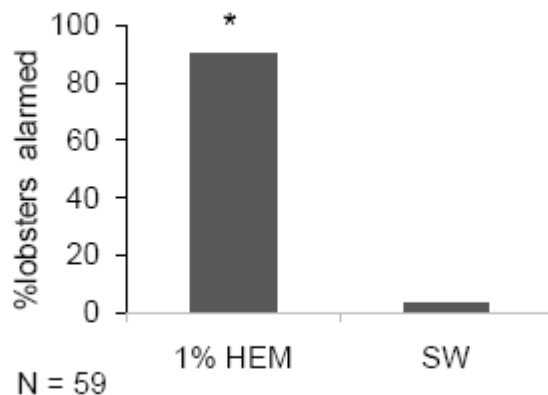


Figure 4-6 Field tests of alarm responses of *P. argus* to conspecific hemolymph. Hemolymph induced alarm responses in a significant percentage of wild lobsters (n = 59). * denotes a significant difference in the percentage of lobsters showing alarm responses to hemolymph (HEM) as compared to sea water (SW), $P < 0.05$, by McNemar test.

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 appetitive

response to food odours (Figs. 2, 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 from *P. argus*, but the responses are either less frequent alarm behaviour or even appetitive feeding. Similar alarm behaviors are also induced by physical threat (S.S. personal observations; 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 field (Parsons and Eggleston, 2005, 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, 1996b). In our study, the behavioural responses to hemolymph changed dramatically after ablation of only the aesthetascs but not after ablation of all other antennular sensilla (Figs. 3, 4, supplementary material Movie

1). In fact, aesthetasc-ablated lobsters respond to hemolymph with very different behaviour than 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 been previously shown that either the aesthetasc or non-aesthetasc pathway can mediate detection, discrimination, and orientation toward food odors (Steullet et al., 2001, 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 aesthetascs pathway is necessary to mediate responses to these intraspecific alarm cues is consistent with previous studies on crustaceans showing that aesthetascs exclusively mediate behavioural responses to conspecific odours. Male blue crabs with ablated aesthetascs show significantly less courtship behaviour to female urine-borne sex pheromones than males with aesthetascs (Gleeson, 1980, 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 odours; and the non-aesthetasc pathway, which together with the aesthetasc pathway mediates responses to food and other general odours. Our study lays the foundation for future studies of neural processing of alarm cues by the olfactory pathway of spiny lobster.

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 rather, 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, shelters, 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 which 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 preemptively 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 whereby this predator risk-assessment system has evolved.

Chemical alarm cues released from injured conspecific 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.

CHAPTER 5

SPINY LOBSTERS USE URINE-BORNE SIGNALS TO COMMUNICATE SOCIAL
STATUS

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Introduction

Many animals compete for social status to gain better access to food, shelters, mates, and other resources (Rowell, 1974; Drews, 1993; Barroso et al., 2000; Petrulis et al., 2004; Burmeister et al., 2005; Gherardi, 2006; Hovland et al., 2008; Izawa and Watanabe, 2008; Val-Laillet et al., 2008). This competition, which is common among gregarious animals (Drews, 1993), often involves fights coupled with signaling between a pair of animals. These dyadic fights between opponents may start symmetrical, with the two acting equally aggressively, but then progress with one showing primarily offensive behaviors or winning and the other defensive behaviors or losing. Those that engage primarily in offensive behaviors are referred to as dominants, whereas those engaging in defensive 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, 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, opponents that chemically communicate may end the fight sooner or not fight at all (Briffa and Williams, 2006; Rutte et al., 2006; Baird et al., 2007).

Competition for shelters or food 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 offensive behaviors and burrow to make shelters, whereas subordinate crayfish engage in defensive 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., 2000; 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). Other decapod crustaceans such as the spiny lobsters *Panulirus argus*, *Panulirus longipes*, *Panulirus cygnus*, and *Jasus lalandei* also show aggression around shelters but differ in sociality from crayfish and American lobsters (Fielder, 1965; Chittleborough, 1974; Berrill, 1975, 1976; Meyer-Rochow and Penrose, 1976; Lozano-Álvarez, 1996; Childress, 2007). Most species of spiny lobsters aggregate in and around shelters, and yet they compete for shelters.

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 bailers, 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 chemosensory structures 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). *P. clarkii* uses its fan organs also to draw odors toward the olfactory organs by creating directed water currents (Brock, 1926, 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 fights. These and other results, especially on crayfish and American lobsters, suggest that urine signals contribute significantly to the decrease in aggression. 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 offensive behaviors and directly in front of the opponent. Zulantz Schneider and Moore (2000) added support to this idea by showing that a pair of crayfish has significantly longer fights when urine release is experimentally blocked by gluing closed the nephropores. 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 familiarization period does not alter the duration of fights in subsequent encounters

(Karavanich and Atema, 1998a, 1998b; Breithaupt and Atema, 2000). Similar results are reported for Norway lobsters (Katoh et al., 2008).

The effect of chemical signals on fight duration 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 *Procambarus clarkii* prevents the decrease in duration of fights in subsequent encounters (Horner et al., 2008a). Olfactory ablation in the 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, 1996b). One is an olfactory pathway, whose receptors are aesthetasc sensilla containing olfactory neurons whose axons project to the olfactory lobes. The other is a non-olfactory pathway, whose receptors are the bimodal ‘non-aesthetasc’ sensilla, which contain both chemo- and mechanoreceptor neurons whose axons project to the lateral antennular neuropils and median antennular neuropil. Although both antennular pathways detect general odors including food chemicals, only the aesthetasc/olfactory pathway carries information about conspecific odors (Gleeson, 1980, 1982; Steullet et al., 2002; Johnson and Atema, 2005; Schmidt and Derby, 2005; Horner et al., 2008a, 2008b).

Caribbean spiny lobsters (*Panulirus argus*) often engage in agonistic interactions when competing for shelters and food, including when aggregating (Fielder, 1965; Berrill, 1975, 1976; Childress, 2007; S. Shabani, personal observations). Aggregation behavior is partly driven by chemical cues released from conspecifics (Nevitt et al., 2000; Ratchford and Eggleston,

1998). Caribbean spiny lobsters prefer shelters scented with urine of conspecifics, and they lose this preference if their olfactory pathway is ablated (Horner et al., 2008b). Choice of shelter is complex and depends heavily on the context in which these chemical cues are transmitted from conspecifics. Spiny lobsters near shelters often engage in fighting and are forced to move into nearby shelters (Fielder, 1965). Thus, spiny lobsters may use urine to influence acquisition of shelters, but they may use it differently than American lobsters and crayfish. We used a series of behavioral experiments to test the hypothesis that spiny lobsters establish social status by releasing urine-borne signals, that urine is a signal of threat, and that these urine signals are detected by the olfactory pathway.

Material and Methods

Animals

Caribbean spiny lobsters, *Panulirus argus*, collected from the Florida Keys with a carapace length between 50-80 mm were held in an enclosed aquarium room at Georgia State University during February and March of 2005 and February of 2007. The aquarium room was kept under fluorescent light in 12 hr light/dark phases. Spiny lobsters were individually held in 80-liter aquaria (60 cm L x 30 cm W x 45 cm H) containing filtered sea water (Instant Ocean®, Aquarium Systems, Mentor, OH) at 25 °C. Each aquarium contained a shelter at one end to provide a refuge. The shelter was 24 cm L x 24 cm W x 25.4 cm H fabricated from plastic egg crate louvers and CPVC pipes, with one side positioned against the aquarium's back wall and with a ramp for spiny lobsters to move up and hide. In one experiment (February 2007), the

shelter was a concrete rectangular block (23 cm x 23 cm x 23 cm). Behavioral experiments were video recorded (Sony DCR PC110) during the dark phase under low red-light conditions.

Ablation of sensilla

To determine if the aesthetasc pathway is necessary or sufficient to mediate responses to urine, we performed behavioral experiments before and after ablations of aesthetasc or non-aesthetasc sensilla. We performed behavioral tests during February and March 2005 on 20 spiny lobsters, first on all animals before treatment ('intact') and then after either ablation of aesthetasc pathway (9 spiny lobsters) or sham treatment (11 spiny lobsters). Next we performed behavioral tests during February 2007 on 26 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 aesthetascs and non-aesthetascs and sham treatments was accomplished according to 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 (2005) and 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 in SPB with 0.02% sodium azide until analysed. To make 0.1 mol l⁻¹ SPB, we dissolved 6.8 g KH₂PO₄ and 21.3 g Na₂HPO₄ in 1 liter deionized H₂O, adjusted 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 aesthetascs.) 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, $99.7 \pm 0.1\%$ (mean \pm S.E.M) of aesthetasc sensilla were ablated. In the process of ablating aesthetascs, $52.1 \pm 3.4\%$ (mean \pm S.E.M.) of the asymmetric sensilla and $3.2 \pm 0.6\%$ (mean \pm S.E.M.) of the guard sensilla were damaged, but none of the 7 other types of non-aesthetasc sensilla were affected. In the non-aesthetasc targeted group, we ablated $97.7 \pm 0.7\%$ (mean \pm S.E.M.) of the asymmetric sensilla and $99.7 \pm 0.1\%$ (mean \pm S.E.M.) of the other 8 types of non-aesthetasc sensilla. In the process of ablating the non-aesthetasc sensilla, $49.8 \pm 6.6\%$ (mean \pm S.E.M.) of the aesthetasc sensilla were damaged.

Collection of urine

To test if urine induced avoidance responses in spiny lobsters and whether urine of spiny lobsters differs when they are disturbed vs. undisturbed, we collected urine from eight spiny lobsters in two contexts: when quiescent and disturbed. Each nephropore was encircled by a tube that provided connection sites for smaller diameter tubes that looped around the carapace and connected to a common tube that exited the aquarium. We first attached a 1-cm long silastic tube (inner diameter 3.2 mm, outer diameter 4.7 mm, wall diameter 0.79 mm) around each nephropore using cyanoacrylate glue (Pacer Technology, Superglue gel). This tube connected to a narrower 6-8 cm long tube (i.d. 1.6 mm, o.d. 3.2 mm, w.d. 0.79 mm). These tubes were looped around the animal's dorsal carapace and joined the two inlets of a 3-way plastic connector glued to the dorsal carapace. The outlet of the 3-way connected was connected to a 45-cm long tube (i.d. 1.6 mm, o.d. 3.2 mm, w.d. 0.79 mm) that exited the aquarium.

Urine from undisturbed spiny lobsters was collected several days in a row. Urine from disturbed spiny lobsters was collected when they were prodded with plastic tongs for 2 min every 20 min over a 4-hr period. These urine samples were pooled from 8 spiny lobsters (both sexes) during March 2005. Urine was similarly collected and pooled from two undisturbed spiny lobsters during February 2007. Urine was frozen at -20 °C until used.

