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Functional Roles of Crustacean Dual Antennular Chemosensory Pathways in Odor Mediated Behaviors

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FUNCTIONAL ROLES OF CRUSTACEAN DUAL ANTENNULAR CHEMOSENSORY PATHWAYS IN ODOR MEDIATED BEHAVIORS

by

AMY JEAN HORNER

Under the Direction of Charles D. Derby

ABSTRACT

Odor signals mediate a variety of behaviors in animals across a diversity of taxa. Despite dramatic morphological differences between animals from different taxa, several important features of olfactory system organization and processing are similar across animals. Because of this similarity, a number of different organisms including mammals, insects, and decapod crustaceans serve as valuable model systems for understanding general principles of olfactory processing.

As in other organisms, including both vertebrates and insects, the chemosensory system of decapod crustaceans is organized into multiple anatomically distinct neuronal pathways. The two main pathways (the aesthetasc/ olfactory lobe pathway and non-aesthetasc/ lateral antennular neuropil pathway) originate in different populations of antennular sensilla and project to different neuropils in the brain. The functional significance of this parallel organization is not well understood in crustaceans or in many other species. Although in some insect species the functions of parallel pathways are clearly delineated by the types of odors processed by each, functional differences between parallel pathways in other organisms are much less distinct. A critical step towards understanding the functional

significance of the multiple chemosensory pathways is to identify the specific behaviors that are driven by each pathway.

Using spiny lobsters and crayfish as model organisms, the importance of each pathway was examined in three different behavioral contexts: (1) orientation to a distant food odor, (2) shelter selection in response to conspecific chemical signals, and (3) determination of conspecific social status. In each study, selective ablations of specific populations of antennular sensilla were performed, and the behavior of ablated animals was compared to that of intact controls. Results show that either the aesthetasc or non-aesthetasc pathway is capable of driving orientation to food odors, suggesting functional redundancy between the pathways in this behavior. In contrast social odors are processed preferentially by the aesthetasc pathway rather than the non-aesthetasc pathway, suggesting a unique role for the aesthetasc pathway in this context. As in other organisms possessing multiple chemosensory pathways, the dual antennular pathways in crustaceans display both unique and overlapping functions depending on the chemicals examined, and the behavioral context in which the signal is presented.

INDEX WORDS: Crustacean, olfaction, chemical senses, lobster, crayfish, aesthetasc, urine, behavior

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To my grandfather, Dr. John W. Coltman, who inspired my interest in science.

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Chapter 1 - General Introduction

The chemical senses are ancient and ubiquitous sensory modalities. Organisms ranging in complexity from bacteria to humans use chemicals signals to drive a diversity of behaviors including food search, predator avoidance, habitat selection, courtship and mating, and many other forms of both inter- and intraspecific interactions. An important step to understanding how chemical signals influence these behaviors is to describe how such signals are detected and processed by chemosensory systems.

Chemosensory systems in complex organisms

Multiple chemosensory systems are present across the body of organisms. In complex organisms such as vertebrates and arthropods, the chemical senses are often divided into the distinct modalities of smell (olfaction) and taste (gustation). Smell and taste are readily distinguished in terrestrial vertebrates by all of the characteristics listed in Table 1-1. Smell and taste are more difficult to define in invertebrates and aquatic organisms because many of the features that clearly distinguish the modalities in terrestrial vertebrates fail to do so in these organisms. For instance, the medium of stimulus transport becomes useless as a distinguishing feature in aquatic organisms because both olfactory and gustatory chemical stimuli are transported in water. The anatomy of the primary sensory receptors fails to distinguish between smell and taste in invertebrates because both olfactory and gustatory systems use bipolar primary sensory neurons. Despite these difficulties, smell and taste in invertebrates and aquatic organisms can be distinguished by other characteristics such as the types of behaviors mediated or the identity of the first order processing centers (Table 1-1).

Table 1-1: Summary of the major characteristics used to distinguish smell from taste in select marine and terrestrial vertebrates and invertebrates.

	Terrestrial				Aquatic			
	Mammal		Insect		Fish		Spiny Lobster	
	Smell	Taste	Smell	Taste	Smell	Taste	Smell	Taste
Medium of stimulus transport	Air	Water	Air	Water	Water	Water	Water	Water
Location of primary sensory receptors	Nasal epithelium and vomeronasal epithelium	Oral cavity, pharynx, esophagus	Chemosensory sensilla on the antennae	Bimodal sensilla on the antennae, palps, mouthparts and legs	Nasal epithelium	Oral cavity, pharynx, esophagus, gills, fins, barbells, entire body surface	Chemosensory and bimodal sensilla on the antennules	Bimodal sensilla on the mouthparts, pereiopods, 2nd antennae and other body surfaces
Anatomy of primary sensory receptors	Bipolar chemoreceptor neurons	Modified epithelial cells (taste buds)	Bipolar chemoreceptor neurons	Bipolar chemoreceptor neurons	Bipolar chemoreceptor neurons	Modified epithelial cells (taste buds)	Bipolar chemoreceptor neurons	Bipolar chemoreceptor neurons
Synaptic connections	Project directly to central targets via the olfactory (I) cranial nerve	Synapse onto primary sensory afferents of the facial (VII), glossopharyngeal (IX), and vagal (X) cranial nerves	Project directly to central targets via the antennal nerve	Project directly to central targets	Project directly to central targets via the olfactory (I) cranial nerve	Synapse onto primary sensory afferents of the facial (VII), glossopharyngeal (IX), and vagal (X) cranial nerves	Project directly to central targets via the antennular nerve	Project directly to central targets
First order processing centers	Olfactory bulb, accessory olfactory bulb	Gustatory areas in the nucleus of the solitary tract in the medulla	Antennal lobe	Subesophageal ganglion, dorsal lobe	Olfactory bulb	Sensory column of the rostral medulla	Olfactory lobes, lateral antennular neuropils	Subesophageal ganglion
Structural organization of the first order processing centers	Glomerular	Non-glomerular	Glomerular	Non-glomerular	Glomerular	Non-glomerular	Glomerular (Olfactory Lobes) and Non-glomerular (Lateral Antennular Neuropils)	Non-glomerular
Behavioral functions	Distance chemoreception; orientation, predator avoidance, inter- and intraspecific communication	Food handling; ingestion; acceptance or rejection of food items	Distance chemoreception; orientation, predator avoidance, inter- and intraspecific communication	Contact chemoreception; food handling; ingestion; acceptance or rejection of food items	Distance chemoreception; orientation, predator avoidance, inter- and intraspecific communication	Orientation and some distance chemoreception; ingestion; acceptance or rejection of food items	Distance chemoreception; orientation, predator avoidance, inter- and intraspecific communication	Contact chemoreception; short range orientation to food odors; food handling; ingestion; acceptance or rejection of food items.

Multiple olfactory pathways

Even within a single sensory modality such as olfaction, there is a great deal of organizational complexity. The noses of many animals are organized into multiple, anatomically distinct neuronal pathways with different peripheral sensors and different central nervous system processing centers. Partitioning the olfactory system in this way suggests that different chemosensory pathways fulfill different functional requirements for odor processing or for driving odor mediated behaviors. Although anatomically separate chemosensory pathways can be functionally distinguished by the types of odors detected and the types of behaviors regulated in some organisms, the functional roles of anatomically separate chemosensory pathways in other organisms are not as well understood.

The functions of anatomically separate chemosensory pathways in the noses of moths and other insects are discrete and are distinguished both by the functional classes of odorants detected and by the types of behaviors mediated. The antennae of male moths contain two anatomically distinct chemosensory pathways. The main olfactory pathway originates in general-odor sensitive neurons innervating antennal sensilla and targets generalist glomeruli within the antennal lobe of the brain (Hansson, 1995; Hildebrand, 1995; Christensen and White, 2000; Hansson and Anton, 2000; Christensen and Hildebrand, 2002; Wyatt, 2003). The accessory olfactory pathway, which is found only in males, originates in pheromone sensitive neurons innervating specialized antennal sensilla and targets a distinct subset of antennal lobe glomeruli called the macroglomerular complex (Hansson, 1995; Hildebrand, 1995; Christensen and White, 2000; Hansson and Anton, 2000; Christensen and Hildebrand, 2002; Wyatt, 2003). The main olfactory pathway of moths mediates the response to general odors such as food or host plant odors, whereas the male-specific pathway is specialized for

processing female sex pheromones (Hansson, 1995; Hildebrand, 1995; Christensen and White, 2000; Hansson and Anton, 2000; Christensen and Hildebrand, 2002). Thus anatomically distinct chemosensory pathways are also functionally distinct in some insect species.

The functional roles of separate chemosensory pathways in the vertebrate nose are not so clearly defined. The noses of many amphibians, reptiles, and mammals contain multiple, anatomically distinct chemosensory pathways. The largest and most well studied of these pathways are the main olfactory system, consisting of the olfactory epithelium and main olfactory bulb, and the vomeronasal or accessory olfactory system, consisting of the vomeronasal organ and the accessory olfactory bulb (Eisthen, 1997; Christensen and White, 2000; Wyatt, 2003; Baxi *et al.*, 2006; Breer *et al.*, 2006; Spehr *et al.*, 2006). Additional presumptive chemosensory areas occur on the nasal septum and in the Grueneberg ganglion, but the function of these regions is not well understood (Breer *et al.*, 2006; Spehr *et al.*, 2006; Storan and Key, 2006). Traditionally the vertebrate main and accessory olfactory systems were believed to be functionally distinct with the main olfactory pathway mediating the response to general odorants and the accessory olfactory pathway playing a more specialized role in processing intraspecific signals or pheromones. However, several studies representing a range of species have demonstrated non-traditional roles for each of these pathways, indicating that strict functional divisions between these odor processing pathways are not universal. The main olfactory system is capable of mediating the response to pheromones in some species (Hudson and Distel, 1986; Dorries *et al.*, 1997; Johnston, 1998, 2000; Restrepo *et al.*, 2004; Lin *et al.* 2005; Baxi *et al.*, 2006; Spehr *et al.*, 2006), and the vomeronasal system is capable of mediating the response to prey odors and other general odorants in other

species (Halpern *et al.*, 1997; Johnston, 1998, 2000; Miller and Gutzke, 1999; Ptacyk and Graves, 2002; Halpern and Martinez-Marcos, 2003; Wyatt, 2003; Baxi *et al.*, 2006; Spehr *et al.*, 2006). The main and accessory olfactory pathways in vertebrates show both complementary and overlapping functions in the types of odorants detected and the types of behaviors mediated (Halpern *et al.*, 1997; Johnston, 1998; Miller and Gutzke, 1999; Johnston, 2000; Sam *et al.*, 2001; Ptacyk and Graves, 2002; Halpern and Martinez-Marcos, 2003; Wyatt, 2003; Halpern *et al.*, 2005; Baxi *et al.*, 2006).

Organization of the crustacean chemosensory system

Similar to insects and vertebrates, the noses (antennules) of decapod crustaceans also contain multiple anatomically distinct chemosensory pathways. The two main antennular chemosensory pathways, called the aesthetasc / olfactory lobe pathway and the non-aesthetasc / lateral antennular neuropil pathway, originate in different populations of sensilla located on the antennular flagella and project to different neuropils in the brain (Schmidt and Ache, 1992, 1996a, b; Schmidt *et al.*, 1992; Schachtner *et al.*, 2005).

The aesthetasc / olfactory lobe pathway is a purely chemosensory pathway that originates in the prominent aesthetasc sensilla. Aesthetascs are a nearly universal feature of crustacean antennules and depending on the species examined can be very densely innervated. The aesthetascs of decapod crustaceans are innervated by the dendrites of up to several hundred olfactory receptor neurons that send axons to paired neuropils in the brain called the olfactory lobes (Laverack and Ardill, 1965; Sandeman and Denburg, 1976; Spencer, 1986; Grunert and Ache, 1988; Mellon *et al.*, 1989; Mellon and Munger, 1990; Schmidt and Ache, 1992, 1996b; Hallberg *et al.*, 1997; Steullet *et al.*, 2000; Derby *et al.*,

2003). The olfactory lobes show the characteristic glomerular organization that typifies the first order olfactory processing centers of both vertebrates and insects (Christensen and White, 2000; Eisthen, 2002; Wyatt, 2003; Ache and Young, 2005).

In contrast to the aesthetasc pathway, the non-aesthetasc/ lateral antennular neuropil pathway is a multimodal pathway that originates in a diverse group of sensilla on the antennular flagella that are collectively called “non-aesthetascs” (Schmidt *et al.*, 1992; Schmidt and Ache, 1996a). Work in the spiny lobster showed that many non-aesthetascs on the antennular flagella are bimodal and innervated by both chemosensory and mechanosensory neurons (Cate and Derby, 2001, 2002a; Schmidt and Derby, 2005) . Backfills of the lobster antennular nerve revealed that the axons of non-aesthetasc chemo and mechanosensory neurons on the antennular flagella project to the paired lateral antennular neuropils (Schmidt *et al.*, 1992; Schmidt and Ache, 1996a). The lateral antennular neuropils have a stratified organization and are considered to be sensory motor integration centers because they receive the afferents of antennular chemo and mechanosensory neurons and also contain the major arborizations of antennular motoneurons (Maynard, 1966; Sandeman *et al.*, 1992; Schmidt *et al.*, 1992; Schmidt and Ache, 1993, 1996a; Schmidt and Derby, 2005).

Although the aesthetasc and non-aesthetasc chemosensory pathways show striking differences in anatomical organization, the roles of each pathway in odor mediated behaviors are not well understood. The work presented in this dissertation examines the roles of the aesthetasc and non-aesthetasc pathways in different odorant and behavioral contexts with the goal of gaining more insight into why the antennular chemosensory systems of crustaceans are partitioned into separate pathways.

Functional roles of the crustacean dual antennular chemosensory pathways

Some of the earliest attempts to define unique functional roles for the two pathways explored the importance of aesthetascs and non-aesthetascs in food-odor mediated behaviors (McLeese, 1973; Reeder and Ache, 1980; Devine and Atema, 1982). In these studies, the relative importance of each pathway was evaluated through antennular ablation and subsequent observation of resulting behavioral deficits. Using this methodology, several studies claimed unique functional roles for the aesthetascs in different aspects of food odor mediated behaviors (Reeder and Ache, 1980; Devine and Atema, 1982). However, in many cases the ablations removed either the entire lateral flagellum or the entire aesthetasc region, including both aesthetasc and non-aesthetasc tuft sensilla. Despite the fact that several types of non-aesthetascs were removed during these ablations, the observed behavioral deficits were attributed to removal of the most populous sensilla, the aesthetascs. However, the true importance of the aesthetascs for food odor mediated behaviors remained uncertain because of the lack of specificity in the ablations.

In a series of experiments using more specific ablations, (Steullet *et al.*, 2001, 2002) showed that behaviors such as food odor discrimination, food odor learning, and activation of searching could be mediated equally well by either the aesthetasc or the non-aesthetasc chemosensory pathway. The results of these studies demonstrated an overlap in the function of the aesthetasc and non-aesthetasc pathways for food odor mediated behaviors in small scale, low flow arenas. These studies did not examine the importance the aesthetasc and non-aesthetasc chemosensory pathways for food odor mediated behaviors occurring over a larger spatial scale or in more natural flow conditions. Odor plumes emanating from sources in realistic flow conditions are spatially and temporally complex (Webster and Weissburg,

2001), and perhaps extracting orientational information from these signals requires a specialized neural pathway.