Visualization of urine release

We visualized the urine release from spiny lobsters by catheterizing the nephropores as described above but with the following modifications (see Supplemental video 1). The small diameter tubes that looped around the carapace were 2-3 cm long, and the large common tube was 3-6 cm long. We placed a small cartridge constructed from silastic tube (o.d. 1.96 mm, i.d. 1.47) filled with 50 mg/ml fluorescein (acid yellow 73, Sigma F-6377) into the common tube. The cartridge was enclosed with alumina-silicate beads at both ends (Davison, Fisher Scientific) and connected to a three-way plastic connector on the dorsal carapace. The cartridge leaked small amounts of concentrated dye continuously into the large tube, and thus fluid within the large tube was colored throughout the experiment, even after daily urine release (see Supplemental video 1). The fluorescing urine in the large tube was prevented from passively leaking out by a one-way sealing rubber valve (valve diameter 3 mm, Da/Pro Rubber, Inc.) attached at the 3-way plastic connector. Thus the common valve opened only when urine was released.

This technique of visualizing urine had an advantage in our studies compared to the technique developed by Breithaupt and Eger (2002) for crayfish, which is to inject fluorescein into animals. Our method allowed visualization of urine for long periods of time, sometimes up

to one month, as compared to the injection method which lasts up to ~ 3 hr after injection. This was important in our study since handling and injection of spiny lobsters stressed the animals and sometimes affected their behavior for 1-2 days afterwards, and thus our technique allowed time for spiny lobsters to recover from this stress and still allow us to visualize urine release.

Behavioral assay of factors influencing urine release

Behavioral tests were performed on spiny lobsters with visualized urine (VU lobsters) to determine if they released urine in three experimental conditions. All behavioral tests were videotaped (Sony DCR PC 110) and analyzed later.

The first condition was to test whether an isolated spiny lobster, i.e. not in a social encounter with other spiny lobsters, will release urine when exposed to stimuli that may be disturbing or threatening. These included hemolymph or urine from conspecifics, and a physical disturbance. Hemolymph and physical disturbance were previously demonstrated to evoke avoidance responses that include ‘retreat’ (Karavanich and Atema, 1998a, 1998b; Shabani et al., 2008), ‘tail-flipping’ (Nauen and Shadwick, 2001), and ‘stridulating’ (Meyer-Rochow and Penrose, 1976; Mulligan and Fischer, 1977) (see Supplemental video 2). Retreat is defined as a spiny lobster curling its tail and walking backwards and away from the stimulus to a corner of the aquarium or inside shelter. Sea water and shrimp juice were tested as neutral and positive control stimuli, respectively. Briefly, VU lobsters ($N = 7$) were presented with 10 ml of sea water and then 5 min later with 10 ml of either urine, hemolymph, or shrimp juice. Urine was collected as described above, hemolymph was collected and used fresh according to Shabani et al. (2008), and shrimp juice was 2 mg tissue per 1 ml sea water. We quantified urine release as the number of pulses, with those pulses being categorized as short and long. Short pulses lasted

for 2-9 sec (see Supplemental video 3) and form small puffs. Long pulses (see Supplemental video 3) lasted on average 100 sec and form clouds. Long pulses were also characterized by a peak, identified by a more intense fluorescein color, within 1-2 sec of the onset of release (see Supplemental video 3). The durations of long and short pulses did not have a normal distribution and the two distributions are significantly different (Kolmogorov-Smirnov test, $P < 0.05$). Approximately 30-60 min after these tests, spiny lobsters were fed a piece of food (ca. 1-2 gm shrimp or squid). Ingestion of food was always followed by release of long pulses of urine and thus served as a demonstration that the spiny lobster was capable of releasing urine and that we could detect it (see Supplemental video 1). These tests were accomplished within a 2-day period for each spiny lobster. On the subsequent day, each spiny lobster received physical disturbance, in which the experimenter prodded the spiny lobster with plastic tongs until the animal retreated and/or tail-flipped; on occasion, the spiny lobsters also stridulated.

The second condition was to determine if a spiny lobster housed with a conspecific will release urine when presented with a stimulus that disturbs or threatens it. This condition was similar to the first condition as described above, using the same VU lobsters, except that another spiny lobster was introduced in the aquarium. The conspecifics were smaller in size than the resident VU lobsters by 10.6 ± 2.1 % (mean \pm S.E.M) in carapace length. Two days later, we tested whether an accompanied VU lobster, i.e. in a social encounter with another spiny lobster, will release urine when exposed to stimuli that may be disturbing or threatening. These included hemolymph or urine from conspecifics, and a physical disturbance. For controls, we used shrimp juice and sea water. We performed the same behavior assay as when VU lobsters were solitary. We quantified urine release after each stimulus delivery as described above.

The third condition was to determine the pattern of urine release during social interactions of a pair of VU lobsters. Members of a pair ($n = 7$) had 4.9 ± 1.4 % (mean \pm S.E.M) difference in carapace length. We video recorded the social interaction during the first hour of pairing and measured duration of urine release and the occurrence of behaviors. The spiny lobster that showed primarily offensive behaviors during agonistic interactions was considered dominant, and the animal that showed primarily avoidance responses was considered the subordinate. Agonistic interactions were marked by four types of common offensive behaviors: locking front-to-front with their antenna or their front legs ('antennae-locking'; see Supplemental video 4), grabbing the antenna or legs from the side using the front legs ('antenna-grabbing' and 'leg-grabbing' respectively; see video 5 and 6), and poking their legs underneath the abdomen ('abdomen-poking'), which often induced tail flips by the opponent (see video 7). Avoidance behaviors included retreat, tail-flipping (see Supplemental video 5-7), and stridulating. Retreat involved spiny lobsters walking backward away from the opponent (see Supplemental video 5-6). Tail-flips and stridulations were sometimes induced when the opponent attacked the spiny lobster by grabbing its legs or antenna, and or poking its abdomen. These offensive interactions were mild in nature and never resulted in injuries. These behaviors performed by the dominant and subordinate spiny lobsters were quantified. We determined social status based on behaviors during the first hour of social interaction. 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 fighting ('fighting'), were less than one-half body length away but not fighting ('in contact'), or were more than one-half body length away ('distant') (see Supplemental video 8 and 9). Two of the seven pairs of spiny lobsters could not be categorized as to which of the pair was dominant: one

pair did not engage in fights, and another pair fought but the social status of the members could not be unequivocally determined.

Behavioral assay of the role of urine in establishing social status

To test whether urine contributes to establishment 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. Thus, we compared social 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'). We also monitored urine release from catheterized spiny lobsters before and after social interactions. 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 from studies on the American lobsters where the eventual social status of paired animals was random if carapace length difference is within 5% (Scrivener, 1971; Karavanich and Atema, 1998a).

Spiny lobsters of all three types were initially placed individually into 80-liter aquaria for at least a week prior to pairing. Urine release from catheterized animals was monitored, and urine was collected and stored in -20 °C. We monitored the volume of urine release during the following conditions: 1) within 1 hr of feeding; 2) 24 hr after feeding; 3) 48-hr after feeding; and 4) every hr for 5 hr during one dark cycle. These data provided baseline measurements of urine release for solitary animals, to compare with release during pairing.

Fights by paired spiny lobsters consisted of combinations of behaviors and ended when opponents disengaged. In virtually all fights, one spiny lobster approached and 'leg-grabbed' the

other spiny lobster. Other offensive behaviors such as ‘antenna-grabbing’, ‘abdomen-poking’, ‘antennae-locking’ were less common. Antenna-locking, which is similar to claw-lock in American lobsters (Johnson and Atema, 2005), virtually always escalated to other offensive behaviors and ended with one opponent retreating. Spiny lobsters that retreated in response to any of the offensive behaviors sometimes also stridulated. Social interactions were scored on number and duration of fights.

The first group of animals (‘Cath’) – catheterized spiny lobsters, which could not release urine into the aquarium – consisted of two animals that were paired for 1 hr and their social interactions and urine release were monitored (same as above). We also determined whether urine was released when the spiny lobsters were in three conditions, as before: ‘fighting’, ‘in contact’, and ‘distant’. Then, a piece of shrimp was given to each spiny lobster by directly placing it at the spiny lobsters’ legs. Ingestion of food and volume of urine released were recorded. We allowed these spiny lobsters to interact for another 24 hr and scored the volume of urine release at the end. At the end of this interaction, we again gave them food and scored ingestion and volume of urine release associated with it.

The second group of animals (‘Cath+Urine’) consisted of pairs of animals that, like those in the first group were catheterized and thus could not release urine, but we released urine during the experiment to determine urine’s role in social interactions. Urine of each of these solitary 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 hr of pairing. Each pulse was ~3.3 ml over ~20 sec, for a total of 10 ml. We delivered 3 pulses since catheterized spiny lobsters with visualized urine often released 3 long pulses. We injected urine primarily when spiny lobsters

were engaging in agonistic interactions or were in close proximity of each other. Urine was injected through a 60-cm long silastic tube (id 1.6 mm, od 3.2 mm, wall diameter 0.79 mm) that was placed in one corner of the aquarium, ~15 cm below the water surface and directed at a 45° angle toward the center of the aquarium. We scored social interactions and the volume of urine release during the first hour of encounter as mentioned above. At the end of the first hour of interaction, we offered shrimp to the legs of each spiny lobster and scored ingestion and volume of urine release. We allowed these spiny lobsters to interact for another 24 hr. The catheterized spiny lobster whose urine was injected in the aquarium had the end of its tube inside the aquarium after the 1st hour of interaction to ensure that urine was released in the aquarium during the next 24 hr. At the end of 24 hr, both spiny lobsters were offered shrimp and ingestion was scored.

The third group of spiny lobsters ('Control') consisted of pairs of animals that were handled identically to the first group except that they were not catheterized. All of the same measurements were taken except, of course, urine release could not be measured.

Behavioral assay of responses to urine

Behavioral assays, conducted according to Shabani et al. (2008), consisted of two phases: acclimation and testing. Briefly, during the acclimation phase, spiny lobsters were acclimated for at least 3-5 days to the aquarium and behavioral testing paradigm. This process included feeding spiny lobsters a piece of shrimp using tongs and delivering sea water or an appetitive stimulus (a small piece of shrimp or 1 ml of 2 mg/ml shrimp juice) with a glass pipette. Spiny lobsters were trained until they did not respond to introduction of sea water and responded with forward movements to the appetitive stimulus.

We measured two types of responses to chemical stimuli, appetitive and avoidance, according to Shabani et al. (2008). Appetitive responses are defined as spiny lobsters moving forward toward the location of the introduced shrimp juice. Retreat response was used as the major dependent measure of avoidance behavior (see Supplemental video 2). Two other dependent measures of avoidance were: 1) time spent in shelter, expressed as a percentage of the total 150 sec of the trial (= % time inside shelter); and 2) suppression of foraging response to food odor, expressed as a reduction in appetitive response to shrimp juice after exposure to urine (= % lobsters with suppressed response to food odor).

Our experimental protocol was to deliver 1-10 ml of 2 mg/ml shrimp juice and observe for 45 sec, then deliver 10 ml of an experimental or control stimulus and observe for 120 sec, and finally deliver another 1-10 ml of shrimp juice and observe for 30 sec. Experimental stimuli were urine from undisturbed or disturbed spiny lobsters. 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. We measured avoidance responses for 120 sec following delivery of either experimental or control stimuli. The avoidance response was quantified using a nominal scale, 1 (alarm) and 0 (no alarm). The intensity of foraging suppression was quantified as the two dependent measures described above: 1) % time inside shelter; and 2) % lobsters with suppressed response to food odor, using a nominal scale, 1 (attractive) and 0 (showing no appetitive response). Differences between control and experimental stimuli were tested for significance using a non-parametric Wilcoxon or McNemar test.

We also performed the same behavior 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 0.0001% full strength. Two groups were tested: one with 1% to 0.1% urine and seawater as control, and another with 0.01% to 0.0001% urine and 1% urine and seawater 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 responses to urine of conspecifics as they do in the laboratory. The experiment was performed in May-August 2006 in the waters near the Florida Fish and Wildlife Conservation Commission facility in Marathon, Florida. Detailed descriptions of the study site and experimental setup are in 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 (mean \pm S.E.M.) 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 seawater collected locally) and the negative control stimulus (filtered sea water) using different tubes. 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 sec. 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 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 the stimulus delivered. Statistical differences between control and experimental stimuli were determined through paired McNemar tests.

Results

Context of urine release

Spiny lobsters released urine under some specific conditions and not others. One important context of urine release was social interactions, and we focus on this in our paper. We also note one non-social context in which spiny lobsters release urine: after ingesting food. This occurred whether animals were solitary or paired with a conspecific (see Supplemental video 1 and 10). Solitary VU lobsters released urine within 32 ± 3.2 sec (mean \pm S.E.M., $N = 7$) of eating food in a long pulse that lasted 71.2 ± 4.1 sec (mean \pm S.E.M., $N = 7$; see Supplemental video 1). VU lobsters paired with conspecifics released urine within 50.5 ± 3.6 sec of eating, with pulses lasting 70.3 ± 5.0 sec (mean \pm S.E.M., $N = 7$). Spiny lobsters did not release urine under most other conditions, including when presented with conspecific odors (hemolymph or urine), food odors (shrimp juice), or physical disturbance (threat), whether they were solitary or paired with a conspecific.

Urine release during social interactions

Paired VU spiny lobsters released urine during the first hour of encounter, especially when in close contact (Fig. 1). Spiny lobsters stayed in ‘contact’ without fighting for most of the first hour (2190 ± 1164 sec: mean \pm S.E.M., or 61% of the hour). During this hour, they engaged in 3.0 ± 0.4 agonistic interactions that lasted for a total of 249 ± 46.2 sec (mean \pm S.E.M., or 7% of the hour). The animal that eventually was identified as the dominant member of the pair almost always initiated the attacks and always engaged in offensive behaviors that included ‘leg grabbing’ and sometimes also ‘antenna grabbing’ or ‘abdomen poking’ (Fig. 1A). The animal that eventually was identified as the subordinate member of the pair almost always engaged in avoidance behaviors in response to the dominants offensive behaviors that included retreat and sometimes tail-flip or stridulation (Fig. 1A).

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. 1B), and those long pulses by dominants lasted 102 ± 12 sec (mean \pm S.E.M.). In fact, all dominants released urine during the first hour of social 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 subordinates (Fig. 1C). Dominants released most of their urine when ‘fighting’ and in ‘contact’ with the subordinate, which includes offensive and close contact behaviors (Fig. 1D). The dominant typically acquired food introduced into the aquarium and subsequently released long pulses of urine (see Supplemental video 10). In the one pair of the six that we were unable to determine social status since both

showed equally often offensive and defensive behaviors, both members of the pair released long pulses of urine during all of their interactions, and both ingested food.

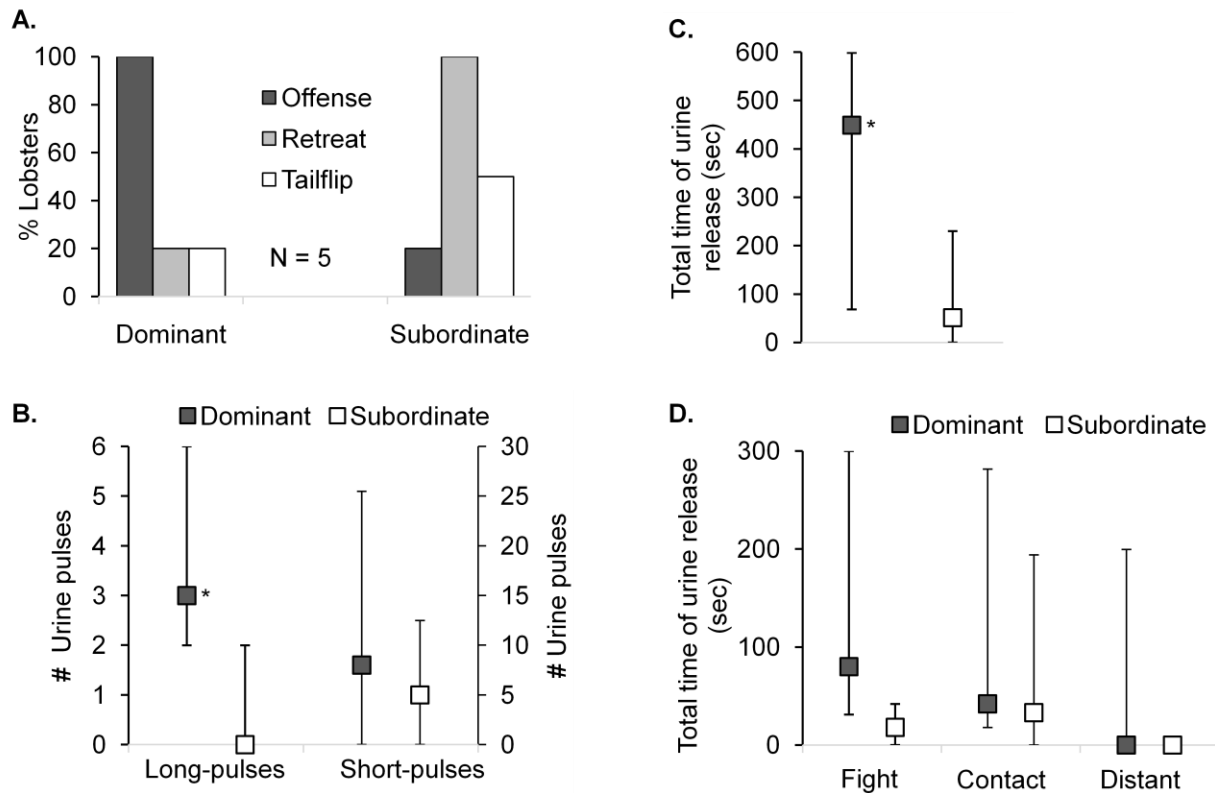


Figure 5-1 Behavior and urine release by paired spiny lobsters. A) % lobsters showing offensive behaviors (leg grabbing, antenna grabbing, abdomen poking) and defensive behaviors (retreat, tail-flip) during a 1-hr social interaction. Dominant lobsters showed more offensive behaviors and subordinate lobsters showed more defensive behaviors. B) Dominants released long pulses of urine significantly more frequently than subordinates. C) Total time of urine release during the social interaction. Dominants released urine significantly longer than subordinates. D) Total time of urine release during three states of social interactions: engaged in fighting ('fight'), within one-half body length away and not fighting ('contact'), or more than one-half body length away ('distant'). Dominants released urine longer than subordinates especially during fights but not significantly different. N = 5 pairs of lobsters.

*denotes significant difference in duration of urine release or number of urine pulses (Wilcoxon matched-paired tests, $P < 0.05$).
Boxes and error bars indicate median and interquartile range.

Role of urine in establishing social status

The behavior of the dominant toward the subordinate was significantly affected by the presence of urine (Fig. 2). In all the first social encounters, regardless of the group, dominants initiated the fight with offensive behaviors, which always ended with retreat or tail-flip by the subordinates. Catheterized spiny lobsters that could not release urine into the aquarium (Cath) engaged in significantly more fights (Fig. 2A) than did either catheterized spiny lobsters paired with experimenter-introduced urine (Cath+Urine) or spiny lobsters that were not catheterized (Control). Consequently, total duration of fighting for the Cath group was significantly longer than either Cath+Urine or Control group (Fig. 2B). For the Cath+Urine group, the member of the pair whose urine was introduced into the aquarium became the dominant spiny lobster in five of the six pairs. In the one exception, the dominant spiny lobster initiated 5 fights that lasted for a total of 257 sec. Furthermore, the subordinate of this pair, even though it was exposed to its own urine, did not release urine. If we exclude this pair from the Cath+Urine group, the median fight number is 1 (interquartile range 1 and 2.5), and the total fight time median is 39 sec (interquartile range 15 and 192).