Earlier studies in *P. argus* (Reeder and Ache, 1980) and *H. americanus* (McLeese, 1973; Devine and Atema, 1982) concluded that the aesthetascs were necessary to mediate distant food search. However, these studies were done with undefined and unnatural flow conditions, and used non-specific ablations that resulted in the elimination of both aesthetasc and non-aesthetasc sensilla on the lateral flagella. Because of the lack of specificity in their ablations, the importance of the aesthetascs for orientation remained ambiguous. The experiments described in Chapter 2 address these limitations by using specific ablations of aesthetasc and non-aesthetasc antennular sensilla to examine the importance of the two pathways for orientation to a 2-m distant food odor in a laboratory flume. In contrast to previous studies using extremely slow flow rates, the flume allowed for the behavior of the animals to be examined under quantifiable and naturalistic flow conditions. Four different bilateral ablations were performed in this study, resulting in the specific inactivation of: (1) aesthetasc chemosensory neurons, (2) non-aesthetasc chemosensory neurons, (3) non-aesthetasc chemo- and mechanosensory neurons, or (4) both aesthetasc and non-aesthetasc chemosensory neurons. The behavior of each group of ablated lobsters was compared to that of intact controls to evaluate the contributions of the aesthetasc and non-aesthetasc chemosensory pathways in food search under naturalistic and defined flow conditions.

Antennular sensilla function not only in food odor mediated behaviors, but in many other types of chemically mediated behaviors as well. Functional differences between the pathways are beginning to emerge in these different behavioral contexts. In other decapod crustaceans, the aesthetasc chemosensory pathway plays a unique role in intraspecific

interactions. Aesthetascs are both necessary and sufficient to mediate the stereotyped courtship display of the male blue crab, *Callinectes sapidus*, in response to female sex pheromones (Gleeson, 1982, 1991). There is also strong but not yet unequivocal evidence that the aesthetasc pathway mediates aspects of courtship and mating in the helmet crab, *Telmessus cheiragonus* (Kamio *et al.*, 2005). In the American lobster, *Homarus americanus*, the aesthetasc pathway is necessary for individual recognition between lobsters that have previously encountered one another (Johnson and Atema, 2005). The results of these studies show that the aesthetasc chemosensory pathway plays an important and unique role in mediating the response to intraspecific signals in the behavioral contexts of mating and individual recognition. Chapters 3, 4, and 5 of this dissertation further explore the importance of the aesthetasc and non-aesthetasc chemosensory pathways in other types of social behaviors including aggregation and shelter selection in the Caribbean spiny lobster and determination of social status in the red swamp crayfish, *Procambarus clarkii*.

Caribbean spiny lobsters display a diversity of gregarious social behaviors, the most prevalent of which is gregarious diurnal sheltering (Childress and Herrnkind, 1997). Following solitary nocturnal foraging trips, spiny lobsters will often aggregate with conspecifics in communal dens (Herrnkind *et al.*, 1975; Kanciruk, 1980). Shelter choice assays conducted in both the field (Nevitt *et al.*, 2000) and laboratory (Ratchford and Eggleston, 1998; Ratchford and Eggleston, 2000) have demonstrated that shelter selection by *P. argus* can be mediated by chemical signals released from conspecifics. Although spiny lobsters are attracted to conspecifics when searching for shelter, the specific source of release and the identity of the attractive signal are currently unknown. Chapter 3 investigates the source of release and specificity of the aggregation signal in *P. argus* through a series of

shelter choice tests conducted in a laboratory flume. In this study, the sheltering behavior of spiny lobsters was examined in response to dilute urine from male and female conspecifics, food odors, and predator odors.

Chapter 4 continues to explore the gregarious sheltering behavior of *P. argus* by assessing the importance of the aesthetasc and non-aesthetasc chemosensory pathways for mediating shelter selection in response to conspecific aggregation signals. In this study, selective ablations of either aesthetasc or non-aesthetasc antennular sensilla were performed and the sheltering behavior of ablated spiny lobsters was compared to that of unablated, control spiny lobsters.

Chapter 5 investigates the importance of the aesthetasc chemosensory pathway in the context of social dominance using the crayfish *Procambarus clarkii* as a model system. When placed together, groups of crayfish readily establish stable social dominance hierarchies. Social dominance is established through agonistic interactions that begin with threat displays and escalate through several levels of increasingly aggressive ritualized behaviors (Bruski and Dunham, 1987; Huber and Kravitz, 1995). The interaction ends when one of the combatants disengages by retreating or tailflipping away. The victor becomes the dominant animal and the retreating animal becomes the subordinate. The dominant and subordinate relationship remains stable over time and the amount of fighting generally decreases upon repeated pairings presumably because the animals are able to recognize the social status of potential opponents and avoid unnecessary and potentially costly interactions (Copp, 1986; Issa *et al.,* 1999; Goessmann *et al.,* 2000; Schneider *et al.,* 2001). Chemical signals in general and urine signals in particular have a strong influence on the dynamics and outcome of agonistic encounters in both crayfish and lobsters (Karavanich and Atema, 1991,

1998; Schneider *et al.*, 1999, 2001; Breithaupt and Atema, 2000; Breithaupt and Eger, 2002). The antennules are known to be important for mediating aspects of agonistic behavior in crayfish and lobsters (Rutherford *et al.*, 1996; Karavanich and Atema, 1998; Bergman *et al.*, 2003; Johnson and Atema, 2005). However, it is not known if this capability is specifically attributable to the aesthetasc sensilla. In this study, the importance of the aesthetasc chemosensory pathway was investigated through selective ablation and subsequent observation of changes in fighting behavior in size matched male *P. clarkii*.

Chapter 2 - Dual antennular chemosensory pathways can mediate orientation by Caribbean spiny lobsters in naturalistic flow conditions

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Introduction

The ability to detect and locate the source of a distant chemical stimulus is an essential process in the lives of benthic crustaceans. Decapod crustaceans, including Achelata (spiny lobsters), Homarida (clawed lobsters), Astacida (crayfish), and Brachyura (crabs) rely on chemical signals to drive a diversity of behaviors ranging from conspecific interactions (Gleeson, 1982, 1991; Atema, 1995; Karavanich and Atema, 1998a; Giri and Dunham, 1999, 2000) and predator avoidance (Berger and Butler, 2001) to den selection (Ratchford and Eggleston, 1998; Nevitt *et al.*, 2000; Ratchford and Eggleston, 2000; Berger and Butler, 2001), grooming behaviors (Barbato and Daniel, 1997; Daniel *et al.*, 2001), and food detection and localization (Kanciruk, 1980; Reeder and Ache, 1980; Devine and Atema,

1982; Dunham *et al.*, 1997; Giri and Dunham, 1999; Keller *et al.*, 2003). Chemical stimuli are detected by a multitude of chemoreceptive structures on crustaceans. Although chemoreceptive sensilla can be found on virtually all body surfaces, they are most concentrated on the appendages, particularly the antennules, antennae, dactyls, and mouthparts (Ache and Macmillan, 1980; Derby, 1982; Schmidt and Gnatzy, 1984; Schmidt, 1989; Cate and Derby, 2001, 2002b; Garm *et al.*, 2003). The antennules in particular have long been considered to be the primary chemoreceptive organ of the spiny lobster (Fig. 2-1).

Each antennule is composed of 4 segments, the most distal of which bifurcates into a lateral flagellum and a medial flagellum. Each flagellum is composed of annuli that bear a complement of chemo- and mechanosensory sensilla that vary in morphology, distribution, and pattern of innervation. Many studies have shown that the antennules are important for distance chemoreception by lobsters (Reeder and Ache, 1980; Devine and Atema, 1982) and other decapod crustaceans (Hazlett, 1971a; Kraus-Epley and Moore, 2002); however, it is not clear which populations of antennular sensilla are involved in this behavior.

Chemosensory information from the antennular sensilla is transmitted to the central nervous system in two parallel pathways: the aesthetasc/ olfactory lobe pathway and the non-aesthetasc/ lateral antennular neuropil pathway (Schmidt and Ache, 1992; Schmidt *et al.*, 1992; Schmidt and Ache, 1996a, b). The aesthetasc/ olfactory lobe pathway originates in clusters of olfactory receptor neurons innervating the prominent aesthetasc sensilla. Aesthetascs are the most numerous sensilla on the antennules of the Caribbean spiny lobster, *Panulirus argus*, and are located exclusively in a distal tuft on the ventral face of each lateral flagellum. Aesthetascs are unique among antennular sensilla characterized thus far because they are innervated exclusively by chemosensory neurons. Each aesthetasc is innervated by

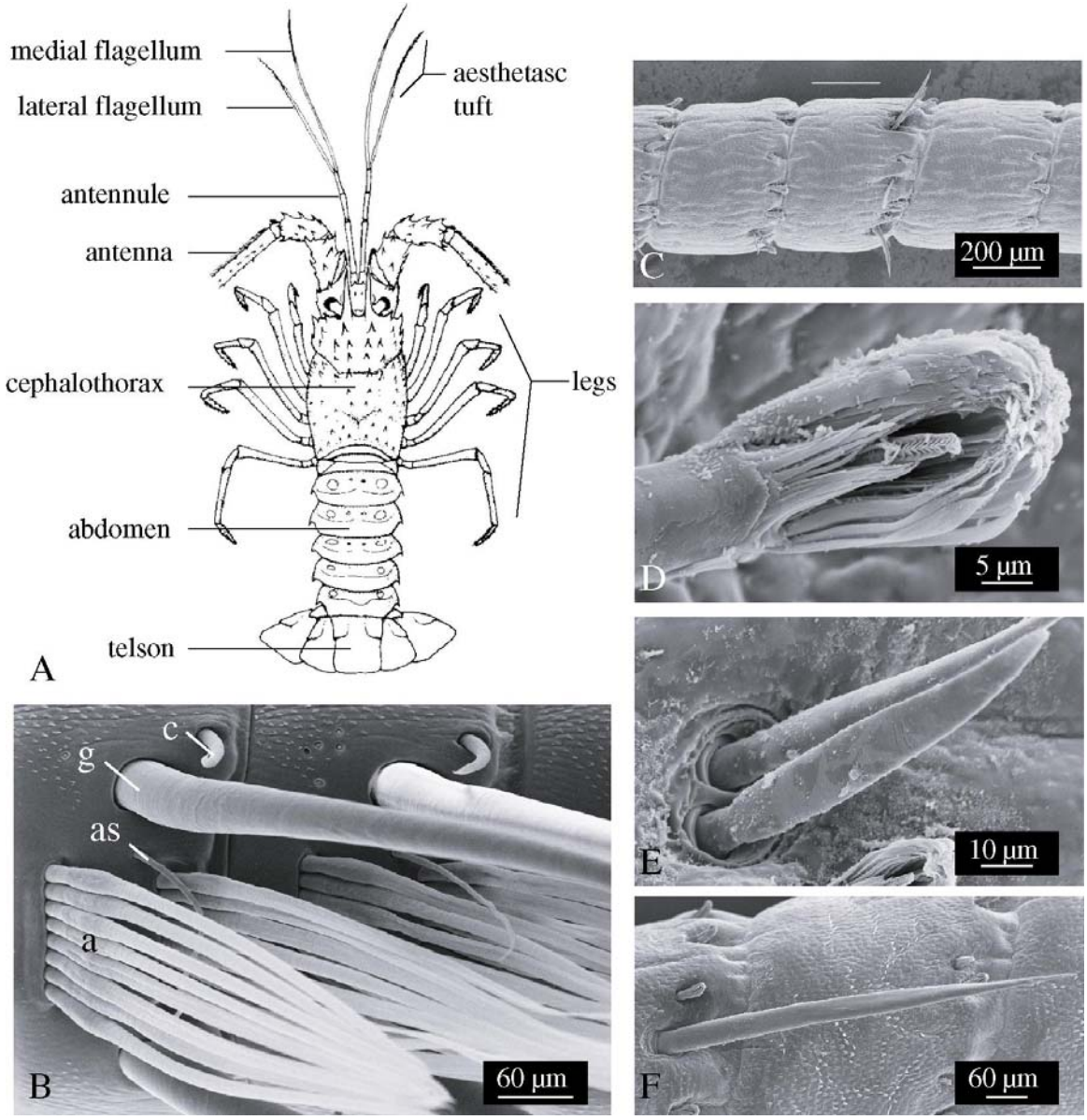

Figure 2-1. Spiny lobster and antennular sensilla. A. Diagram of the spiny lobster showing the major components of the chemosensory system. B. High magnification scanning electron micrograph of a portion of the aesthetasc tuft region of the lateral flagellum. Aesthetasc (*a*), guard (*g*), companion (*c*) and asymmetric sensilla (*as*) are visible. C. Top: scanning electron micrograph of a portion of the medial flagellum showing various types of non-aesthetasc sensilla. Bottom: high resolution scanning micrographs of 3 bimodal chemo-mechanosensilla. From left to right: hooded sensillum, medium simple sensillum, long simple sensillum. Modified from Cate and Derby (2001).

the dendrites of approximately 300 olfactory receptor neurons (Grunert and Ache, 1988; Steullet *et al.*, 2000; Derby *et al.*, 2003) whose axons project to glomeruli within the paired olfactory lobes of the brain (Schmidt and Ache, 1992, 1996b; Sandeman and Mellon, 2002). Aesthetascs were traditionally believed to be the most important structures for detecting, discriminating, and localizing odors because of their great numbers and extensive innervation by chemosensory neurons. Indeed several studies have shown that ablation of the lateral flagellum impairs odor mediated behaviors (Reeder and Ache, 1980; Devine and Atema, 1982; Giri and Dunham, 1999; Kraus-Epley and Moore, 2002; Wroblewska *et al.*, 2002). These behavioral impairments were often attributed exclusively to the loss of aesthetasc sensilla because they are the most numerous sensillar type on the lateral flagellum. However, more recent work has shown that the aesthetascs are not the only structures on the antennule capable of driving food-odor mediated behaviors (Derby *et al.*, 2001; Steullet *et al.*, 2001; Steullet *et al.*, 2002).

Nine other types of sensilla, collectively referred to as “non-aesthetascs”, are widely distributed on the antennules of *P. argus*, and at least four of these (hooded, long simple, medium simple, and asymmetric sensilla) are bimodal and innervated by distinct populations of chemoreceptive and mechanoreceptive neurons (Cate and Derby, 2001, 2002a; Schmidt *et al.*, 2003). Backfills of the antennular nerve have revealed that presumptive chemo- and mechanosensory neurons innervating non-aesthetasc sensilla on the antennular flagella project to the stratified lateral antennular neuropils, while those on the proximal segments and statocysts project to the unstructured median antennular neuropil (Schmidt *et al.*, 1992; Schmidt and Ache, 1993, 1996a; Cate and Roye, 1997), thus forming the non-aesthetasc chemosensory pathway.

Furthermore, these pathways remain anatomically distinct at the next synaptic pathway. Output interneurons from the olfactory lobes and from the lateral antennular neuropil project to different regions of the terminal medullae (Sullivan and Beltz, 2001). It should be noted, however, that there is some connectivity between these two neuropils; for example, local olfactory interneurons exist that connect the ipsilateral olfactory lobe and lateral antennular neuropil (Mellon and Alones, 1994; Schmidt and Ache, 1996b).