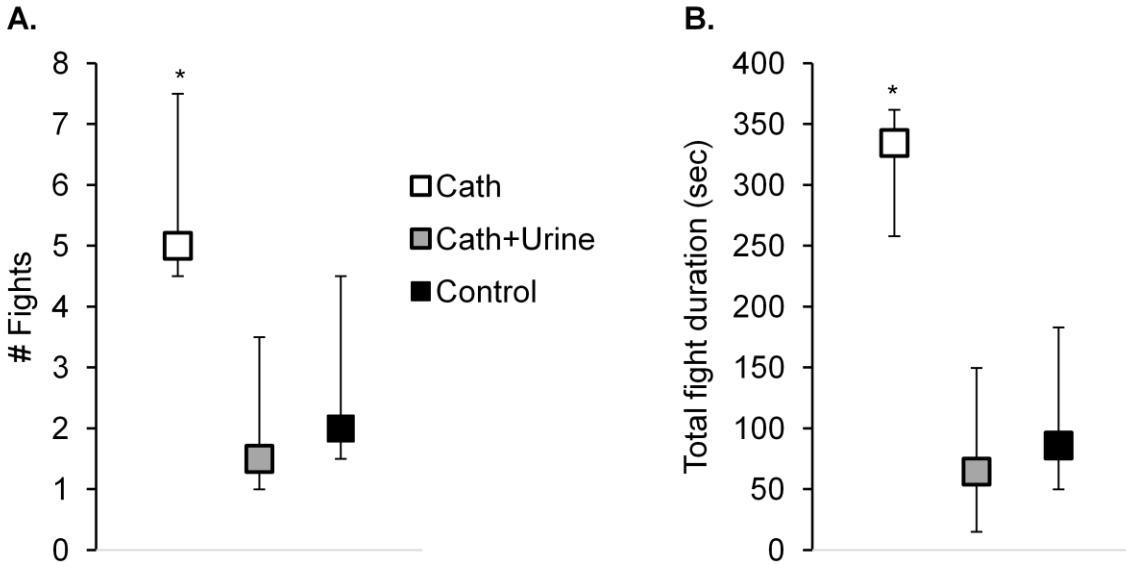


Figure 5-2 Effect of urine on fighting of paired lobsters. ‘Cath’ are catheterized lobster pairs that could not release urine into the aquarium ($n = 6$ pairs). ‘Cath+Urine’ are catheterized lobster pairs that could not release urine into the aquarium but urine of one member of the pair was introduced into the aquarium by the experimenter ($n = 6$ pairs). ‘Control’ are lobsters that were not catheterized ($n = 5$ pairs). ‘# Fights’ and ‘Total fight duration’ are the number of fights and the time during which animals fought, respectively, during the 1-hr experimental period. * denotes a significant difference in # of fights or in total fight duration between ‘Cath’ group and the other two groups (Mann-Whitney test, $p < 0.05$). Boxes and error bars indicate median and interquartile range.

Pairing significantly increased the release of urine by spiny lobsters. Solitary animals were less likely to release urine during any given 1-hr period compared to the dominant, but not subordinate, animal of a pair during the first hour of social interaction (Fig. 3A). During the first hour of social interaction in the Cath and Cath+Urine group, a significantly greater percentage of dominants than subordinates released urine (Fig. 3B). Dominants released urine most often when fighting and in contact with subordinates (Fig. 3B). Half of these dominant catheterized spiny lobsters, regardless of which group, released 5.2 ± 0.2 ml of urine per long pulse, which on

average lasted 28.6 ± 4.2 sec (mean \pm S.E.M.). The other half of dominant spiny lobsters released small puffs of urine (<0.5 ml) at different times, for a total volume of ~ 1 ml. Two subordinates released long pulses of urine that were similar in volume and duration to those of dominants.

Dominants were significantly more likely than subordinates to grab food offered to their legs 1 hr after social interactions (Fig. 3C). A similar difference in food acquisition between dominants and subordinates was observed on the second day, but this difference was not significant. These differences in food acquisition between dominants and subordinates were similar among the three groups of spiny lobsters (Cath, Cath+Urine, Control).

After 24 hr of social interactions, dominant spiny lobsters released significantly more urine than the subordinate partly explained by the fact that they acquired more food than subordinates (Fig. 3D). During solitary period (before pairing), when fed 24 hr prior, dominant and subordinates released similar amounts of urine. In contrast, when fed 48 hr prior, solitary dominants and subordinates released significantly less than when fed 24 hr prior. Immediately after feeding, both solitary dominants and subordinates released similar volumes of urine per pulse (5.0 ± 0.8 ml vs. 5.5 ± 1.0 ml respectively). These dominants and subordinates released similar amounts of urine per pulse when ingesting food during social interactions (5.8 ± 0.6 ml vs. 6.5 ± 1.8 ml respectively).

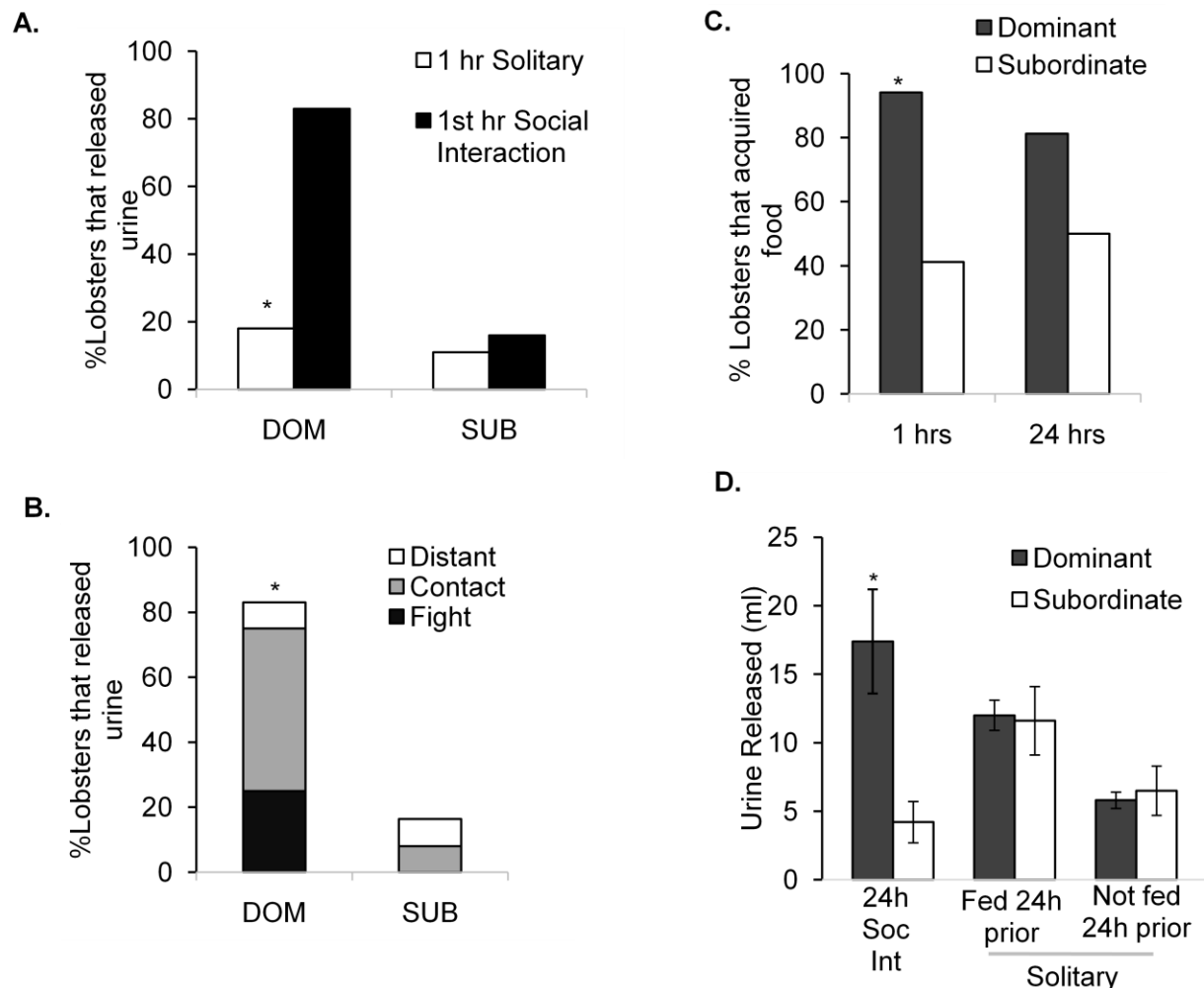


Figure 5-3 Effect of social status on urine release during social interactions. A) During any given 1-hr period when animals were solitary, only a small % of either dominants or subordinates released urine. However, during social interactions (after pairing), % of dominants that released urine increased significantly, while subordinates remained the same. These data were from Cath+urine and Control groups. B) A significantly higher percentage of dominants (DOM) than subordinates (SUB) released urine during the 1st hour of social interaction (N = 12 pairs); also a significantly higher percentage of dominants released urine during the first hour of social interaction than during any random 1 hr of solitary days in either dominants (N = 6) or subordinates (N = 6). C) Dominants acquired food preferentially over subordinates, especially in the 1st hour of social interaction (N = 17 pairs). Dominants also (D) released a larger volume of urine in the first 24 hr of social interaction than subordinates (N = 6). D) The same lobsters, regardless of social status, when solitary released greater amounts of urine when fed 24 hr prior than when not fed. * denotes a significant difference between dominant and subordinate animals (Wilcoxon matched-pair test and McNemar test, $p < 0.05$). In D, bars and error bars denote mean and standard error of mean. N = 17 pairs of animals.

Effect of urine on avoidance responses of laboratory and wild spiny lobsters

In the laboratory, spiny lobsters showed the full range of avoidance behaviors in response to urine of conspecifics (Fig. 4). Urine evoked significantly more avoidance responses than did sea water, expressed either as percentage of animals showing avoidance responses ($n = 53$, Wilcoxon and McNemar test, $p < 0.05$) (Fig. 4A), time spent inside a shelter (Wilcoxon and McNemar test, $p < 0.05$) (Fig. 4B), or percentage of animals with a suppressed appetitive response to food odor (Wilcoxon 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 either disturbed or undisturbed spiny lobsters induced the same responses, we tested only urine of undisturbed spiny lobsters in the rest of the tests. The response of laboratory spiny lobsters to urine was dependent on concentration (Fig. 5). Urine at 1% and 0.1% of full strength (when presented in the aquarium, and thus without factoring in any further dilution in the aquarium) induced significant avoidance responses ($n = 24$, 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, $p > 0.05$).