Although the two pathways have distinct anatomical arrangements, the functional significance of this dual organization remains unclear. To date, no published studies have conclusively demonstrated unique functions in food-odor mediated behaviors for either pathway in spiny lobsters. In fact, previous work has generally found an overlap in the functions of the aesthetasc and non-aesthetasc pathways for behaviors such as odorant activation of searching behavior, odor learning, and discrimination of food odors in small-scale, low-flow arenas (Steullet *et al.*, 2001; Steullet *et al.*, 2002).

The importance of each pathway for behaviors over a larger spatial scale in more complex flows, such as those occurring during orientation to distant food-odor stimuli, has not been as thoroughly studied. Odor plumes emanating from sources in realistic flow conditions are spatially and temporally complex (Webster and Weissburg, 2001), and perhaps extracting orientational information from these signals requires a specialized neural pathway. Previous studies examining orientation behavior in flumes have focused more on uncovering the organism's method of orientation (e.g. tropotaxis, odor-gated rheotaxis) or on the role of entire antennular flagella rather than on determining the specific sensilla or chemosensory pathways involved in orientation (McLeese, 1973; Reeder and Ache, 1980; Devine and Atema, 1982; Atema, 1995; Beglane *et al.*, 1997; Weissburg, 2000; Kozlowski *et*

al., 2001). In several of these studies, entire flagella (including both aesthetasc and non-aesthetasc sensilla) were ablated, while in others, ablations were only performed unilaterally. Because the ablations were not specific to a single population of sensilla, the importance of each pathway for orientation remains unknown.

Therefore, the goal of this work is to determine whether the aesthetasc pathway or the non-aesthetasc pathway is necessary and sufficient for locating the source of a distant food odor stimulus. To assess the importance of each pathway for this task, we systematically ablated different populations of antennular sensilla and compared the behavior of ablated animals to that of intact controls. Under the conditions tested, both the aesthetasc and non-aesthetasc pathways were sufficient for orientation, but neither pathway alone was necessary. Overall, the results suggest that there is an overlap in the function of the pathways and that food searching is not a unique function of either pathway alone.

Methods

Animals

Caribbean spiny lobsters, *Panulirus argus* (Latreille, 1804), ranging in carapace length from 48-74 mm (mean ± S.E.M. = 62.3 ± 0.86 mm, N = 70) were collected in the Florida Keys, shipped to Georgia State University, and held in 800-L aquaria containing aerated, recirculated, filtered artificial seawater (Instant Ocean®, Aquarium Systems, Mentor, OH, USA). Animals were maintained on a 12h: 12h light: dark cycle and fed shrimp or squid 3 times a week. Intermolt animals (as determined by the method of Lyle and MacDonald, 1983) were selected for the behavioral assays if they approached and consumed a piece of shrimp that had been dropped into the aquarium. At least three days prior to the

start of the trials, experimental animals were transported to holding aquaria (0.90 m long x 0.58 m wide x 0.67 m tall) at Georgia Institute of Technology where they remained throughout the course of the experiment.

Ablations

To assess the importance of different populations of antennular sensilla for orientation, we performed four bilateral ablations, which are described below and summarized in Table 2-1. The four ablations have been used previously, and their effectiveness has been confirmed both morphologically and electrophysiologically (Steullet *et al.*, 2001; Steullet *et al.*, 2002). All ablations were performed on non-anesthetized spiny lobsters immobilized on a plastic restraining device within a shallow container of artificial seawater. Ablations requiring surgical removal of sensilla were performed once, at least three days prior to the start of a series of experimental trials using a hand-tooled narrow blade (0.2 mm wide) (Steullet *et al.*, 2001). Chemical ablations were performed with distilled water within 24 hr of the start of each trial. At the conclusion of each series of experimental trials, ablated antennules were excised and the efficacy of ablation was evaluated by using light microscopy to count the number of sensilla that remained intact on each antennule. This analysis confirmed that shaving was a highly reliable method for removing sensilla. Shaving removed 99.8 ± 0.04 % (mean ± S.E.M., N= 13) of all aesthetascs on the antennule, which is similar to values obtained in other studies (Steullet *et al.*, 2001). This corresponds to 1-2 intact aesthetascs per animal for the animals that we used in this study.

Table 2-1: Summary of the effects of each ablation type on antennular and non-antennular sensilla.

		Ablation			
Sensilla Type	Control	Aesthetascs Ablated	Non-aesthetasc chemoreceptors ablated	Non-aesthetasc chemo- and mechanoreceptors ablated	All antennular chemoreceptors ablated
Aesthetasc	+	-	+	+	-
Non-aesthetasc chemoreceptors	+	+	-	-	-
Non-aesthetasc mechanoreceptors	+	+	+*	-	+*
Non antennular chemo- and mechanoreceptors	+	+	+	+	+

+, Intact/ Functional; -, Ablated/ Non-functional; * some types of mechanoreceptors are more susceptible to distilled water ablations than others, and thus may have been inactivated during treatment. See text for details.

(1) Control

Control animals were immobilized in the plastic restraining device in the same manner as ablated animals, but no sensilla were removed or inactivated.

(2) All Antennular Flagellar Chemoreceptors Ablated

Aesthetasc and non-aesthetasc chemosensory neurons on both antennules were chemically ablated by immersing the lateral and medial flagella of each antennule in a tube of distilled water for 15 min. Distilled water functionally inactivates chemosensory neurons by disrupting the osmotic balance of the outer dendrites (Derby and Atema, 1982; Gleeson *et al.*, 1997). The ablation is temporary and reversible, lasting only about 24 hr before the neurons once again respond to chemical stimuli (Derby and Atema, 1982; Steullet *et al.*, 2001). Because of the ephemeral nature of this ablation, it was performed within 24 hr of each experimental trial. Distilled water effectively inactivates chemosensory neurons, but may also affect the function of some types of mechanosensory neurons. Mechanosensory

neurons with dendrites projecting up the length of the sensillum may be exposed to and inactivated by the distilled water environment (Derby and Atema, 1982); Garm and Derby personal observation).

(3) Aesthetascs-Ablated

All aesthetasc sensilla on both lateral flagella were surgically removed at the base using a hand-tooled blade. Asymmetric setae, which are located laterally to the aesthetasc rows (Gleeson *et al.*, 1993; Cate and Derby, 2001), were also removed during this ablation. Removal of aesthetascs in this manner obliterates the chemosensory dendrites of the sensillum, which results first in unresponsiveness to odors, followed by death and degradation of their receptor neurons (Harrison *et al.*, 2001a).

(4) Non-Aesthetasc Chemo and Mechanoreceptors Ablated

All visible non-aesthetasc sensilla were surgically removed from the entire length of the lateral and medial flagella of both antennules. The flagella were then coated with a thin layer of cyanoacrylate glue (Super Glue Corp., Rancho Cucamonga, CA) to prevent stimulus access to any remaining, unseen non-aesthetasc sensilla. Covering the antennules with cyanoacrylate glue effectively prevents stimulation of both non-aesthetasc chemosensory neurons as well as mechanosensory neurons that are responsive to hydrodynamic and some tactile stimuli (Derby and Atema, 1982).

(5) Non-Aesthetasc Chemoreceptors Ablated

This ablation was designed to specifically eliminate the function of non-aesthetasc chemoreceptors while maintaining the integrity of at least some non-aesthetasc mechanoreceptors. Non-aesthetasc sensilla were surgically removed from annuli located within the aesthetasc-region of each lateral flagellum. The shaved region was then coated with a thin layer of cyanoacrylate glue to prevent stimulus access to any remaining non-aesthetasc sensilla. The rest of the antennule (medial flagellum and proximal region of lateral flagellum) was then immersed in distilled water for 15 min to ablate non-aesthetasc chemoreceptor neurons in these regions. The aesthetasc region on each lateral flagellum was maintained in seawater during this process. Although the shaving and gluing inactivated mechanoreceptor neurons within the aesthetasc region, at least some of the mechanoreceptors along the medial flagellum and proximal portion of the lateral flagellum likely remained intact and functional (see above – All Antennular Flagellar Chemoreceptors ablated – for explanation).

Odor Stimuli

Three different odor stimuli were used in the experiments. Control stimuli consisted of artificial seawater (Instant Ocean®) taken directly from the flume before the start of trials, and experimental stimuli consisted of two concentrations of shrimp extract. Shrimp extract is a potent feeding stimulus for spiny lobsters (Carr, 1988; Derby, 2000) and was prepared by homogenizing frozen shrimp in artificial seawater with a blender, and then collecting and freezing the raw extract in 10-ml aliquots. The final concentration of the raw extract was approximately 300 g/L. We then made dilutions of this stimulus by mixing raw shrimp

extract in artificial seawater taken directly from the flume. Each stimulus was thoroughly mixed by shaking and filtered through Whatman #5 filter paper to remove large pieces of shrimp material. Preliminary experiments showed that shrimp concentrations of 3 and 0.3 g/L were effective in attracting lobsters to the odor source, so they were used in subsequent trials and were called 'high' and 'low' concentrations respectively.

Experimental Setup

To simulate semi-natural flow conditions where fluid flow and boundary layers conditions could be controlled, all trials were conducted in a recirculating 5,000-L flume housed at Georgia Institute of Technology (Fig. 2-2). (See Webster and Weissburg, 2001; Keller *et al.*, 2003; Weissburg *et al.*, 2003) for descriptions of the flume and its use in examining chemosensory behavior of other animals.) The flume measured 12.5 m long, 0.75 m wide, and 0.35 m high, and the 2 m working section for this study began 10 m downstream of the entry way and ended 0.5 m upstream of the reservoir (Fig. 2-2). The floor of the flume was covered with a 1-cm deep layer of fine-grained quartz sand, and the sidewalls were covered with black panels to eliminate confounding visual cues. The flume was filled with artificial seawater (Instant Ocean®) at around 22^{o}C. Flow velocity was 4.9 cm s^{-1} ± 0.08 (mean ± SD) as measured by an acoustic-doppler flow meter with a water depth of 23.0 cm ± 0.348 (mean ± SD) controlled by a vertical tailgate. At this flow speed, the boundary layer shear velocity u^*, calculated using the Law-of-the-Wall equation, and boundary layer structure conformed well to expectations for turbulence in open channel flows (Keller *et al.*, 2003). The near-bed flow was smooth (Reynold's number $Re^* = 2.65$) with a shear velocity, u^*, of 3.1 mm s^{-1}. A cage (0.32 m long x 0.31 m wide x 0.18 m high) constructed out of

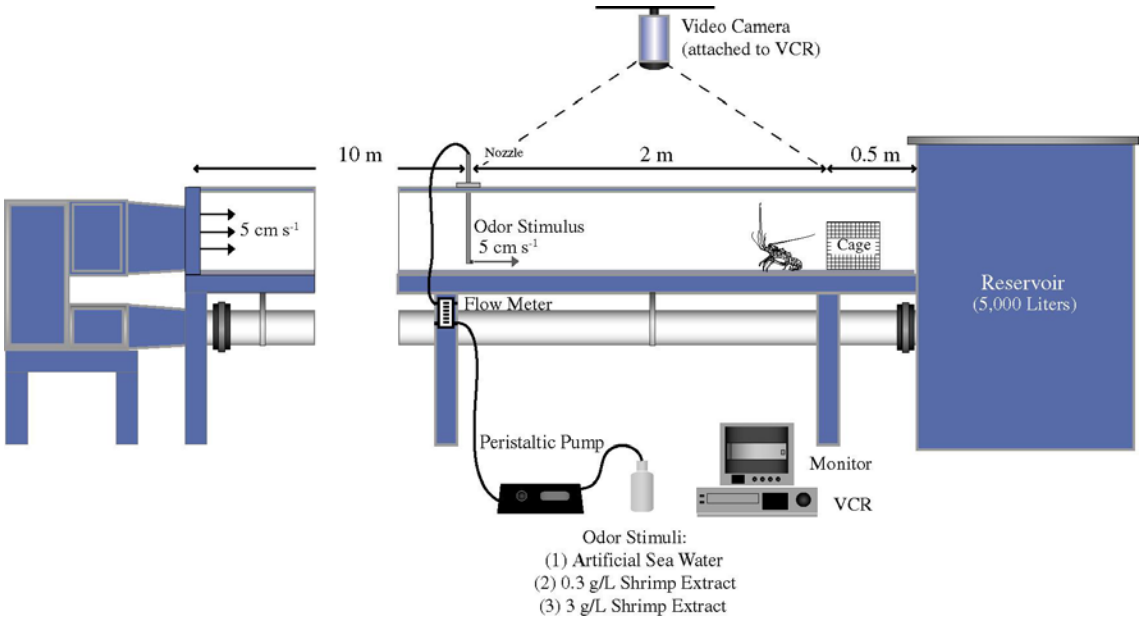

Figure 2-2. Diagram of the flume setup at Georgia Institute of Technology. See text for explanation.

plastic grating (1 cm x 1 cm grate size) was placed at the downstream end of the working section. Odor stimuli were released parallel to the flow 2 m upstream from the cage and 2.5 cm above the bed from a 4.7 mm diameter brass nozzle with a fairing to minimize the flow perturbation. Control and experimental odors were introduced into the flume by a peristaltic pump, which pushed the stimuli through the nozzle at approximately the same velocity as the background flow (i.e., isokinetic release of the stimulus).

All trials were conducted during the day under low light conditions. Although *P. argus* is nocturnal in the natural environment, in the laboratory spiny lobsters will search when presented with food odors during the day if light levels are low enough. A video camera mounted above the flume was used to track the two-dimensional movements of the

animals. Prior to the start of a trial, each animal was fitted with a watertight silicone (Sylgard) backpack containing two red light emitting diodes to facilitate tracking (Weissburg *et al.*, 2002; Keller *et al.*, 2003). The backpack was attached to the animal by a strip of Velcro® that had been glued to the carapace. The presence of the backpack had no apparent effect on the behavior of the animal.

Fifteen minutes before the start of each trial, the lobster was fitted with the backpack and placed in the cage. This was done in order to acclimate the animal to the flume conditions and to provide a constant starting point for each trial. At the end of this acclimation period, the odor stimulus was introduced into the flow, and 30 sec later the cage door was opened, allowing the animal to exit and move freely around the flume. The task was for the animal to exit the cage, track the odor plume to its source, and physically grab the nozzle.

Each trial lasted a maximum of 10 min. Each spiny lobster had 5 min to completely exit the cage. If the animal did not exit the cage within 5 min, the trial was terminated immediately. If the animal did exit the cage within the first 5 min, it was then given an additional 5 min to locate the odor source and grab onto the nozzle. A trial ended either when the animal successfully located the odor source and held onto the nozzle, or when the additional 5-min period had expired. Lobsters were offered a piece of shrimp at the conclusion of every trial as a test of motivational state. The lobster was removed from the experiments and not included in the final data set if it failed to take the shrimp. This was done to insure that an unsuccessful search attempt was due to sensory deficits rather than lack of interest in food.

Each animal was tested a total of 3 times over the course of 3 days (once each day in one of the three stimulus concentrations). The order of stimulus presentation was randomly determined for each animal prior to the start of the experiment. The three trials were not necessarily run on consecutive days, but all were conducted within a two-week period so that no animal was housed at Georgia Tech for more than two weeks.

Immobilization of 2nd Antennae

When spiny lobsters search for the source of an odor stimulus, they typically walk with their second antennae positioned perpendicularly to the long axis of the body. During the course of our experiments, we observed that some lobsters walked towards the source with one antenna in constant contact with the sidewall of the flume. We were concerned that this additional contact might enhance search efficiency and mask any possible deficits caused by antennular ablations. To identify any possible confounding effects of physical contact between the flume walls and second antennae, we conducted a series of trials using animals with and without their second antennae immobilized. We chose to immobilize rather than remove the second antennae because immobilization was a less severe treatment that retains some sensory function of the antennae and limits non-specific effects. The second antennae of five lobsters were positioned above and parallel to the long axis of the body, and secured in this position by binding the two antennae together and then to the horns above each eye with plastic-coated wire. This arrangement restricted the movement of the second antennae and thus prevented the animals from extending them perpendicularly from the body. If the animals were relying on physical contact with the wall to move towards the source, then we

they would have to move further from the center of the flume in order to bring their antennae in contact with the wall.

Results

Success rate for locating the odor source

Antennular sensilla are necessary for locating the odor source, but either the aesthetascs alone or the non-aesthetascs alone are sufficient to mediate this behavior (Fig. 2-3). *Control* animals successfully located the source regularly when challenged with both the high (68%, n=22: Fig. 2-3) and low (45%, n=22: Fig. 2-3) concentrations of shrimp stimulus. However, when tested under control conditions with seawater as an odorant, none of the animals located the odor source (Fig. 2-3). Thus, the presence of chemical stimuli is necessary for spiny lobsters to locate and grab the nozzle.

In contrast to *control* animals, animals with *all antennular flagella chemoreceptors ablated* generally did not locate the odor source when exposed to either of the shrimp extract stimuli or the seawater control (Fig. 2-3). Post-test feeding responses to shrimp showed that the lack of response in this group of animals was not due to low motivation. Less than 16% of the animals in this treatment group failed to respond to the post-test shrimp. Similar responses levels were observed with the other treatment groups, and there was no difference in the percentage of animals that did not respond to the post-test shrimp between the five treatment groups (chi-square [0.05, 4] = 5.48001; $p>0.05$). Thus, functional antennular chemosensilla are necessary for locating the odor source. However, neither the aesthetasc alone nor the non-aesthetascs alone are required to mediate this behavior.

Aesthetasc ablated animals responded similarly to *control* animals (Fig. 2-3), and there were no significant differences between the percentage of *control* versus *aesthetasc ablated* animals finding the source. *Aesthetasc ablated* animals found the source frequently when high (77%, n=13) and low (69%, n=13) concentrations of shrimp extract were used as an odorant, and rarely left the cage, let alone found the source, when seawater was used as an odorant. Thus, aesthetascs alone are not necessary, and non-aesthetascs alone are sufficient to drive this behavior.

Non-aesthetasc chemo and mechanoreceptors ablated animals also responded similarly to the *control* animals (Fig. 2-3). They also located the source regularly in response to both the high (64%, n =11) and low (45%, n = 11) concentrations of shrimp stimulus, and they did not locate the source with seawater. The success rate of *non-aesthetasc chemoreceptor ablated* animals was not significantly different from that of the *non-aesthetasc chemo and mechanoreceptor ablated* animals (Fig. 2-3), although there were fewer animals in this treatment (n=8).

The combination of these results suggests that antennular flagellar chemoreceptors are necessary for spiny lobsters to locate an odor source but that either the aesthetascs alone or the non-aesthetascs alone are sufficient to accomplish this task. Additionally, over the short time frame of these experiments, we saw no evidence that non-antennular chemoreceptors may be able to compensate for the loss of antennular chemoreceptors, as has been shown over a longer time period for other species (Hazlett, 1971b).

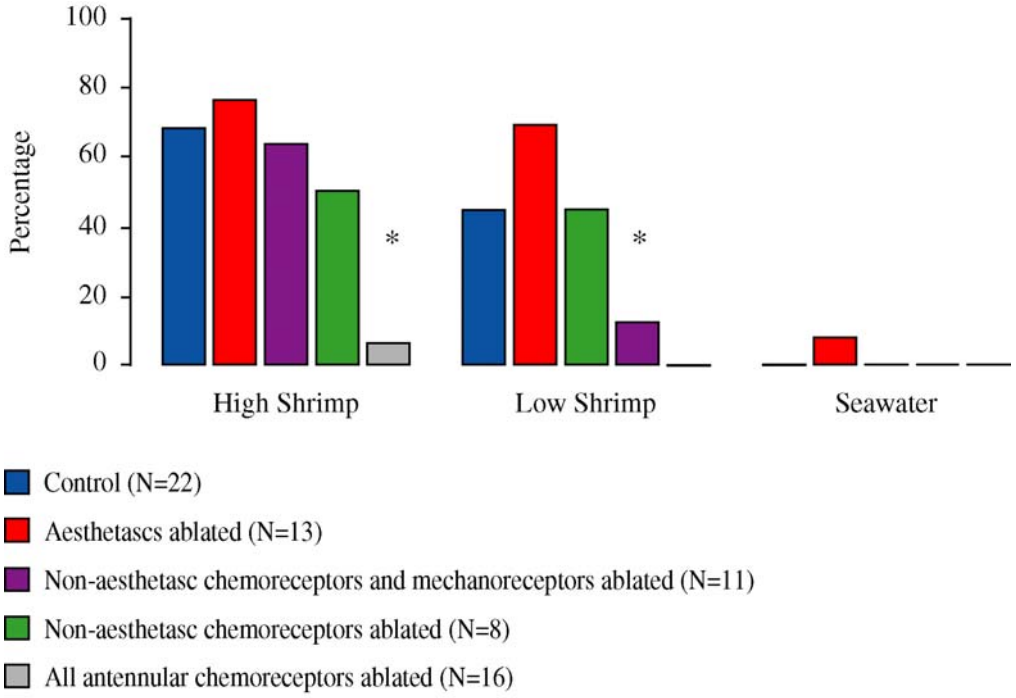

Figure 2-3. Percentage of spiny lobsters that successfully located the odor source. In each of the 3 stimulus groups, only those ablation conditions with an asterisk differed significantly from the Control (unablated) animals (Fisher exact test with Bonferroni correction, $p<0.0125$)

Search Efficiency

In addition to recording the overall success rate of animals in each treatment group, we also examined the efficiency of successful searches to identify more subtle influences of the ablations. Search efficiency was quantified using four parameters that are commonly used in orientation experiments (Devine and Atema, 1982; Moore *et al.*, 1991; Moore and Grills, 1999; Kraus-Epley and Moore, 2002; Keller *et al.*, 2003). The parameters were mean time to

locate the odor source (Fig. 2-4A), net to gross displacement ratio (Fig. 2-4B), mean walking speed (Fig. 2-4C), and mean heading angle with respect to the source (Fig. 2-4D). Because our analysis of efficiency is limited only to successful searches, we did not include completely ablated animals or searches with seawater as a stimulus (since animals did not locate the source under these conditions).

Mean time to locate the source (Fig. 2-4A). The mean time to locate the odor source was calculated as the average time difference between exiting the cage and grabbing the source for all animals in the treatment group. For animals tested in both the high and low shrimp stimulus concentrations, the time to locate the odor source was not different for control and ablated groups (Fig. 2-4A). All four groups of animals found the source within 96 sec in the high concentration and 176 sec in the low concentration (Fig. 2-4A).

Net to gross displacement ratio (Fig. 2-4B). The net-to gross displacement ratio (NGDR) was used to describe the directness of a search path. The ratio was calculated as the Euclidean distance from the cage to the nozzle divided by the total distance traveled by the animal. Ratios approaching 1 represent more direct paths to the source whereas values approaching 0 represent increasingly more tortuous paths to the source. The NGDR values of the control and the three ablated groups were not different when the animals were tested in the low concentration of shrimp extract (Fig. 2-4B). In contrast, when tested in the high stimulus concentration, there was a significant difference in the NGDR between *control* and ablated groups (*aesthetascs ablated, non-aesthetasc chemo- and mechanoreceptors ablated*) (Fig. 2-4B). *Control* animals took very direct paths to the source (NGDR= 0.82, n=14). Compared to

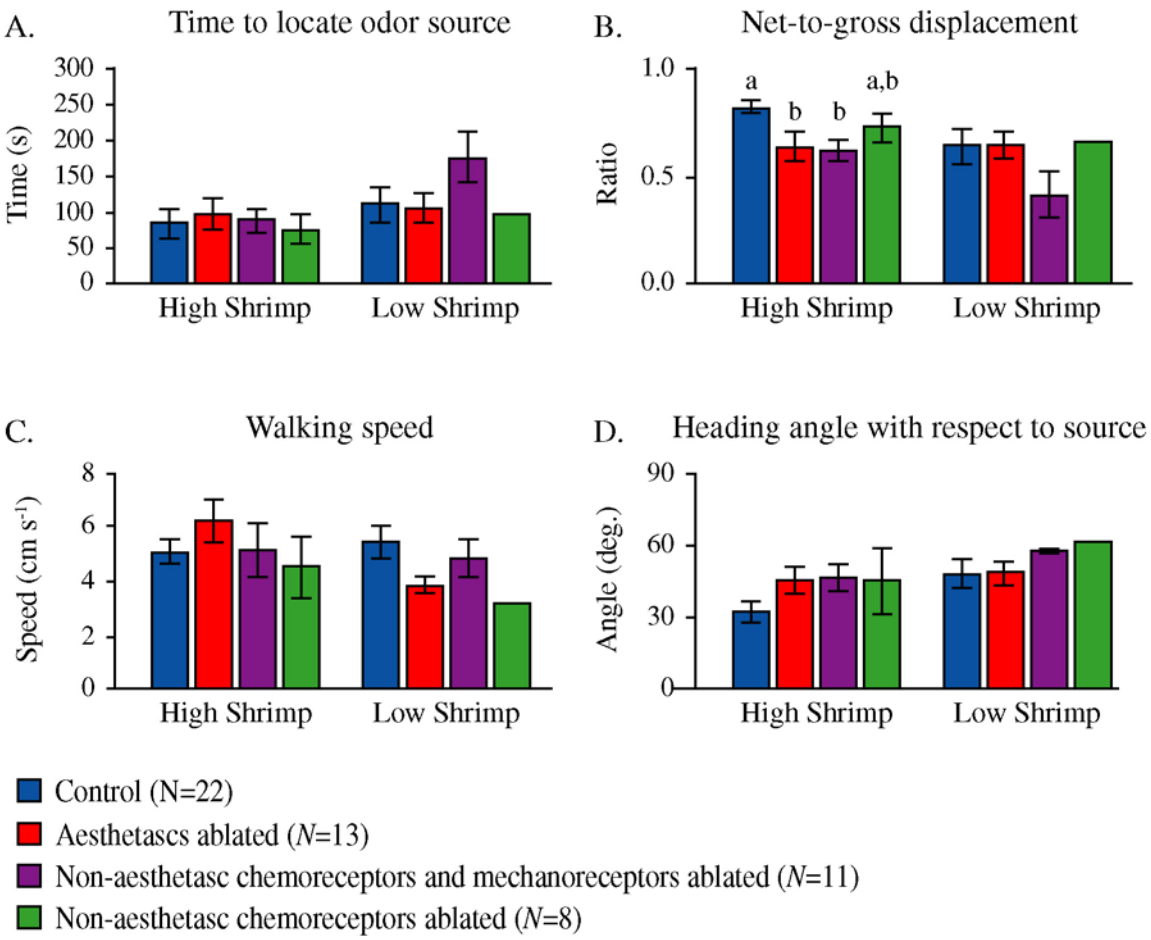

Figure 2-4. Efficiency of successful searches. Mean ± S.E.M. of time to find the odor source (A), net to gross displacement ratio (B), walking speed (C), and heading angle with respect to odor source (D). For behaviors in A, C, and D, there were no differences in responses of the ablated animals at either shrimp concentration (A, C: ANOVA, $p>0.05$; D: Watson-Williams test, $p>0.05$ (Zar, 1996)). For B, at high shrimp concentration only, there was a significant ablation effect (ANOVA, $F_{[3,30]}=3.50$, $p=0.027$), and ablation conditions whose bars have different letters are significantly different (LSD test, $p<0.05$).

control animals, *aesthetasc-ablated* and *non-aesthetasc chemo- and mechanoreceptors ablated* animals took significantly more tortuous paths to the source, with NGDR values of 0.63 (n=10) and 0.62 (n=6) respectively (Fig. 2-4B). Interestingly, although these ablated groups differed from the control group, they did not significantly differ from one another. Thus, the different ablations produced a similar deficit in this measure of search efficiency.

Mean walking speed. The mean walking speed for each orientation path was calculated by averaging the speed of the animal over 1-sec intervals. There were no significant differences between the walking speed of control and ablated animals in either of the stimulus concentrations (Fig. 2-4C). In fact, the average walking speed of all the groups remained relatively constant over all trials regardless of stimuli being tested. Animals tested with seawater as a stimulus walked at similar speeds (in the range of 3-6 cm/sec) to those tested with the shrimp extracts.

Heading angle with respect to the odor source (Fig. 2-4D). Heading angle with respect to the odor source was determined using the methodology of Moore *et al*. (1991). Heading angle was calculated as the absolute value of the angle between a straight line connecting the lobster's current position on the search path (based on the location of the first LED of the backpack) and the nozzle, and a straight line connecting the lobster's current position on the search path and the lobster's next position on the search path. Values ranged between 0^o and 180^o, with 0^o heading directly towards the source and 180^o heading directly away from the source. There were no significant differences between the heading angles of control and ablated animals in either of the stimulus concentrations (Fig. 2-4D).

Effects of stimulus concentration. The behavior of the lobsters was somewhat dependent on the concentration of shrimp odor extract. Animals tended to find the odor source more successfully in high than low shrimp concentration (Fisher exact test, p=0.06). Animals performed more efficient searches in the high compared to the low concentration of shrimp for two of behaviors as suggested by the higher NGDR and lower heading angles in high vs. low plumes (Fig 2-4B, ANOVA, $F_{[1,57]}$=5.42, p=0.023 for NGDR; Fig 2-4D, Watson-Williams test, $F_{[1,57]}$=6.909, p=0.025 for heading angle). Additionally, there was a strong trend for animals in the high vs. low plumes to locate the odor source more quickly (Fig 2-4A, ANOVA, $F_{[1,57]}$=3.45, p=0.068) and a weak trend for them to walk faster (Fig 2-4C, ANOVA, $F_{[1,57]}$=1.95, p=0.168).

Effects of mechanical stimulation of the second antennae (Fig. 2-5). There was no difference between the percentages of animals locating the odor source with free or immobilized antennae (Fig. 2-5A). Both groups of animals found the source regularly when tested with high concentration of shrimp extract, and neither group located the source with seawater as an odorant (Fig. 2-5A). Additionally, in all measures of search efficiency (mean time to source, net-to-gross displacement, mean walking speed, mean heading angle), the two groups of animals did not differ (Fig. 2-5B-E). Thus, contact between the second antennae and the wall of the flume does not significantly influence the success or efficiency of search in our flume.