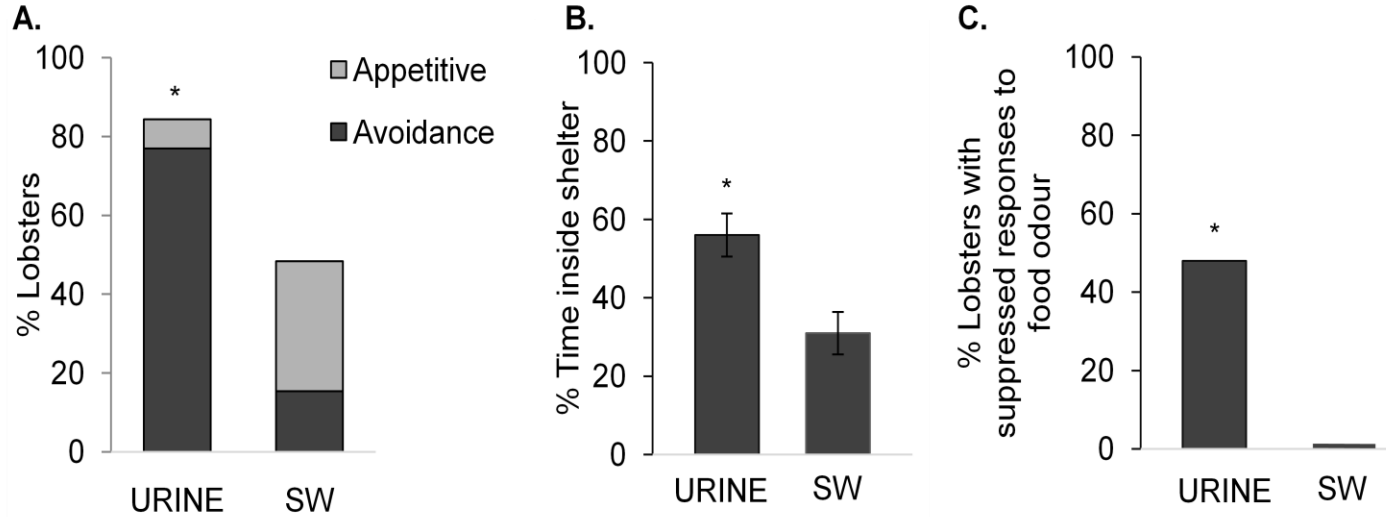


Figure 5-4 Effect of urine on behavioral response of solitary spiny lobsters. (A) Urine induced avoidance responses in a significantly greater percentage of lobsters than did sea water (SW). In contrast, urine and SW induced a similarly low frequency of appetitive responses. (B) Urine caused spiny lobsters to spend significantly more time inside shelter than did SW. (C) Urine suppressed the appetitive response to shrimp odor in significantly more lobsters than did SW. Results are based on 53 lobsters. * denotes a significant difference between Urine and SW during nominal data (McNemar tests, $P < 0.05$) as described in the Methods; evaluation of the results based on ordinal data using Wilcoxon matched-pairs test showed similar results.

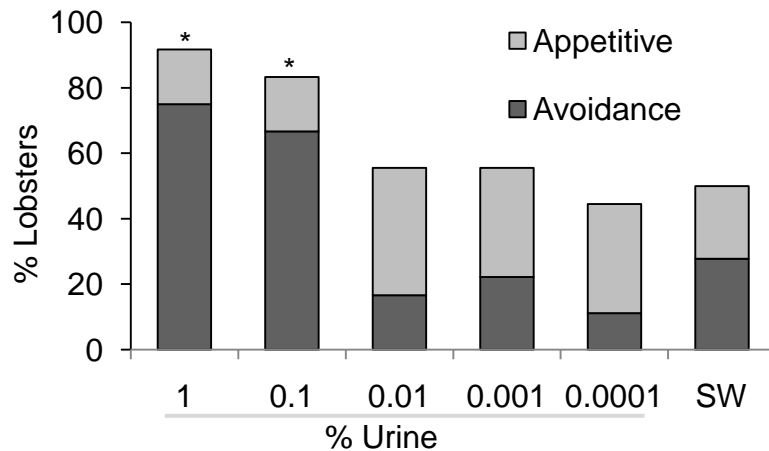


Figure 5-5 Effect of urine concentration on behavioral responses of spiny lobsters. A significant percentage of lobsters showed avoidance responses (retreat) to 0.1% and 1% urine ($n = 24$) but not lower ($n = 18$). * denotes a significant increase in percentage of animals showing avoidance response using nominal data and McNemar tests ($P < 0.05$). Evaluation of the results based on ordinal data using Wilcoxon matched-pairs test showed similar results.

In the field, wild spiny lobsters responded to urine in a similar way as did laboratory animals (see Supplemental video 11). 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.

Sensory basis for responses to urine

Ablating the aesthetasc sensilla abolished avoidance responses to urine. Instead, these animals responded to urine with appetitive responses ($n = 9$; Fig. 6A) and spent significantly less time inside the shelter (Fig. 6B). They also tended to show less suppression of responses to food odor

after urine, though this effect was not statistically significant (Fig. 6C). On the other hand, ablation of non-aesthetasc sensilla did not significantly affect responses to urine. Ablated animals continued to respond to urine with avoidance behaviors ($n = 10$; Fig. 6A), sheltering (Fig. 6B), and suppression of responses to food odor (Fig. 6C) at levels significantly similar to pre-ablation levels, albeit at somewhat reduced levels. Sham-treated animals showed no appreciable changes in behavior after treatment ($n = 21$; data not shown).

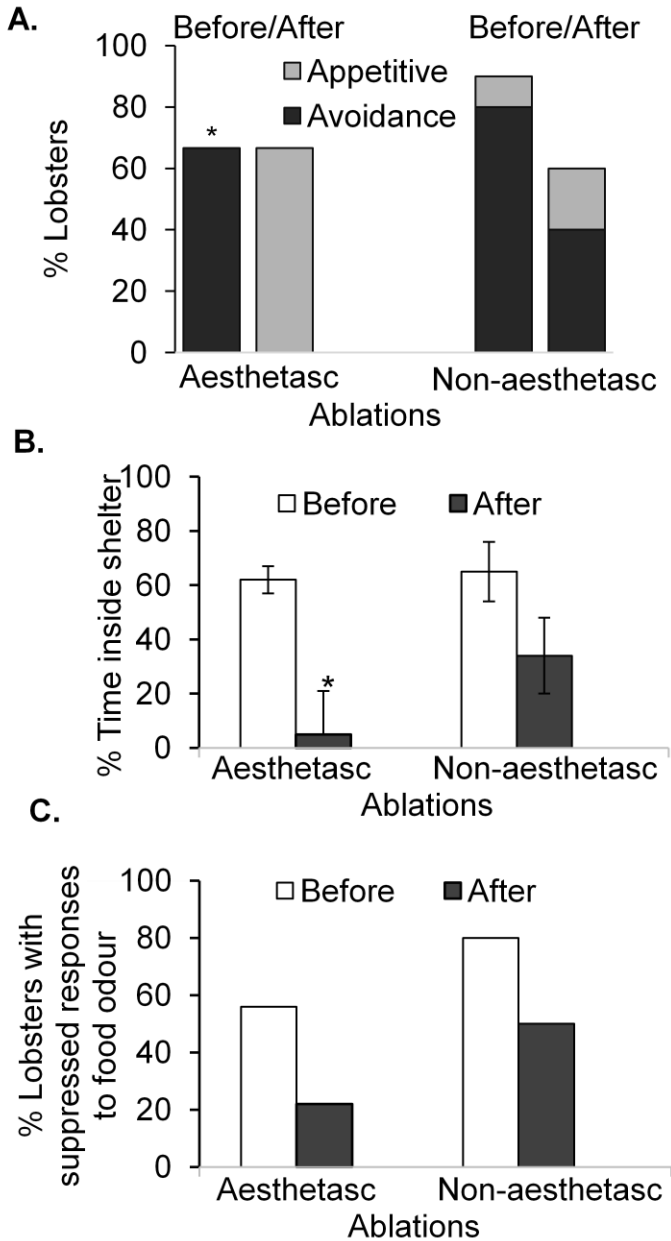


Figure 5-6 Ablation of aesthetasc ($n = 9$ spiny lobsters) but not non-aesthetasc sensilla ($n = 10$ spiny lobsters) eliminated all forms of avoidance responses to urine in solitary spiny lobsters. (A left bars) Before ablation of aesthetasc sensilla, a significantly higher percentage of experimental lobsters (left graph) showed avoidance responses to urine than after ablations. The percentage of experimental lobsters showing appetitive responses to urine increased significantly, to 67%, after ablation. (A right bars) Ablating non-aesthetasc sensilla did not significantly change behavior. (B) Aesthetasc-ablated lobsters spent significantly more time inside shelter in response to urine before than after ablation. Non-aesthetasc-ablated lobsters did not spend significantly more time inside shelter in response to urine before than after ablation, though there was a strong trend. (C)

Ablation of either aesthetasc or non-aesthetasc did not significantly reduce the % lobsters with a suppressed appetitive responses, though there was a strong trend in this direction. * denotes a significant increase in percentage of animals showing avoidance response after either ablation using nominal data and McNemar tests ($P < 0.05$) as described in the Methods. Evaluation of the results based on ordinal data using Wilcoxon matched-pairs test showed similar results.

Discussion

Urine borne-signals communicate social status

We show here that Caribbean spiny lobsters use urine-borne signals to communicate social status. These urine-borne signals, primarily released by the dominants, have a significant effect on the behavior of both dominants and subordinates. Dominants increase their offensive behavior significantly when urine is not released during social encounters. Subordinates respond to urine-borne signals with avoidance and with suppression of three behaviors: appetitive responses to food odor, food intake, and urine release. Urine is effective in inducing avoidance responses only at high concentrations, i.e. 0.1% full strength or greater, indicating that it is used in signaling only for animals in close proximity to each other. These urine effects are mediated mainly by the olfactory pathway and its aesthetasc sensilla. Some of these effects of urine were validated by being observed in wild spiny lobsters in the field.

Context of urine release

The release of urine during encounters of spiny lobsters depends on the animal's social status. Dominants released urine more frequently and in greater amounts compared to subordinates (Figs. 1, 3). Spiny lobsters coupled their urine release with physical contact with conspecifics, whether or not that contact included offensive behaviors. In American lobsters,

dominants also released urine more frequently than did subordinates (Breithaupt et al., 1999; Breithaupt and Atema, 2000). However, unlike spiny lobsters, American lobsters coupled the release of urine especially with offensive behaviors. The results from American lobsters are more similar to those in crayfish (Breithaupt and Eger, 2002). This difference in urine release could be accounted for by 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 escalation of offensive behaviors since they seek each other's company anyway.

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 by physical objects or to urine or hemolymph from conspecifics. Furthermore, they responded with the same avoidance behaviors to urine of either undisturbed or disturbed conspecifics, which is similar to previous findings on American lobsters (Breithaupt et al., 1999; Breithaupt and Atema, 2000) but unlike some findings reported for crayfish (Hazlett, 1989, 1990; Zulantz Schneider and Moore, 2000). These studies on crayfish concluded that urine of stressed or disturbed crayfish provide disturbance signals to conspecifics. However, some control experiments that would strengthen this argument were not reported. For example, Zulantz Schneider and Moore (2000) did not test urine from undisturbed crayfish, which would be important to show that the signals were specific to disturbed animals. Studies by Hazlett (1989, 1990) did not test urine itself but water collected from disturbed and undisturbed crayfish. Additionally, walking and lowered posture were used as dependent measures in these studies rather than behaviors that would be more specific to a directed avoidance response. Thus, whether crayfish differ from American lobsters and spiny lobsters by having urine-based disturbance signals requires more analysis for a more definitive answer.