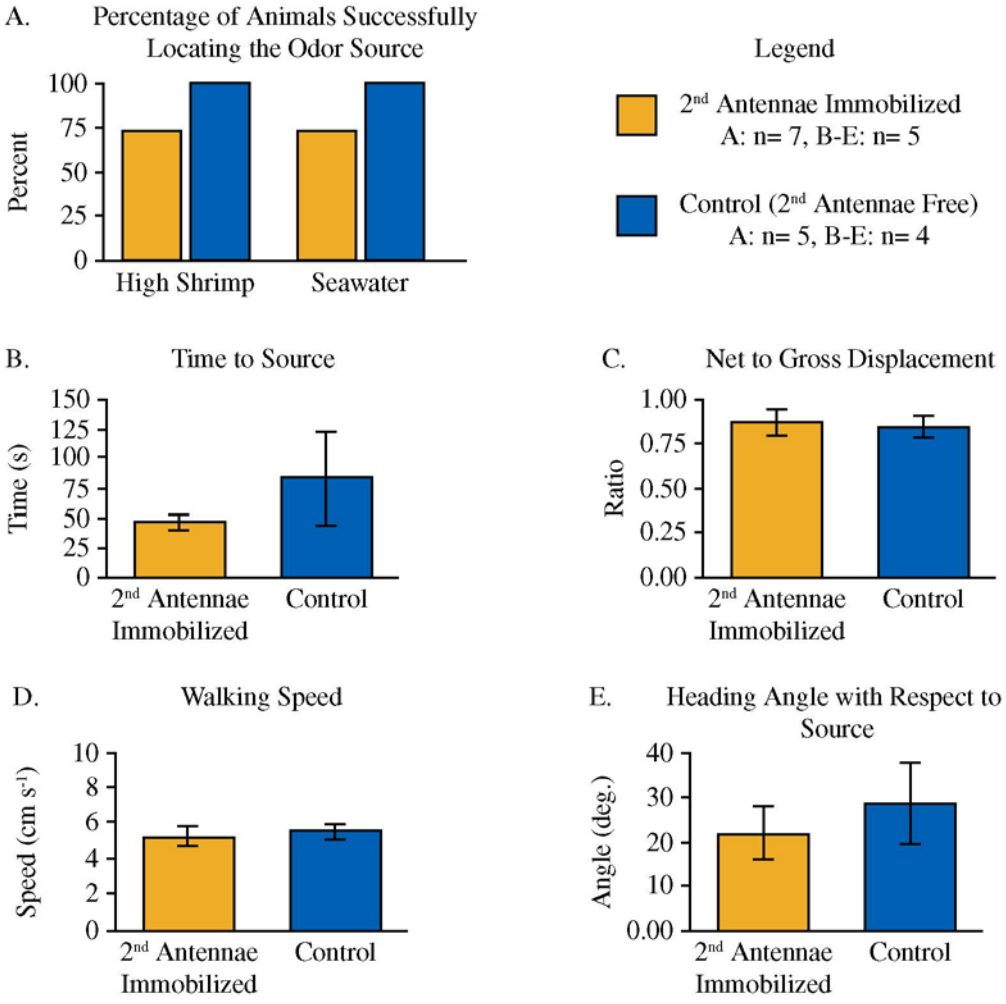

Figure 2-5. Success rate and search efficiency of animals with free and immobilized second antennae. A. Percentage of animals finding the odor source with shrimp and seawater as odorants. Mean ± S.E.M. of time to find the odor source with shrimp as an odorant (B), net to gross displacement ratio (C), walking speed (D), and heading angle with respect to odor source (E). For each of the 4 behaviors, there were no differences in responses of the ablation conditions (A-D, ANOVA, $p>0.05$; E: Watson-Williams test, $p>0.05$).

Discussion

The purpose of this study was to investigate the roles of the dual antennular chemosensory pathways in the spiny lobster during orientation to a 2-m distant food odor source. Our results show that although antennular sensilla in general are necessary for food localization, either the aesthetasc pathway or the non-aesthetasc pathway alone is sufficient to drive the behavior. Food localization was mediated equally well by either the aesthetasc or the non-aesthetasc pathway in our assay, indicating a high degree of functional overlap in the pathways for this task.

Either aesthetasc or non-aesthetasc chemosensory neurons can mediate food localization behavior

Several previous studies have demonstrated that distance chemoreception in decapod crustaceans is mediated primarily by antennular chemoreceptors (Hazlett, 1971a; Reeder and Ache, 1980; Devine and Atema, 1982; Kraus-Epley and Moore, 2002), and the results of this study also support this conclusion. When all antennular flagellar chemoreceptors were ablated, spiny lobsters lost the ability to locate the source of a 2-m distant food odor stimulus (Fig. 2-3). They did, however, respond to a piece of shrimp brought into contact with their legs, indicating that the impairment was due to sensory deficit rather than lack of motivation to feed. Thus, chemosensory input from antennular sensilla in general is necessary for orientation. However, the presence of only a subset of functional chemoreceptors is sufficient to enable orientation. Aesthetasc ablated lobsters were as successful as control animals in locating the odor source (Fig. 2-3). The same pattern of behavior was seen in non-aesthetasc ablated animals (Fig. 2-3). Thus, either of the two chemosensory pathways – the aesthetasc

pathway or the non-aesthetasc pathway– is alone sufficient to allow orientation. Although it did not affect the percentage of animals that successfully located the odor source, ablation of a single pathway did affect search efficiency in some cases. For example, when the aesthetasc pathway was ablated, animals took more circuitous paths to the odor source than control animals in the high shrimp stimulus condition (Fig. 2-4B). Interestingly, the same deficit was seen when the non-aesthetasc pathway alone was ablated (Fig. 2-4B), further suggesting an overlapping role for the pathways in our assay.

The results of this study demonstrate that the two antennular chemosensory pathways are equally able to mediate the behavior under the current experimental conditions. There were no statistically significant differences in the percentage of animals locating the source (Fig. 2-3) or in the search efficiency (Fig. 2-4A-D) between aesthetasc-ablated and non-aesthetasc ablated animals. Thus, the aesthetasc and non-aesthetasc pathways have overlapping functions in this behavioral assay. Possible reasons for the observed overlap and potentially unique roles for each pathway in odor-mediated behaviors are discussed in later sections.

Non-antennular sensors and non-odor stimuli are not sufficient to mediate food localization behavior

In addition to the antennules, chemosensilla are concentrated on several other body regions of the spiny lobster including the walking legs, mouthparts, and second antennae (Derby and Atema, 1982; Cate and Derby, 2002b; Garm *et al.*, 2003). Work on other decapod crustaceans has shown that leg chemoreceptors in particular can aid in orientation as the animal approaches the source of an odor stimulus (Devine and Atema, 1982; Moore *et*

al., 1991; Keller *et al.*, 2003). In our experiments however, inputs from chemosensilla on the legs or other regions of the body were not sufficient to allow the lobster to overcome the sensory deficits caused by antennular ablation. Spiny lobsters with all antennular chemoreceptors ablated did not locate the odor source even though all other non-antennular chemoreceptors were intact (Fig. 2-5). Although our results show that non-antennular chemosensory inputs are not sufficient to drive orientation behavior, they do not suggest that these inputs are unimportant or unnecessary. Spiny lobsters likely use a combination of receptor inputs in the natural environment to locate prey efficiently and avoid unnecessary exposure to predators.

Additionally, visual, hydrodynamic, and tactile cues were not sufficient to allow the lobsters to locate the odor source in the absence of chemical stimulation of the antennules. Spiny lobsters did not locate the nozzle when seawater was used as a stimulus, even though visual and hydrodynamic cues would have been comparable between seawater and shrimp odorant trials. Thus, the lobsters in our study were not simply locating the nozzle by moving upstream in the flow; the presence of a chemical signal was necessary.

Although flow cues alone were not sufficient for lobsters to locate an odor source in our assay, lobsters may use these cues in combination with chemical cues to orient efficiently to the source of an odorant. Hydrodynamic stimuli can provide potentially valuable information about the direction and spatial arrangement of stimuli in the environment, and crustaceans are known to respond to strong local flows and also to more general cues like wave surge (Breithaupt *et al.*, 1995; Nevitt *et al.*, 1995; Wilkens *et al.*, 1996). However, decapod crustaceans do not rely exclusively on flow cues to locate the source of an odor stimulus; they also extract important information directly from the spatial or temporal

properties of the chemical signal (Weissburg and Dusenbery, 2002). Blue crabs, for instance, employ a search strategy that incorporates both chemical and flow cues (odor-gated rheotaxis) to locate the source of an odor (Weissburg and Zimmer-Faust, 1993, 1994; Weissburg, 2000; Webster and Weissburg, 2001; Weissburg and Dusenbery, 2002; Weissburg *et al.*, 2002; Keller *et al.*, 2003). The concurrent use of both hydrodynamic and chemical cues results in more efficient searches with more direct paths and fewer course corrections (Weissburg and Dusenbery, 2002; Keller *et al.*, 2003). The spiny lobsters in our experiments may have also used flow cues to efficiently orient to the odor source after the chemical signal had been detected. However, because this experiment was designed specifically to examine the chemosensory pathways involved in odor guidance, we cannot definitively identify the searching strategy employed by the animals in our assay.

Tactile stimulation resulting from physical contact between the second antennae and the sidewalls of the flume also did not alter the ability of spiny lobsters to locate the odor source. The overall success rate and search efficiency of animals with immobilized antennae was not different from that of animals with free antennae (Fig. 2-5). Lobsters with immobilized antennae generally walked straight down the center of the flume without attempting to contact the sidewall, suggesting that physical contact with the sidewall does not necessarily enhance their success rate or search efficiency.

Why have multiple chemosensory pathways?

The results of our experiments strongly suggest that there is a high degree of functional overlap between the dual antennular pathways for food localization behavior. Functional overlap is an important feature of many sensory systems and can benefit an

organism in several important ways (Derby and Steullet, 2001). Possession of multiple, overlapping sensors allows an animal to continue to function normally in the event of loss or damage to a subset of sensors (Derby and Steullet, 2001). Lobsters missing part or the entire aesthetasc region occur in both the field and laboratory (Harrison *et al.*, 2001a). Because the acquisition of food is crucial for survival, it is not surprising that lobsters can use other chemosensory structures besides the delicate aesthetascs to mediate this important behavior.

A multiplicity of receptors can also extend the range of stimuli that a lobster is able to detect, and increase the sensitivity and resolution of the system (Derby and Steullet, 2001). Electrophysiological studies have demonstrated that aesthetasc and non-aesthetasc chemoreceptor neurons respond to the same types of odorants and have similar response thresholds (Fuzessery, 1978; Thompson and Ache, 1980; Cate and Derby, 2002a). The combination of inputs from these two pathways may allow for much greater sensitivity than either pathway alone could provide, as suggested by some of the results of this study. When

han either group of partially ablated animals (aesthetascs ablated and non-aesthetasc chemo and mechanoreceptors ablated), suggesting that the combined input of both chemosensory pathways provides more information than either pathway alone. Although each pathway alone is sufficient to drive the behavior in this instance, the performance of the lobster is enhanced by the combined activity. Although this combination can have important benefits, it is likely that the aesthetasc and non-aesthetasc chemosensory pathways also have specialized roles that would emerge under different experimental conditions. Despite the lack of experimental demonstrations of specific roles for each pathway in complex behaviors, both the

organization of the pathways and the results of behavioral studies done with other species of decapod crustaceans provide some possibilities.

The aesthetasc pathway originates in the olfactory receptor neuron innervating each aesthetasc on the antennule. The axons of these neurons synapse onto olfactory interneurons within the olfactory lobes of the deutocerebrum (Schmidt and Ache, 1992, 1996b; Sandeman and Mellon, 2002). The paired olfactory lobes have a glomerular organization and are structurally analogous to the olfactory bulbs of vertebrates and the antennal lobes of insects (Sandeman and Denburg, 1976; Mellon and Munger, 1990; Sandeman *et al.*, 1992; Schmidt and Ache, 1992, 1996b). Glomeruli are typical features of first order olfactory processing centers (Hildebrand, 1995; Eisthen, 2002) and are thought to play an important role in determining odor quality. Indeed, behavioral experiments show that the aesthetascs are sufficient to mediate olfactory discrimination of relevant food odor mixtures (Steullet *et al.*, 2002). Although they are not necessary for analyzing food odors (at least at the concentrations tested), aesthetascs may be important in determining the quality of other types of odor stimuli. In the male blue crab *Callinectes sapidus*, aesthetascs are essential for mediating the response to courtship and mating signals Gleeson (Gleeson, 1982, 1991). It is possible that the aesthetasc pathway also functions in spiny lobster intraspecific communication perhaps by mediating the response to aggregation signals.

In contrast, the organization of the non-aesthetasc pathway suggests that it may play a role in detecting spatial aspects of a chemical stimulus. The non-aesthetasc pathway contains both chemosensory and mechanosensory afferents, including those from bimodal non-aesthetasc sensilla on the antennular flagella. Although this pathway is thought to be involved primarily in driving sensory-motor reflexes and movements of the antennules

(Maynard, 1966; Schmidt and Ache, 1993), more recent work indicates that it also functions in a variety of odor mediated behaviors (Steullet *et al.*, 2001; Steullet *et al.*, 2002); this paper). It has been hypothesized that the bimodal non-aesthetasc sensilla, which allow spiny lobsters to detect both chemical and hydrodynamic characteristics of an odor stimulus, may provide the animal with information about the location of stimulation on the antennule. Additionally, the stratified organization of the lateral antennular neuropils (one pair of target neuropils in this pathway) has been hypothesized to represent a spatial map of sensory inputs on the antennule (Schmidt and Ache, 1996a). Although it has not been demonstrated experimentally, the non-aesthetasc pathway may detect spatial aspects of an odor stimulus through the integration of chemosensory and mechanosensory cues. The fact that the output interneurons from the lateral antennular neuropils and from the olfactory lobes project to distinctly different regions of the protocerebrum (Sullivan and Beltz, 2001) supports the notion that these pathways have some divergent functions.

Possession of multiple chemosensory pathways with redundant as well as complementary functions may allow a lobster to detect and discriminate over a much broader range of chemical stimuli than would be possible with only a single chemosensory pathway. Although unique behavioral roles for either chemosensory pathway in the Caribbean spiny lobster have not yet been conclusively demonstrated, several possibilities for specialized functions exist. Ongoing experiments in our laboratory are focused on these possibilities in order to understand the functional significance of the dual chemosensory pathways of the Caribbean spiny lobster.

Chapter 3 - Source and specificity of chemical cues mediating shelter preference of Caribbean spiny lobsters (*Panulirus argus*)

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A. J. Horner designed and conducted the behavioral trials, analyzed the behavioral data and wrote the manuscript with guidance from C. D. Derby. S. P. Nickles assisted with animal care and behavioral trials. M. J. Weissburg provided the flume facility and helpful comments on early drafts of the manuscript.

Introduction

Many species of palinurid lobsters display gregarious social behaviors (Atema and Cobb, 1980). In the Caribbean spiny lobster, *Panulirus argus*, this sociality is evident in a variety of behaviors including the formation of long, single file migratory queues, and defensive rosettes and aggregations (Herrnkind, 1969, 1970; Berrill, 1975; Atema and Cobb, 1980; Herrnkind, 1980; Herrnkind *et al.*, 2001). However, the most ubiquitous example of their sociality is gregarious sheltering (Childress and Herrnkind, 1997). After solitary nocturnal foraging trips, spiny lobsters often aggregate with conspecifics in dens where they

remain sheltered throughout the day (Herrnkind *et al.*, 1975; Kanciruk, 1980). Both male and female spiny lobsters shelter gregariously (Herrnkind *et al.*, 1975; 2001), suggesting that this form of aggregation is not a sex specific behavior (Zimmer-Faust *et al.*, 1985).