Function of urine release

Urine enforces social status in both dominants and subordinates. The number and duration of fights in spiny lobsters were significantly affected by the urine-borne signals released by the dominant. Thus, urine of dominants has two functions. It communicates social status to the subordinate and it provides feedback to the dominant.

Urine of dominants enforces the effects of offensive behaviors, by activating avoidance behaviors on subordinates. Thus, urine may foremost represent a signal of threat when presented in close proximity, in high enough volumes, and in the appropriate context. Similar to offensive behaviors of dominants, urine alone induced avoidance behaviors. In our laboratory and field experiments, a significant number of spiny lobsters engaged in avoidance behaviors when presented with sufficient volumes of conspecific urine. A high percentage of field spiny lobsters responded to urine by moving to another shelter nearby. Furthermore, animals exposed to conspecific urine had suppressed appetitive responses to food odors. This is in line with our observation that subordinates yielded to dominants when offered food. Importantly, in all of our laboratory assays, ~30-40% of spiny lobsters showed no avoidance responses. In fact, some spiny lobsters displayed antenna whipping in response to urine, perhaps indicating that urine-borne signals mediate different aggression-related behaviors depending on the animal's social status. Furthermore, these effects of urine in our study, which are in line with studies on American lobsters (Karavanich and Atema, 1998a, 1998b), suggest that subordinates exposed to urine of dominants are less likely to challenge dominants.

Urine release by a dominant may provide sensory feedback to itself. Offensive behaviors by spiny lobsters were virtually always initiated by one opponent – the dominant – regardless of whether urine was present or not. In all the social interactions, the opponent that initiated the

first fight initiated almost all the other fights. Those that were attacked almost always retreated from the fight. This is similar to American lobsters and crayfish (Karavanich and Atema, 1998a, 1998b; Issa et al., 1999). In our study, the increased number and duration of fights in spiny lobsters that were prevented from releasing urine is partly explained by the dominant's increase in offensive behavior. This increase in offensive behavior indicates that there is a feedback mechanism such that urine-borne signals affect dominants that release them.

Dominants enforce social status on subordinates through two means: urine-borne signals and offensive behaviors. Both likely provide feedback for the dominant. However, if one of these feedback mechanisms is prevented, it may induce a compensation mechanism by the feedback mechanism still present. Therefore, use of urine-borne signals is adaptive for both dominants and subordinates, because reduction of aggression by dominants also benefits subordinates. These benefits might include reduced stress and thus increased survival. Furthermore, for dominants it may be more cost effective energetically to employ urine signals as apposed to offensive behaviors.

Urine signals are probably functioning only at close distance. We found responses to 0.1% urine but not to lower concentrations (Fig. 5). 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 other animals (Figs. 1-3).

Conspecific cues and their sensory pathways

The avoidance responses to urine are very similar to avoidance behaviors that spiny lobsters show in response 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). Thus, urine-borne signals mediate the avoidance behaviors through the same risk-assessment pathway as hemolymph alarm cues. 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 conspecifics. 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, 2006; Behringer et al., 2006; Bouwma, 2006; Horner et al., 2008b).

Both urine-borne signals that communicate social status to spiny lobsters (our study) and hemolymph cues that induce alarm responses (Shabani et al., 2008) are largely or exclusively detected through the olfactory (aesthetasc) pathway (Fig. 6). 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-significantly) lower level. This reduction may indicate that non-aesthetasc sensors play a minor role in mediating responses to urine, much less than aesthetascs. Alternatively, the reduced response in non-aesthetasc ablated animals may be due to collateral damage to aesthetascs that resulted during the surgical elimination of the non-aesthetasc sensilla, wherein ca. 50% of the aesthetascs were damaged. In either case, it is clear that aesthetascs 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 aesthetascs 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 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 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; Horner et al., 2006, 2008b; Childress, 2007; 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 Herrnkind, 2001). Ablating aesthetasc sensilla eliminates this preference for shelters (Horner et al., 2008b). Urine is also important in mating. Female American lobsters show greater preference for shelters occupied by dominant males than subordinate males; however, this preference by females is lost if urine release of males is blocked (Bushman and Atema, 2000). Male blue crabs with ablated aesthetascs no longer perform courtship display behavior to the pheromone of reproductive females (Gleeson, 1982), and male helmet crabs with ablated distal half of 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 is present in other animals as well. For example golden hamsters use flank-glands both for individual recognition and identification of sex and reproductive state (Johnston, 2003).

Conclusions

Our findings in Caribbean spiny lobsters and others' studies of 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 a fight not just because they induce avoidance behavior on the subordinate but also because they provide feedback to the dominant. Furthermore, we show that this communication is directly mediated by a common risk-assessment pathway with an alarm cue system. To activate this risk-assessment pathway, spiny lobsters control their release of urine during specific types of social interactions. Therefore, our study provides new information about the mechanism of social competition in spiny lobsters. Furthermore, because spiny lobsters are gregarious animals, they provide an excellent model to contrast mechanisms of urine-borne communication during competition and aggregation, as well as with competition among other decapods that are solitary.

Chapter 6

General Discussion

Discussion

Chemical information permeates food webs and can have strong impacts in behavior, ecology, and community structure and dynamics. Chemicals affect the interactions of animals with each other and with environmental resources. Some interactions such as those with predators have immediate consequences on survival. Chemicals can be employed as defenses, alarm cues, and communication signals. They can directly affect chemosensory system of animals and often through very specific chemosensory structures. This dissertation explores the mechanisms of chemicals used as chemical defenses, alarm cues, and communication signals from the chemosensory perspective of a decapod crustacean, the spiny lobster. It identifies specific chemosensory structures involved in processing this information.

Mechanisms of chemical defenses at the chemosensory level

Many plants and animals use chemical defenses against herbivores and predators, which act through various mechanisms including through those animals' sensory systems. Most chemical defenses act by producing aversive responses, being toxic or harmful, or a combination of effects (Derby, 2007; Glendinning, 2007). Many plants have a diversity of chemical defenses that function in these ways against herbivores. It follows that most studies on mechanisms of chemical defenses are on herbivores and in particular insects (Bernays and Singer, 2005; Conner et al., 2007; Glendinning et al., 2002; Glendinning, 2007; Schar et al., 2001; Stowe et al., 1995). Many defensive chemical compounds in plants and their mechanisms of action at the sensory

level are known (Mustaparta, 2002; Glendinning, 2007). However, in aquatic environments, although many defensive chemical compounds are known, our knowledge of their mechanisms of action at the sensory level is limited.

Chemical defenses can induce immediate aversive responses through olfactory and/or taste organs. Olfactory organs can detect low concentrations of deterrent compounds at a distance from the source (Glendinning, 2007; Kobayakawa et al., 2007). Many poisonous insects and plants generate unique odors that are detected through olfaction and that may facilitate persistent memories in predators (Rowe and Guilford, 1999; Rothschild et al., 1994). The effects of chemical defenses on behavior at the sensory level are better characterized for taste systems (Glendinning, 2007). Taste organs play a major role in the decision as to whether to ingest or reject food. In vertebrates, taste organs mediate reflexive behaviors (Derby and Sorensen, 2008; Lamb and Finger, 1995; Scott, 2005). Most poisonous or toxic compounds are aversive, meaning they are distasteful or unpalatable, even though there is a considerable variation in their chemical structure and means of detection (Chandrashekar et al., 2006; Scott, 2005). Cells that detect these aversive compounds are for the most part broadly tuned and induce aversive responses (Meyerhof, 2005). A family ~30 types of receptors, all G-protein-coupled receptors known as T2Rs, mediate bitter taste in mammals (Mueller et al., 2005). Thus, bitter tasting cells detect a large number of aversive compounds without necessarily discriminating between them.

Insect taste organs are composed of sensilla. These sensilla are located on various body parts and often in the mouthparts, legs, and sometimes even wings and genitalia (Amrein and Thorne, 2005). Insects, like vertebrates, show aversions to a range of deterrent compounds, and although they can discriminate between these compounds with high specificity they show similar

aversion responses. In insects, deterrent compounds are detected by gustatory receptor cells very different from those of vertebrates (Ishimoto and Tanimura, 2004). Insect gustatory cells are located in specific sensilla in the legs, labellum, wings, genitalia, and some other appendages. They typically respond preferentially to sugars, salt, and water, but some cells are strongly affected by the deterrent compounds. Most of the deterrent compounds in plants are secondary metabolites.

The use of chemical defenses by marine plants and animals is well documented by chemical ecologists, including demonstrations that defensive chemicals can have enormous impacts on communities and ecosystems (Derby, 2007; Hay, 1996; Hay and Kubanek, 2002; Kicklighter and Hay, 2006; Kicklighter et al., 2004; Long and Hay, 2006; Parker et al., 2005; Pohnert et al., 2007). Chemical defenses of marine plants are often secondary metabolites. These metabolites are shown through feeding assays to affect the palatability of herbivores and predators. However, limited studies in marine systems have shown so far the sensory mechanisms whereby these deterrent compounds function (e.g. Kem and Soti, 2001).

Chemical defenses of marine animals have been extensively studied on predatory fish and arthropods (Kicklighter et al., 2003; Long and Hay, 2006; Ritson-Williams and Paul, 2007). Usually chemical compounds are isolated from chemical defenses and tested for deterrence through feeding assays. Chemical compounds are mixed with known feeding stimulants and tested for feeding suppression (Cruz-Rivera and Hay, 2003). These tests are highly effective in testing the feeding suppression (Lindquist, 2002) but not for the mechanism of chemical defenses as a whole. Thus in real case scenarios even if some chemicals play a greater role in survival than others, they usually are presented to predators with the other chemical compounds which can have a major effect on behavior and chemosensory system.

Mechanism of action of the chemical defenses of sea hares

The sea hare mollusk *Aplysia californica* employs defensive secretions against the crustacean predator *Panulirus interruptus* in a more complex way than usually described in other systems. First and foremost, ink and opaline stick to multiple chemosensory organs and thus activate more than just one chemosensory pathway. Second, ink and opaline induce multiple conflicting behaviors: feeding, grooming, and avoidance. Thus, ink and opaline protect sea hares via multiple mechanisms. One mechanism that is also commonly used by other animals is through deterrent compounds. However, other mechanisms also play a role in defending sea hares. These include phagomimicry and sensory disruption. Phagomimicry refers to distracting predators using feeding stimulants in chemical defenses, consequently allowing sea hares to escape (Johnson, 2002). I showed that feeding stimulants in ink and opaline affect both the behavioral and chemosensory responses of spiny lobsters. Ink and opaline as well as their artificial mixtures composed of their constituent feeding stimulants activated a large number of chemosensory neurons, and this activation is likely prolonged in real scenarios because ink and opaline stick to the chemosensory organs. The effect of ink and opaline was similar in both olfactory and gustatory organs. This massive and prolonged activation of chemosensory neurons suggests that it plays a role in the evoking behaviors such as extensive grooming, digging, and even tail flipping. This phenomenon is referred to as sensory disruption. Behavioral responses to ink and opaline are partly explained by their high acidity. Acidity has a major impact on the chemosensory activity in both olfactory and taste organs of spiny lobsters. Thus, chemical defenses protect sea hares via multiple mechanisms.

Other questions remain unanswered that would increase our understanding the mechanism of chemical defenses. An important question is whether ink and opaline target

specific chemosensory pathways in predators such as decapod crustaceans. Ink and opaline had a variable effect on the olfactory receptor neurons (ORNs) by either suppressing or enhancing their activity; but only excite the chemosensory neurons in the mouthparts and other chemosensory neurons in the antennules (Chapters 2 and 3). These results suggest that ORNs in aesthetasc sensilla process ink and opaline differently from chemosensory neurons in other sensilla. Thus, the olfactory and other chemosensory pathways may be targeted by ink and opaline. In conjunction with these studies, identifying deterrent compounds would further unravel the mechanism of ink and opaline in affecting the chemical senses of spiny lobsters.

Avoidance of active predators through intraspecific chemical cues

Predatory behavior affects not only the sought after or captured prey but also the behavior of nearby prey. Prey live in the same habitat as predators and respond adaptively when they perceive predators as posing a risk. Cues released during predation events can indicate an honest and immediate risk to prey. To minimize risk, prey engage in immediate adaptive responses when they detect these cues (Breed et al., 2004; Chivers and Smith, 1998). Information in the form of chemical cues released by prey is particularly important since these cues can linger long after prey die, disperse over large areas, and facilitate learning for future encounters (Chivers and Smith, 1998; Smith, 1992). When attacked by predators, prey actively or passively release chemicals that evoke immediate alarm and related anti-predatory behavior by nearby conspecifics. Furthermore, although the mechanisms of releasing these chemicals vary, the sensory pathways and the effect on behavior are fairly conserved across taxa (Galizia et al., 1999; Hamdani and Døving, 2007; Yamagata et al., 2006, 2007).

Active release of alarm pheromones is extensively studied in insects. The mechanisms of release and reception of these alarm pheromones are known in many species. The mechanism of release is especially well documented in eusocial insects. Honeybees, for example, release their alarm pheromones primarily from two sources: the Koschewnikow gland and glandular areas within the stinging apparatus, and mandibular gland (Breed et al., 2004; Hunt, 2007). When the colonies are threatened, honeybees attack the intruder and autotomize their stinging apparatus onto the intruders. The autotomized sting continuously releases alarm pheromones in addition to harmful chemical compounds to the intruder, becoming a target for other honeybees to attack. Aphids use their cornicle gland to secrete a sticky chemical defense that also contains alarm pheromones. The released alarm pheromones induce alarm behavior in nearby aphids. Other insects use specialized glands, and they too can show alarm behavior (Blum, 1985; Wyatt, 2003). These examples involve eusocial insects that form complex and genetically related social groups. Therefore, communication through alarm pheromones is beneficial to both senders and receivers.

Aquatic animals also release chemical alarm cues that induce alarm behavior in conspecifics, but their mechanism of release is different than that of insects. Furthermore, while receivers benefit from these alarm cues, senders may not. This is because while many aquatic animals that have chemical alarm cue systems form social groups, they are not genetically related. Second, the chemical alarm cues are released after fluid leakage from injured wounds instead of secreting them from specific glands as is the case in insects. Fish, sea urchins, sea snails, sea anemones, and crustaceans release alarm cues through fluids leaked after injury (Howe and Sheik, 1975; Jacobsen and Stabell, 2004; Snyder, 1967; Snyder and Snyder, 1970; Zimmer-Faust et al., 1985). In all these examples, conspecifics flee rather than fight against the

active predators, thus mainly benefiting receivers. This is also the case with spiny lobsters, which flee the area scented with alarm cues (Chapter 4).

Spiny lobsters use injury-related chemicals to assess risk of predation. They forage predominantly at night under low light conditions. For most animals foraging involves the highest risk of predation (Lima and Dill, 1990). During the night, spiny lobsters make several round trips of foraging often hundreds of meters from their original den. After foraging, they seek shelter in the same general area but not necessarily in the same den. Spiny lobsters often remain in their shelters throughout the night (Weiss et al., 2008). Furthermore, they leave their dens shortly after disturbance by intruders or when exposed to injury-related conspecific odors (Herrnkind et al., 1975; Bouwma, 2006). To find dens, spiny lobsters usually rely on chemical cues released by conspecifics, which serve as guide posts (Childress and Herrnkind, 2001). Conspecific chemical cues minimize both the time necessary to find refuge and the risk of predation. Therefore, depending on the source of conspecific odors, spiny lobsters may choose to aggregate with other resident lobsters or avoid them if they indicate events of predation.

Injury-related conspecific odors are contextually specific to predation events and thus are critical for risk assessment in spiny lobsters. Risk of predation is not only high when spiny lobsters are in the open but also when they return to their shelters. Predators such as octopuses often attack spiny lobsters inside shelters. For this reason, spiny lobsters often avoid shelters that contain octopus odor and or injury-related conspecific odors (Berger and Butler, 2001; Bouwma, 2006; Chapter 4). Alarm cues released from injured conspecifics can also linger for a long time and facilitate not only avoidance but learning of novel predators. For example, some predators such as octopuses consume spiny lobsters over several hours (Weiss et al., 2008). In some species of predatory fish, chemical alarm cues can also end up unaltered in their bodily excreta (Chivers

and Smith, 1998). Furthermore, these alarm cues when paired with a novel potential predator odor or other cues can mediate learning in fish and gastropods (Brown, 2003; Long and Hay, 2006; Wisenden, 2000). Although these phenomena in fish are not tested in spiny lobsters, I speculate that they occur in spiny lobsters. Previous studies have indicated that spiny lobsters learn quickly to avoid odors associated with danger though in the context of discrimination tasks (Derby, 2000; Derby and Sorensen, 2008).

Alarm cues or pheromones often are small in molecular weight and not species specific (Wyatt, 2003). In many insects, the alarm cues have small molecular weight and presumably disperse and dilute rapidly from the environment (Bossert and Wilson, 1963; Hölldobler and Wilson, 1990). Thus once danger is gone, it may be beneficial for prey to resume foraging or other activities. In fact, some species rely on visual stimulation as well chemical alarm cues to fully assess the risk (McCormick and Manassa, 2008). In reef fish, chemical alarm cues induced the same anti-predator responses as did visual stimuli, and the combination of the two induced the strongest alarm responses. This idea would explain why in cases where alarm cues linger, prey may overestimate predation risk and thus other modalities could complement the risk assessment (Turner and Montgomery, 2003). In my study, chemical alarm cues in spiny lobsters induce similar alarm responses as threatening visual stimuli, but the combination of the two was not tested. The alarm cues or pheromones provide less specificity than sex pheromones (Wyatt, 2003). Furthermore, the released alarm cues do not require much privacy as say sex pheromones do. Thus it may be beneficial for heterospecifics to respond to alarm cues especially if they face predation pressures from the same predators. According to my preliminary data on the molecular identity of alarm cues in hemolymph of spiny lobsters, the alarm cues are also of low

molecular weight. Furthermore, these alarm cues are not species specific, though they induce the best behavioral responses in conspecifics as apposed to heterospecifics.

My data suggest that phylogeny is a good predictor of alarm responses to hemolymph. This idea is supported by recent results (Briones-Fourzán and Lozano-Álvarez, 2008) showing that fluids from damaged *Panulirus guttatus*, a close relative sympatric to *P. argus* (Ptacek et al., 2001), and fluids from *P. argus* induce similar alarm responses in *P. argus*. In some species, although phylogeny plays an important role in responses to heterospecific chemical cues, so does sympatry (Chivers and Smith, 1998; Chivers et al., 1995). For example, in the freshwater gastropod *Lymnaea stagnalis*, responses to alarm cues diminish with increasing phylogenetic distance, but the degree of sympatry is also a factor (Dalesman et al., 2007). Thus local adaptation to common predators can have an impact on behavioral responses to alarm cues in phylogenetically related species. This phenomenon is partly explained by the fact that two species may associate each other's chemical stimuli with predation events conducted by the same predators (Chivers and Smith, 1998). Resolution of this issue of the importance of phylogeny and sympatry in alarm cue responses in spiny lobsters must await molecular identification of the alarm cues.

The risk assessment system in spiny lobsters likely evolved because of their mechanisms of escape from predators. Spiny lobsters, like many other crustaceans, autotomize their limbs when attacked by predators. Limb autotomy benefits the individual because it enhances the escape (Fleming et al., 2007; Juanes and Smith, 1995). In addition, limb autotomy can benefit nearby conspecifics, by leaking alarm cues even autotomy functions to reduce loss of hemolymph. This might be considered as a form of predator tagging similar to that in honeybees.

Risk assessment pathway

The olfactory pathway in spiny lobsters is critical for risk assessment. Animals make decisions affecting their fitness on moment-to-moment basis. Death limits the fitness permanently, which is why animals often cease all other activities temporarily to minimize risk of death. This phenomenon is viewed as a series of threat-sensitive trade-offs between predator avoidance and foraging, mating, and feeding (Brown, 2003; Helfman, 1989; Lima and Bednekoff, 1999). Avoidance of predators is most beneficial when the risk from predation is highest. Therefore, these trade-offs are maximized in animals with well-developed risk assessment systems (Lima and Bednekoff, 1999).

Insects detect alarm pheromones through their olfactory pathway. The olfactory pathway in insects involves the antennal lobe, which is analogous to the olfactory bulb in vertebrates. These olfactory structures are glomerular in nature and contain axon endings of olfactory receptor neurons and dendrites of projection neurons which send information to specific brain centers. The antennal lobe mediates detection of complex odors from food and from conspecific odors. Alarm pheromones activate a specific set of glomeruli in the antennal lobe of honeybees (Galizia et al., 1999). This activation is highly conserved across individuals. Similar processing of alarm pheromones occurs in ant (Yamagata et al., 2007).

Alarm cues in many aquatic animals, including fish, are detected by the olfactory pathway. Some early studies speculated that solitary chemosensory cells may also play a role in the responses to chemical alarm cues, though no studies have confirmed this claim (Kotrschal et al., 1989). One subpathway of the fish olfactory system detects and processes alarm cues (Chivers and Smith, 1993; Hamdani et al., 2000; Hamdani and Døving, 2002, 2003; Zippel et al., 2000).