Although spiny lobsters are often aggregated in shelters, the extent of gregarious sheltering in any particular area is variable and influenced by several factors including conspecific density, predation levels, and the number, availability, and size of suitable shelters (Eggleston and Lipcius, 1992). However, multiple occupancy of a shelter occurs more often than expected by random chance (Herrnkind *et al.*, 1975; Kanciruk, 1980). The primary benefit of gregarious sheltering is believed to be a reduction in overall predation levels, which could be accomplished in several ways: through group defense, dilution effects, or via the guide effect, which suggests that spiny lobsters can minimize the amount of time spent searching for a shelter (thus minimizing their exposure to predators) by homing in on cues released from sheltered conspecifics (Eggleston and Lipcius, 1992; Childress and Herrnkind, 1997, 2001a, b).

An essential first step to understanding how aggregation occurs is to identify the proximal cues that attract spiny lobsters to sheltering conspecifics. Shelter choice assays conducted in both the field and laboratory (Ratchford and Eggleston, 1998; Ratchford and Eggleston, 2000) demonstrated that shelter selection by *P. argus* can be mediated by chemical signals released from conspecifics. In these studies, spiny lobsters sheltered in dens from which conspecific odor (water in which a conspecific was housed) was emanating significantly more often than they sheltered in unscented control dens. Conspecific attraction also facilitates shelter selection and aggregation in the California spiny lobster, *Panulirus interruptus*, a cold water congener of *P. argus* (Zimmer-Faust *et al.*, 1985; Zimmer-Faust and

Spanier, 1987), and seasonal aggregation in *Panulirus guttatus*, which is sympatric to *P. argus* (Briones-Fourzan and Lozano-Alvarez, 2005).

Although spiny lobsters are attracted to conspecifics when searching for shelter, the specific source of release and the identity of the attractive signal are currently unknown. Studies done by Ratchford and Eggleston (2000) demonstrated that although spiny lobsters are continuously receptive to the aggregation signal, the release of the signal is temporally regulated. They also showed that spiny lobsters of various sizes release the signal in a mass-dependent manner (Ratchford and Eggleston, 1998). These two release characteristics suggest that the aggregation signal may be contained within the urine.

Urine is often an important carrier of chemical information in intraspecific interactions between decapod crustaceans. Urine-borne signals mediate several aspects of courtship and mating behavior (Ryan, 1966; Christofferson, 1978; Gleeson, 1980; Bushmann and Atema, 1994; Atema, 1995; Bushmann and Atema, 1997; Bushmann and Atema, 2000; Kamio *et al.*, 2000; Hardege *et al.*, 2002; Kamio *et al.*, 2002; Raethke *et al.*, 2004) and also play an important role in the determination of social status and individual recognition in other species of decapods (Breithaupt and Atema, 1993; Atema, 1995; Karavanich and Atema, 1998a, b; Breithaupt *et al.*, 1999; Breithaupt and Atema, 2000; Schneider *et al.*, 2001; Breithaupt and Eger, 2002).

The goals of the current study were to develop a naturalistic but relatively rapid laboratory bioassay for examining chemically-mediated sheltering behavior in *P. argus*, and to use it to examine the source and specificity of the aggregation signal.

Methods

Animals

Intermolt Caribbean spiny lobsters, *Panulirus argus* (Latreille, 1804) (carapace length: mean ± S.E.M.= 56.9 ± 1.0 mm, *n*= 76), were collected in the Florida Keys, shipped to Georgia State University, and held in 800-l aquaria containing aerated, recirculated, filtered artificial seawater (Instant Ocean®, Aquarium Systems, Mentor, OH, USA). For the population from which these lobsters were obtained, females as small as 57 mm carapace length can be reproductive (Bertelsen and Matthews, 2001). However, none of the animals used in this study were gravid or bore a spermatophore. Judging from their size, these animals were subadults or young adults. Animals were maintained on a 12 h : 12 h light: dark cycle and fed shrimp or squid three times a week. At least two days before being tested in the behavioral assay, experimental animals were transported to holding aquaria (0.90 m long x 0.58 m wide x 0.67 m tall) at Georgia Institute of Technology, where they were maintained throughout the course of the experiments when not being tested.

Odor stimuli

Control stimulus

Control stimulus was artificial seawater taken directly from the flume before the start of trials.

Urine stimuli

Urine was collected from four subadult male (carapace length: mean ± S.E.M. = 64 ± 5 mm) and four subadult female (carapace length: mean ± S.E.M. = 59.8 ± 7.2 mm) *P. argus*

housed singly in 40-l aquaria. Lobsters were catheterized using a modified version of the technique developed by Lindstrom (1991) and employed by Breithaupt and Atema (1993). Briefly, animals were immobilized on a Plexiglas® restraining device, and the area surrounding the nephropore was blotted dry. Tygon ® R3603 flexible tubing (inner diameter: 1.6 mm; outer diameter 3.2 mm, Saint-Gobain Performance Plastics, Akron, OH) was affixed over each nephropore using cyanoacrylate glue (Quicktite Super Glue Gel ©, Loctite Corp., Manco, Inc. Avon, OH, or Zap-a-Gap ©, Pacer Technology, Rancho Cucamonga, CA) and a catalytic accelerator (Zip Kicker ©, Pacer Technology). The tubes enclosing the nephropores were secured to the dorsal side of the animal and connected to a collection vial via a T-connector and an additional length of tubing. The collection vial was a 50-ml plastic centrifuge tube surrounded by a polystyrene ring, which kept the vial afloat at the water surface. As urine was released, it moved through the tubes and accumulated in the collection vial. Collection vials were emptied daily, and the collected urine was frozen and stored at -20^{o}C. The amount of urine produced by an individual lobster varied greatly, but generally ranged from 0 to 20 ml over the course of the day.

Previous research showed that the release of the aggregation signal in *P. argus* is discontinuous (Ratchford and Eggleston, 2000), which suggests that the signal (if contained in the urine) may not necessarily be present in all urine samples. To maximize our chances of collecting some volume of the aggregation signal, lobsters were continuously catheterized, and the urine output from multiple animals collected over the course of several days was combined into a single sample.

Collected urine was thawed and diluted 1:10 or 1:100 in artificial seawater taken directly from the flume for use in the experimental trials. Conspecific urine stimuli consisted

of samples pooled from all 8 catheterized *P. argus* (including both males and females). Sex specific urine stimuli consisted of either pooled male urine (collected from all 4 catheterized male *P. argus*) or pooled female urine (collected from all 4 catheterized female *P. argus*).

Other odor stimuli

We also examined the sheltering behavior of spiny lobsters in response to three additional odor stimuli: shrimp extract, whole shrimp, and octopus tank water. This was done to insure that any sheltering behavior exhibited by the lobsters in response to urine was specific to that stimulus, and not simply a generalized response to any novel odorant introduced into the flow.

Shrimp extract

Shrimp extract is a potent feeding stimulus for spiny lobsters (Carr, 1988; Derby, 2000) and was prepared by homogenizing frozen penaeid shrimp in seawater in a blender and then collecting and freezing the raw extract in 10-ml aliquots. The final concentration of the raw extract was approximately 300 g/l. The raw extract was diluted 1:10 (30 g/l) in artificial seawater taken directly from the flume and filtered to remove large pieces of shrimp material.

Whole shrimp

We also examined the sheltering behavior of lobsters when a piece of penaeid shrimp was placed on the floor of the shelter. This was done to ensure that any lack of preference seen in the previous treatment was because shrimp is an ineffective stimulus for choosing a

shelter, and not because the concentration of shrimp extract was too low to influence sheltering behavior.

Octopus odor

Octopus odor consisted of artificial seawater taken from an 80-l aquarium in which a single small *Octopus briareus* (approximately 30 cm from arm tip to arm tip) was living for several weeks. *P. argus* and *O. briareus* are sympatric and utilize the same types of crevice shelters, but *O. briareus* is a competitor and potential predator of *P. argus* (Berger and Butler, 2001). Previous research indicates that lobsters avoid shelters that emanate octopus odors (Berger and Butler, 2001). Therefore we also expected the lobsters would not associate with and might avoid shelters emanating the scent of a live octopus in our shelter choice assay.

Experimental setup

One experimental goal was to develop a laboratory assay that was more rapid than previous assays of sheltering behavior, which required many hours (e.g. Ratchford and Eggleston, 1998; Ratchford and Eggleston, 2000), and that placed the animals in natural flow dynamics. Our shelter choice assay was performed in a 5,000-l seawater flume located at Georgia Institute of Technology (Fig. 3-1). The flume measures 12 m long x 0.75 m wide x 0.35 m high with a downstream working section measuring 2 m long x 0.75 m wide x 0.35 m high. When in operation the flume itself (not including the reservoir) contains over 2,500 l of seawater. Details on the flow dynamics of this flume and its use in other behavioral experiments are described elsewhere (see (Webster and Weissburg, 2001; Keller *et al.*, 2003;

Weissburg *et al.*, 2003). All trials in this study were conducted with a background flow rate of 5 cm/s. The flume water was filtered through biological, particulate, activated carbon and UV filters between trial days.

Two concrete blocks were used as shelters for the lobsters (block dimensions: 39.5 cm tall x 19.5 cm wide x 19 cm deep, opening size: 14 cm tall x 13 cm wide x 19 cm deep). The blocks were placed at the upstream end of the working section 5 cm from the wall of the flume and 26 cm apart. The area between each block and the sidewall of the flume was filled with a small section of plastic grating (1 cm x 1 cm) to prevent the lobsters from sheltering in this area. A larger piece of plastic grating spanning the width of the flume was placed behind the blocks to prevent the lobsters from escaping the working section. We secured two handmade L-shaped plastic pipettes to the plastic grating such that the opening of each pipette was centered in the opening of each concrete block, 1 cm above the floor of the block opening. Odor stimuli were introduced into the flow by a dual channel peristaltic pump (Masterflex, Cole Parmer Instrument Company, Vernon Hills, IL) which moved the odorants through a pair of Teflon tubes that had been threaded through each of the plastic pipettes. During control trials, artificial seawater was simultaneously pumped through both shelters. During experimental trials, the experimental odorant was pumped through one shelter while seawater was simultaneously pumped through the other shelter as a control. The experimental odor stimulus was always paired with a seawater control. We never directly tested one experimental odor against another because we were only interested in whether a particular stimulus influenced sheltering behavior, not in the relative strength of its influence. We randomly chose which shelter would release which odorant, and switched the site of odorant release between trials. Control and experimental stimuli were pumped into the flume

at approximately 15 ml/h. (Thus with a 1:100 dilution, only about 150 µl of urine is released into the flow over the course of 1 h). This rate and dilution was used to conserve our odor stimuli, and because preliminary experiments indicated that these conditions were sufficient to elicit sheltering behavior.

All trials were conducted with the room lights on and with a 60W light mounted at the downstream end of the flume to provide constant illumination in the working section. In the natural environment, lobsters typically search for shelter in the early morning. However, previous research has shown that lobsters will shelter in the presence of aggregation signal regardless of where they are in their circadian cycle (Ratchford and Eggleston, 2000). Therefore we did not make special efforts to run the trials at specific points in the lobster's natural light-dark cycle.

Trials began when a lobster was placed in an acrylic and plastic grate cage (30.5 cm long x 21 cm wide x 20.5 cm high) 1.5 m downstream from the face of the concrete blocks for a 5-min acclimation period. Odor stimuli were pumped into the flume during this acclimation period. After 5 min, the cage was lifted and completely removed from the flume, thus forcing the lobster to explore the working section. Each trial lasted for 1 h, and the movements of the lobsters were recorded by a video camera mounted above the flume. Trial length was set at 1 h because we wanted to establish a short but reliable bioassay, and our preliminary experiments indicated that this was a sufficient period of time for lobsters to establish a clear preference for one shelter over the other.

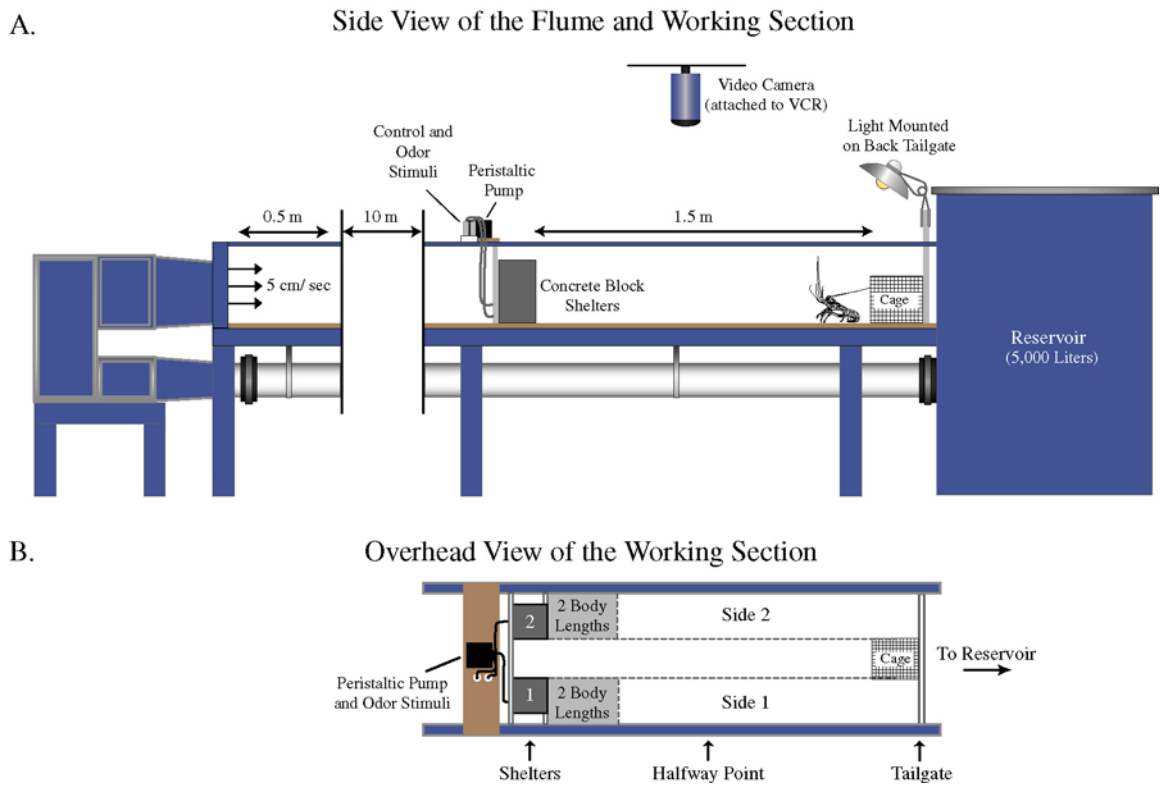

Figure 3-1. Condensed diagram of the seawater flume at Georgia Institute of Technology showing a side view (A) and overhead view (B) of the working section and setup for the behavioral assay.

Video analysis

Videos were analyzed by an individual unaware of which shelter contained the experimental odorant. To be included in the data set, a lobster had to explore both sides of the flume and walk more than halfway upstream. These criteria were set to ensure that lobsters had the opportunity to sample odors eluting from both shelters, and that they were healthy and motivated to explore the flume and shelters. A majority of lobsters tested with each odorant met the criteria to be included in the data set (Table 3-1). The sheltering

behavior of the lobsters was quantified by recording the number of times each shelter was entered and by measuring the total amount of time spent inside (all body parts except antennae completely within the shelter) or within 30 cm (roughly the equivalent of two body lengths of the smallest lobsters used in the trials) of each shelter. Each trial thus produced two values for total sheltering time: one for time spent in or around the control shelter, and the second for time spent in or around the experimental odor shelter. Each trial also produced two values for the number of entries into each shelter. Sheltering time was defined to include the time spent within two body lengths of each shelter because animals sometimes were outside of the shelter but behaved as if they were sheltered inside the block. For instance, in several trials, the lobster approached the shelter head on, turned around, and then backed into the corner formed by the plastic grate and sidewall of the flume immediately adjacent to the shelter. In this position, the lobster's abdomen was partially protected on two sides, while its antennules were still able to sample water passing though the shelter. Although these animals were not inside the shelter, they still secured protection from the block.

Table 3-1. Number and percentage of spiny lobsters meeting criteria for each odor tested.

							Female Lobsters		Male Lobsters	
	Seawater	1:10 Urine	1:100 Urine	1:10 Shrimp	Whole Shrimp	Octopus	Female Urine	Male Urine	Female Urine	Male Urine
No. Lobsters Tested	10	15	36	14	10	9	14	10	10	11
No. Meeting Criteria	8	13	31	14	10	9	13	9	10	11
% Meeting Criteria	80	86	86	100	100	100	93	90	100	100

Statistical analysis

For each of the odorants examined (seawater, conspecific urine, shrimp extract, whole shrimp, and octopus odor), we used a two-tailed Wilcoxon matched pairs test to determine if there were statistically significant differences in the amount of time spent inside or within two body lengths of the control shelter *versus* the shelter emanating the experimental odorant. Data from all lobsters that met criteria were included in the statistical analysis. The same analysis was used to examine differences in the number of entries into the control shelter *versus* the shelter emanating the experimental odorant.

We performed a different statistical analysis for the sex specificity treatments. In this analysis, we subtracted the amount of time spent within the control shelter from the amount of time spent within the experimental shelter. This yielded a single sheltering value for each *trial* (instead of one value for each *shelter*). We then used a Mann-Whitney U test to identify any statistically significant differences between male and female lobsters in the computed values for male urine trials *versus* female urine trials. If there is any sex specificity to the signal or response, then we would expect to see different patterns of sheltering behavior by male and female lobsters in response to male and female urine signals.

Results

Response to seawater

Spiny lobsters generally spent most of their time exploring the flume when tested with seawater emanating from both shelters. All of the lobsters tested explored both sides of the flume, and 8 out of the 10 lobsters tested walked upstream and explored the area around the shelters during the trial (Table 3-1). The two lobsters that did not walk upstream (and

were subsequently not included in the data set) spent most of the trial walking back and forth across the flume near the back tailgate, which formed the downstream border of the working section. These results indicate that the typical behavior of healthy lobsters is to explore the working section after being placed in the flume, thus affirming our criteria for inclusion in the data set. Although a majority of the lobsters explored the shelters at some point during the trial, only half of these lobsters actually entered the shelters (data not shown). Overall the animals did not show a significant preference for either of the two shelters. There were no significant differences in either the number of entries into each shelter (Fig. 3-2B) or in the amount of time lobsters spent inside or around each shelter (Fig. 3-2A).

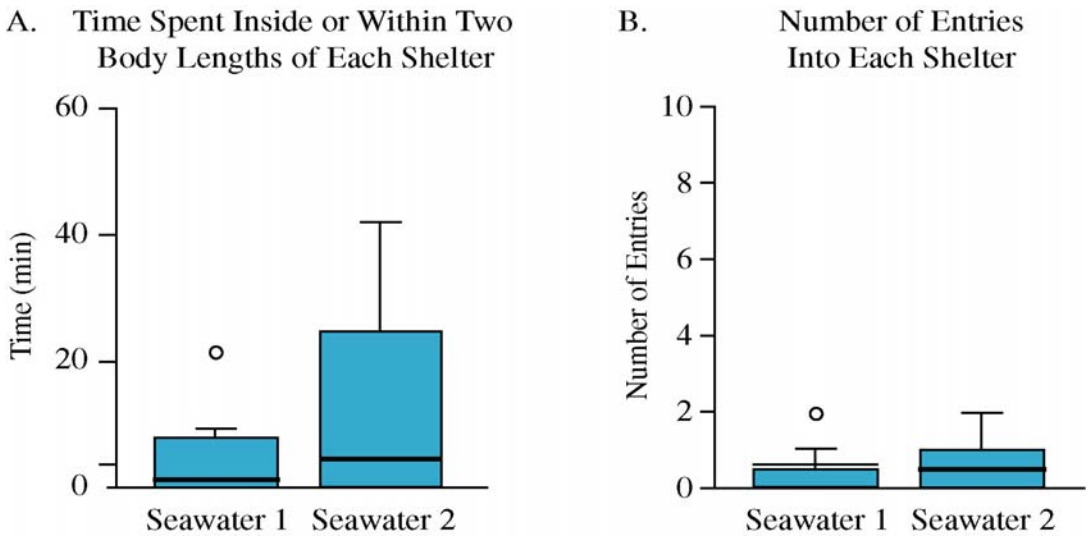

Figure 3-2. Sheltering behavior of spiny lobsters in response to artificial seawater. Box plots represent median (black line) and interquartile range (box length) for time spent inside or within two body lengths of each shelter (A) and number of entries into each shelter (B). Outliers (cases between 1.5 and 3 box lengths away from the upper or lower edge of the box are indicated by open circles (o). All data, including outliers, were included in the statistical analysis. Sample sizes are n = 8 for both A and B. No statistically significant differences were observed between the responses to either shelter in any of the treatments. Wilcoxon matched pairs test, two tailed, $P > 0.05$.

Response to diluted conspecific urine

In contrast to the behavior of the spiny lobsters tested with seawater only, lobsters that were given the choice between a shelter emanating seawater and a shelter emanating diluted conspecific urine showed a significant overall preference for the shelter emanating urine. The same pattern of behavior was observed regardless of whether the lobsters were tested with the 1:10 (Fig. 3-3A, B) or 1:100 (Fig. 3-3C, D) dilution of conspecific urine. Approximately 86% of the lobsters met the criteria to be included in the data set in both the 1:10 and 1:100 dilution trials (Table 3-1). Lobsters in both sets of trials entered the shelter emanating conspecific urine significantly more often than the control shelter (Fig. 3-3B, D) and spent significantly more time inside or within two body lengths of this shelter than the control shelter (Fig. 3-3A, C). Thus, our 1-h assay meets our goal of being sufficiently long to reveal odor-mediated sheltering preference but also has the experimental advantage of being much shorter than previous laboratory and field assays of sheltering.

Response to male and female urine signals

In the previous section, both male and female spiny lobsters responded to dilutions of conspecific urine pooled across sexes (Fig. 3-3). We tested separately the response of subadult male and female lobsters to subadult male and female urine signals to confirm that shelter selection in our assay was not a sex specific behavior. There was no statistically significant difference in the shelter preference of female lobsters tested with male *versus* female urine (Fig.3- 4A). There was also no statistically significant difference in the shelter preference of male lobsters tested with male *versus* female urine (Fig. 3-4B). There were also no statistically significant differences between the shelter preference of male and female

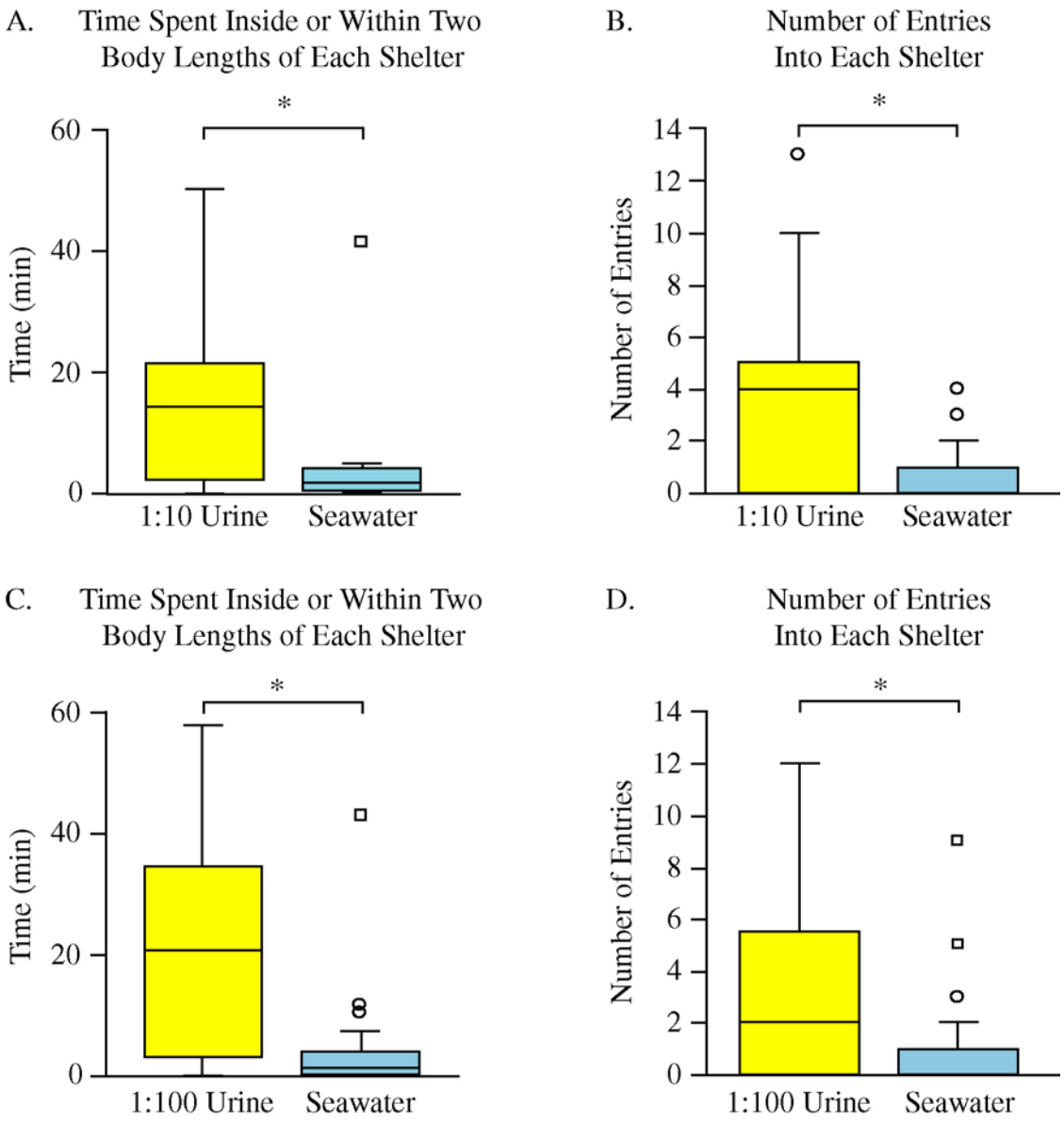

Figure 3-3. Sheltering behavior of spiny lobsters in response to 1:10 (A and B) and 1:100 (C and D) dilutions of conspecific urine in artificial seawater. Box plots show median and interquartile range for time spent inside or within two body lengths of each shelter (A and C) and number of entries into each shelter (B and D). Outliers are indicated by open circles (○) and extremes by open squares (□); both were included in the statistical analysis. Sample sizes are n = 13 for 1:10 dilution of urine in seawater, and N= 31 for 1:100 dilution of urine in seawater. Statistically significant differences are indicated by "*"; Wilcoxon matched pairs test, two-tailed, $P < 0.01$.

lobsters tested with male urine, nor were there any statistically significant differences in the shelter preference of male and female lobsters tested with female urine (data not shown).

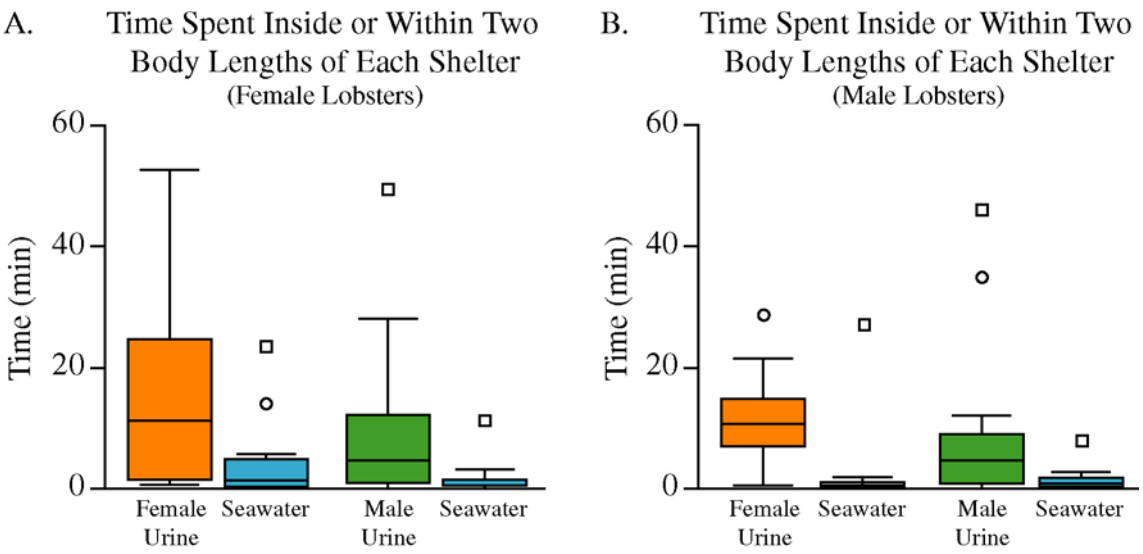

Figure 3-4. Sheltering behavior of female (A) and male (B) spiny lobsters in response to female and male conspecific urine. Box plots show median and interquartile range for time spent inside or within two body lengths of each shelter. Outliers are indicated by open circles (○) and extremes by open squares (□); both were included in the statistical analysis. Sample sizes are n = 13 female lobsters tested with female urine, n = 9 female lobsters tested with male urine, n = 10 male lobsters tested with female urine, and n = 11 male lobsters tested with male urine. There were no statistically significant differences in the response of female lobsters to shelters emanating male urine versus seawater or female urine versus seawater. There were also no statistically significant differences in the response of male lobsters to shelters emanating male urine versus seawater or female urine versus seawater. Mann-Whitney U test, $P > 0.05$.

Response to shrimp odor

All of the spiny lobsters tested with shrimp extract *versus* seawater met criteria to be included in the data set. Lobsters did not show a statistically significant preference for the shelter emanating shrimp odor over the control shelter. The number of entries into each shelter was similar (Fig. 3-5B), as was the amount of time spent inside or around each shelter (Fig. 3-5A).

Response to whole shrimp

All of the spiny lobsters tested with a whole piece of shrimp met criteria for inclusion in the data set (Table 3-1). Although lobsters tended to show more interest in the shelter containing the piece of shrimp *versus* the control shelter, this difference was not statistically significant. A few lobsters that located the shrimp stayed within the shelter after they had consumed this food. Most animals only entered the shelter to obtain the shrimp, which they often grabbed or partially consumed before exiting quickly to resume exploration. There were no statistically significant differences between the behavioral responses to the control shelter and the shelter containing the shrimp piece (Fig. 3-5C, D).

Response to live octopus odor

All of the spiny lobsters tested with live octopus odor *versus* seawater met the criteria for inclusion in the data set (Table 3-1). Although lobsters tended to show more interest in the control shelter than the shelter emanating octopus odor, this difference was not statistically significant. There were no statistically significant differences in either the time spent in and around each shelter (Fig. 3-6A) or in the number of entries into each shelter.