In my study, I found that as in many insects and fish, the olfactory pathway is the main risk assessment pathway in crustaceans. Decapod crustaceans including spiny lobsters have multiple chemosensory pathways but only the olfactory pathway mediates the responses to alarm cues and the urine-borne signals. The olfactory lobe pathway and the non-olfactory chemo-mechanosensory pathway detect general odors including food chemicals (Gleeson, 1980, 1992; Horner, 2007; Horner et al., 2008a, 2008b; Johnson and Atema, 2005; Schmidt and Derby, 2005; Steullet et al., 2002). In addition, the olfactory pathway uniquely mediates detection of urine-borne signals in some social and sexual contexts of decapod crustaceans (Chapter 5; Gleeson, 1980, 1992; Horner, 2007; Horner et al., 2008a, 2008b; Johnson and Atema, 2005). Ablating the aesthetasc sensilla has a dramatic effect on behavioral responses to conspecific odors. Thus spiny lobsters and other decapods use urine-borne signals for multiple functions: aggregation, reproduction, and social interactions (Chapter 5; Gleeson, 1980, 1992; Horner, 2007; Horner et al., 2008a, 2008b; Horner, 2007; Johnson and Atema, 2005). This is similar to mammals and insects where a single source of conspecific odor can have multiple functions in behavior (Blum, 1996; Johnston, 2003; Wyatt, 2003).

My findings in spiny lobsters support the view that decapod crustaceans have two functionally distinct antennular chemosensory pathways. The non-olfactory pathway mediates specific grooming behavior (Schmidt and Derby, 2005) and responses to conspecific odors (Chapters 4 and 5; Horner, 2007). I showed that the non-olfactory pathway is sufficient to mediate appetitive behaviors to feeding stimulants in prey of lobsters as well as in the alarm cue source – hemolymph – or urine (Chapters 4 and 5). Spiny lobsters show alarm responses to hemolymph, but this changes to appetitive responses if their olfactory pathway is blocked. This reversal in behavior is likely because the information from the two chemosensory pathways is

processed differently starting from the periphery to the higher brain centers. Furthermore, these two chemosensory pathways likely activate functionally different brain centers.

In addition to other functions, the olfactory pathway in spiny lobsters and likely other decapods functions as a risk-assessment pathway. Risk of injury to an individual animal can come from predators and competitive conspecifics (Rutte et al., 2006; Wyatt, 2003). In spiny lobsters, both hemolymph and urine-borne signals, which are used in very different contexts, mediate risk-assessment behaviors (Chapters 4 and 5). These behaviors are compromised dramatically if the olfactory sensilla are ablated (Chapters 4 and 5). Therefore, spiny lobsters rely heavily on their olfactory system in making critical decisions directly related to threat-sensitive-trade-offs between predators avoidance, foraging, and social communication.

Communication of social status

Animals across taxa compete with conspecifics to gain an advantage over resources (Barroso and Boza, 2000; Burmeister et al., 2005; Drews, 1993; Gherardi, 2006; Gilmour et al., 2005; Hovland et al., 2008; Izawa and Watanabe, 2008; Petrulis et al., 2004; Rowell, 1974; Val-Laillet et al., 2008). This competition involves agonistic interactions between individuals, which can sometimes lead to injuries. However, injuries are often avoided by establishing dominance hierarchies. This involves the dominant animal consistently winning in agonistic interactions and the subordinate losing (Drews, 1993). Consequently, dominants often have greater access to resources (Wilson, 1975). Dynamics of these agonistic interactions depend on many factors, including agonistic behaviors and chemical communication. Bodily odors are often used to help establish the dominance and even maintain it. For example, many mammals actively use scent marks to defend territory ownership from competitors (Hurst and Baynon, 2004).

Rodents fight for social status through both aggression and chemical communication to gain and withhold resources (Hurst and Baynon, 2004; Petrulis et al., 2004). Fights, which occur primarily between males, end after one opponent retreats. The one that retreats, referred to as subordinate, experiences long-lasting physiological changes coupled with pronounced avoidance behaviors toward familiar and sometimes unfamiliar conspecifics. Dominants increase scent-marking by releasing urine around their immediate territory, while subordinates decrease scent-marking (Johnson, 1973; Wyatt, 2003). Scent marks of dominants are thought to facilitate and enforce the social status of subordinates. Thus, even if the owner of the territory is not present at specific locations, its identity and competitive abilities are advertised to the intruder. Urine scent mark signatures by dominants are partly fixed by the non-volatile major urinary proteins (MUPs) that are genetically determined. These MUPs bind to low molecular weight and volatile molecules in the urine, thereby slowing the evaporation of volatile molecules. For example, house mice flee areas that contain fresh scent marks of dominants (Hurst and Beynon, 2004). These dominants also countermark frequently any intruder's scent-marks within their territory. These responses in house mice are triggered by MUPs and their bound ligands (Humphries et al., 1999). The function of this phenomenon is mainly for advertising the owner's identity and its competitive ability to third parties in the vicinity (Rich and Hurst, 1999). In fact, most mammals use some form of scent-marking for defending their territories (Johnston, 2003).

These scent-marking odors are thought to mediate their effect through both the main olfactory pathway (Mandiyan et al., 2007) and the vomeronasal pathway (Chamero et al., 2007). The low molecular weight compounds in these scent marks release their behavioral effect primarily through the olfactory pathway. House mice for example can flee the area by sniffing only volatile compounds. The non-volatile MUPs however mediate their effects through the

vomeroneasal organ. Non-volatile compounds are necessary for the release of countermarking behavior (Hurst and Baynon, 2004). Furthermore, removing the vomeronasal organ can eliminate male aggression to an intruder's countermarking scents (Maruniak et al., 1986; Wysocki and Lepri, 1991). Thus, both olfactory and vomeronasal organ process scent marks in an integrative way.

Use of urine-borne signals is also common in aquatic animals when competing for resources (Barata et al., 2007; Breithaupt et al., 1999; Breithaupt and Atema, 2000; Breithaupt and Eger, 2002). Fish such as the Mozambique tilapia (*Oreochromis mossambicus*) fight for social status and in the process release urine. These fish increase their release of urine significantly with aggressive behavior but not with non-aggressive behaviors. Furthermore, dominants release consistently more urine than subordinates (Barata et al., 2007).

These results in fish are similar to those in decapod crustaceans as shown by my results and others. My findings on Caribbean spiny lobsters and others' studies of other decapod crustaceans indicate that decapods actively use urine-borne signals to communicate social status. My studies support the idea that urine-borne signals released mostly by dominant animals affect the length of a fight. This effect is not just because urine-borne signals induce avoidance behavior by subordinates but also because they provide feedback to the dominants. The avoidance behaviors on subordinate spiny lobsters, like those in mammals (Hurst and Baynon, 2004), are directly mediated by a common risk-assessment pathway with an alarm cue system – the olfactory pathway. To activate this risk-assessment pathway, spiny lobsters control their release of urine during specific social interactions. Therefore, my study provides new information about the mechanism of social competition in spiny lobsters and the chemosensory pathway involved in this social competition.

The social status in the Caribbean spiny lobsters is communicated through both agonistic behavior and urine-borne signals (Chapter 5). Dominant spiny lobsters showed primarily offensive behaviors, whereas subordinate spiny lobsters showed primarily defensive or submissive behaviors. In this communication, dominants frequently released urine, whereas subordinates suppressed it. If urine release in both dominants and subordinates is prevented, they increased the number and duration of fights. This increase was primarily driven by the dominant's increase in attacks on the subordinate. If urine of dominants is reintroduced in the aquaria, the number and duration of fights drop to normal levels. Thus, when one means of communication – urine release – is prevented, spiny lobsters compensate by increasing the other means of communication – offensive behavior. Urine-borne signals induced various avoidance behaviors in spiny lobsters in both laboratory and field conditions. In addition, urine-borne signals also induced approach-type responses in a small percentage (~20%) of lobsters, presumably dominant lobsters. This variance in behavioral responses was likely because of previous experiences that these lobsters may have had or innate predisposition. The avoidance responses to urine were eliminated if the olfactory sensilla are ablated, but not if the non-olfactory sensilla were ablated. Thus the urine-borne signals are exclusively detected by the olfactory pathway and its aesthetasc sensilla.

Function of urine-borne signals

Urine contains a complex mixture of chemical compounds and is used parsimoniously for social signaling in multiple contexts across taxa (Johnston, 2003; Wyatt, 2003). Mammals are well documented for their parsimonious use of urine signals in social recognition, aggression, and reproduction (Brennan and Zufall, 2006; Chamero et al., 2007; Hurst and Beynon, 2004;

Johnston, 2003). Fish use urine signals for reproduction and social status communication (Appelt and Sorensen, 2007; Barata et al., 2007; Sorensen et al., 1988). Female goldfish regulate their urine release to indicate spawning readiness to males (Appelt and Sorensen, 2007). Male Mozambique tilapia regulate their urine release according to social status and context of social interaction (Barata et al., 2007). Urine is also used for multiple purposes in decapod crustaceans (Bushman and Atema, 2000; Gleeson, 1982; Horner et al., 2006, 2008b).

My results also support the view that decapod crustaceans use urine for multiple purposes. Urine is used for social status communication, aggregation, and mating (Chapter 5; Breithaupt and Atema, 1999; Breithaupt et al., 2000; Bushman and Atema, 2000; Gleeson, 1982; Horner et al., 2006, 2008b; Johnson and Atema, 2005; Karavanich and Atema, 1998a, 1998b). In my study and in other studies on decapods, urine-borne signals are used for communicating social status (Chapter 5; Breithaupt and Atema, 1999; Breithaupt et al., 2000; Karavanich and Atema, 1998a, 1998b). Gregarious spiny lobsters prefer shelters scented with urine and lose this preference if the olfactory pathway is ablated (Horner et al., 2006, 2008b). Urine is also important in mating. Female American lobsters prefer shelters occupied by dominant vs. subordinate males; however, if urine release of males is blocked, females lose this preference (Bushman and Atema, 2000). Male blue crabs perform courtship display behavior to the urine of reproductive females, but not when olfactory sensilla are ablated (Gleeson, 1982). Similarly male helmet crabs with ablated distal half of lateral flagellum, which harbors the olfactory sensilla, do not respond to female signals (Kamio et al., 2005). Although spiny lobsters and other decapod crustaceans use urine-borne signals in multiple contexts, no molecules responsible for these various effects on behavior are identified yet. Identification of chemical compounds in conjunction with behavioral manipulations will improve understanding of the mechanisms of

urine-borne signals. Furthermore, because spiny lobsters are gregarious animals, they provide an excellent model to contrast mechanisms of urine-borne communication during competition, aggregation, and reproduction with other decapods that are solitary.

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