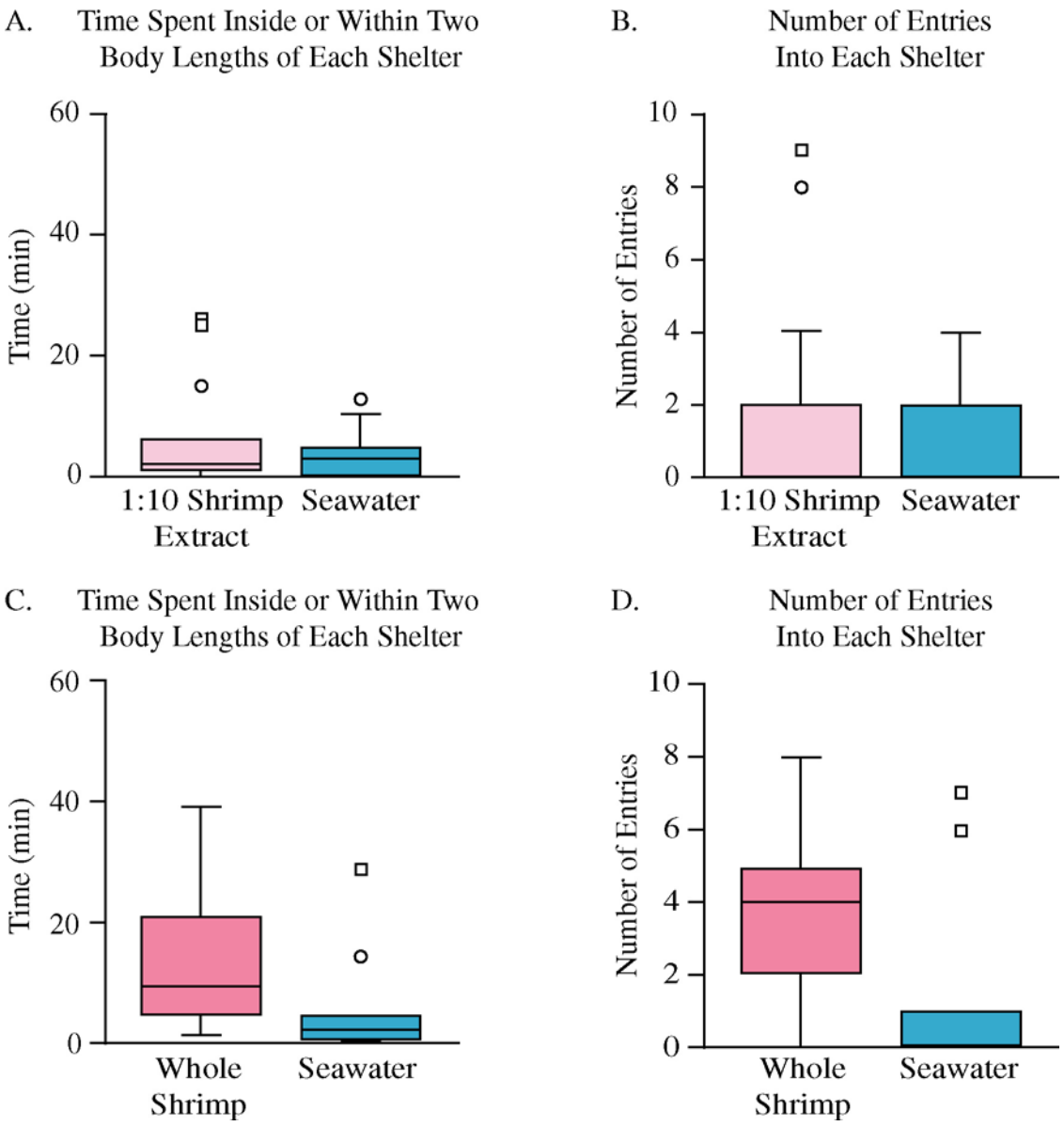

Figure 3-5. Sheltering behavior of spiny lobsters in response to 1:10 shrimp extract (A and B) and whole shrimp (C and D). Box plots show median and interquartile range for time spent inside or within two body lengths of each shelter (A and C) and number of entries into each shelter (B and D). Outliers are indicated by open circles (○) and extremes by open squares (□); both were included in the statistical analysis. Sample sizes are N= 14 for 1:10 shrimp extract, and N= 10 for whole shrimp. No statistically significant differences were found between the responses to either shelter in any of the treatments. Wilcoxon matched pairs test, two-tailed, P> 0.05.

(Fig. 3-6B). Rather than spending more time in and around the control shelter, lobsters tended to avoid octopus odor by spending time at the downstream end of the working section away from both shelters.

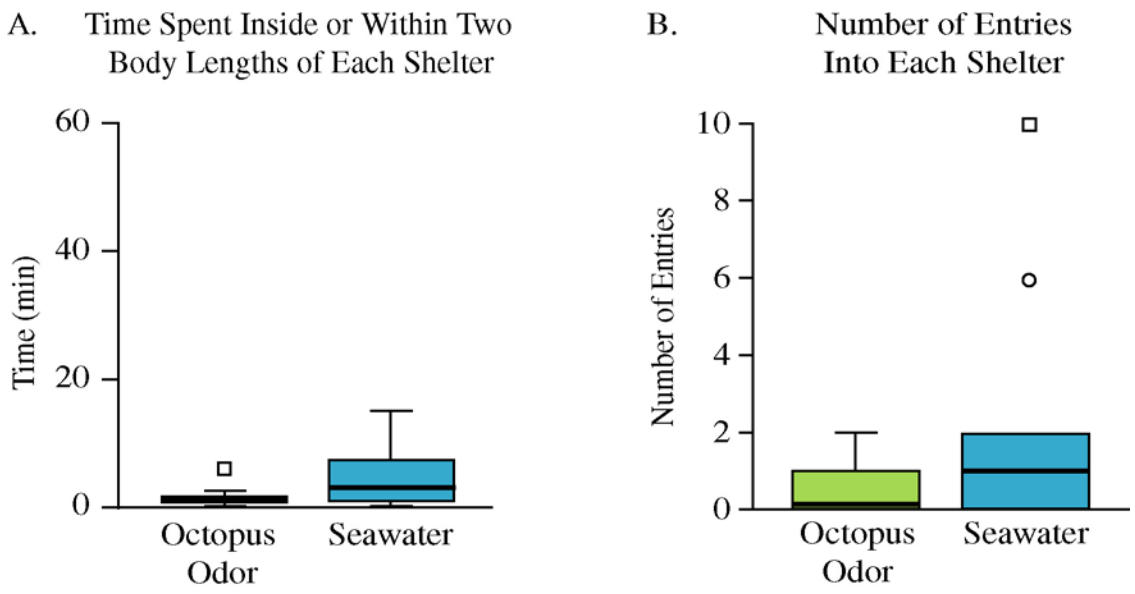

Figure 3-6. Sheltering behavior of spiny lobsters in response to octopus odor. Box plots show median and interquartile range for time spent inside or within two body lengths of each shelter (A) and number of entries into each shelter (B). Outliers are indicated by open circles (○) and extremes by open squares (□); both were included in the statistical analysis. Sample sizes are N= 9 for both A and B. No statistically significant differences were observed between the responses to either shelter in any of the treatments. Wilcoxon matched pairs test, two-tailed, $P > 0.05$.

Discussion

The aim of this study was to examine the source and specificity of the chemical signals mediating gregarious sheltering in Caribbean spiny lobsters. We developed a relatively rapid but naturalistic bioassay and used it to show statistically significant preferences for shelters emanating conspecific urine signals, regardless of the sex of the urine donor or the sex of the responder. Spiny lobsters did not shelter preferentially with food or predator odors. These results demonstrate that dilute urine is sufficient to mediate rapid shelter selection, and strongly suggest that urine is at least one source of the aggregation signal in this species.

Urine is a source of the sheltering cue

When presented with two shelters, both of which emanated seawater, spiny lobsters were not strongly motivated to shelter (*i.e.*, they did not enter or spend much time inside the shelters) and they did not display a clear preference for either refuge (*i.e.*, they spent similar amounts of time inside and around both shelters) (Fig. 3-2). In contrast, lobsters displayed completely different behaviors when presented with one shelter emanating seawater and another shelter emanating conspecific urine. Lobsters spent significantly more time inside or around the shelter emanating conspecific urine than the control shelter, and they also entered this shelter significantly more often when compared to the control shelter (Fig. 3-3).

Even very low concentrations of conspecific urine were sufficient to mediate shelter selection. Both 1:10 and 1:100 dilutions of conspecific urine elicited the same pattern of sheltering behavior. Although it is impossible to determine when the animals detected the urine stimulus, they almost certainly detected it at even lower concentrations than the initial

1:10 or 1:100 dilution. In roughly equivalent flow situations, peak odor concentrations are commonly 10% or less than the source concentration within a few 10s of cm from the source, and are well below 1% of the initial concentration by 1 m downstream (Webster and Weissburg, 2001). Thus, the release of even a small volume of urine by sheltering conspecifics is sufficient to attract lobsters to the den.

The sheltering cue and response are not sex specific

There does not appear to be any sex specificity to the urine signal in the context of shelter selection in the subadult or young adult spiny lobsters tested in this bioassay. Although some of the lobsters tested in the study were potentially of reproductive age, neither the sex nor reproductive state of either the lobster producing or responding to the urine appeared to have any effect on the behavior. Previous research has shown that lobsters as small as 15 mm carapace length are capable of producing and responding to conspecific aggregation signals, even though animals in this size class are clearly not reproductive (Ratchford and Eggleston, 1998). In our assay, lobsters of both sexes and of various sizes preferred shelters emanating either male or female conspecific urine over control shelters (Fig. 3-4). This finding mirrors the results of other laboratory studies (Zimmer-Faust *et al.*, 1985) and observations in the field that find both male and female lobsters of various sizes aggregated in a single den (Herrnkind *et al.*, 1975; Herrnkind *et al.*, 2001).

Specificity of the sheltering cue

In our bioassay, conspecific urine was the only cue that elicited a statistically significant preference for the shelter releasing it; odors from food (dead shrimp) and

predators (live octopus) did not. Spiny lobsters showed no clear preference for either shelter when shrimp extract was the test stimulus in spite of the fact that this odor is a potent feeding stimulus for lobsters (Fig. 3-5). Even the presence of an obtainable food item – a piece of shrimp – on the floor of the shelter was not sufficient to induce significant sheltering behavior by the lobsters (Fig. 3-5). Most animals only entered the shelter to obtain the shrimp. Lobsters tended to prefer the control shelter over the shelter emanating the odor of a competitor and potential predator of spiny lobsters – live *Octopus briareus,* although the difference was not statistically significant (Fig. 3-6). This trend to avoid octopus odor in our assay was expected since previous research showed that lobsters avoid shelters scented with octopus odor (Berger and Butler, 2001).

The results of these experiments show that a strong preference for a particular shelter does not occur simply in response to any novel odorant released into the flow. The preference for one shelter over another was much more specific in our assay. Statistically significant differences in shelter preference were observed only with conspecific urine. Thus, urine appears to be at least one source of the aggregation signal in this species. An allopatric spiny lobster, *Panulirus interruptus,* and a sympatric spiny lobster, *Panulirus guttatus,* also use chemical aggregation cues (Zimmer-Faust *et al.*, 1985; Zimmer-Faust and Spanier, 1987; Briones-Fourzan and Lozano-Alvarez, 2005). Testing the species specificity of these signals and responses would be informative.

Urine as a source of conspecific cues

Facilitation of aggregation and gregarious sheltering is just one example of the importance of urine signals in decapod crustacean social interactions. Decapod crustaceans

use urine signals to mediate a variety of intraspecific interactions. For example, urine-borne signals mediate many aspects of courtship and mating in species including *Homarus americanus* (Bushmann and Atema, 1994; Atema, 1995), *Jasus edwardsii* (Raethke *et al.*, 2004), *Callinectes sapidus* (Gleeson, 1980), *Carcinus maenas* (Bamber and Naylor, 1997; Hardege *et al.*, 2002), *Telmessus cheiragonus* (Kamio *et al.*, 2000, 2002), *Portunus sanguinolentus* (Ryan, 1966; Christofferson, 1978), and others. Urine signals also play an important role in individual recognition and the determination of social status in *H. americanus* (Breithaupt and Atema, 1993; Atema, 1995; Karavanich and Atema, 1998a, b; Breithaupt *et al.*, 1999; Breithaupt and Atema, 2000) and regulate the dynamics of agonistic interactions in crayfish *Astacus leptodactylus* (Breithaupt and Eger, 2002) and *Orconectes rusticus* (Schneider *et al.*, 2001).

Although the results of the current study strongly suggest that urine is one source of the aggregation signal in *P. argus*, it is not necessarily the only source of the aggregation signal in this species. Other crustaceans emit social signals concurrently in urine and other sources (Bamber and Naylor, 1997; Bushmann, 1999). There is evidence in both *Callinectes sapidus* and *Carcinus maenas* that sex pheromones are released from other sources in addition to urine (Bamber and Naylor, 1997; Bushmann, 1999). It is possible that there are additional sources of the aggregation signal in the spiny lobster. Ratchford (1999) found that *P. argus* is attracted to catheterized conspecifics, but the specific source of the attractant in this case is unknown. Specific non-urine conspecific odors have not yet been examined in *P. argus*.

The behavioral response to chemical signals in urine may also change depending on the particular context in which the urine is presented. In a different behavioral assay,

Ratchford (1999) reported a potential alarm response to conspecific urine by *P. argus*. Potential alarm responses were also noted in *P. argus* when presented with conspecific urine in bioassays in small (80-l) aquaria (Shabani *et al.*, 2006). These differences in behavior are probably attributable to differences in experimental design, and perhaps differences in the quality (*i.e*. presence or concentration of the aggregation signal) of the collected urine. At present, we know virtually nothing about the chemical identity or the release dynamics of the aggregation cue contained within the urine. Although previous research showed that the aggregation signal is released discontinuously (Ratchford and Eggleston, 2000), the cause of this intermittency is unclear. For instance, the signal may always be present in the urine but appear discontinuous because urine release is intermittent. Alternatively, the aggregation signal itself may only be present in the urine intermittently. In addition, the release of other substances into the urine along with the aggregation signal might modify the response of receiving lobsters. In any case, the manner in which the urine is collected and pooled could have a considerable influence on its odor quality, which in turn could affect the behavior of the lobsters. Further research into the chemical identity of the specific substances within the urine that influence shelter preference, aggregations, and alarm responses will help us to understand better how social behaviors are mediated in *P. argus*.

Chapter 4 - The olfactory pathway mediates sheltering behavior of Caribbean spiny lobsters in response to urine signals

Introduction

Chemical signals are used by organisms to drive diverse social behaviors including courtship, mating, aggregation, recognition, agonism, social dominance, and other forms of intraspecific interactions. An important step to understanding how chemical signals influence social behaviors is to describe how such signals are detected and processed by chemosensory systems.

Many organisms, both invertebrate and vertebrate, have multiple, anatomically distinct neuronal pathways for processing chemosensory information. In some organisms, the functions of multiple chemosensory pathways are discrete and can be distinguished by the types of odorants that they process. For example, in many species of lepidopteran insects, the males have two anatomically distinct chemosensory pathways: a main olfactory pathway that detects general host plant odorants, and a secondary pathway specialized for the detection of species specific female sex pheromones (Hansson, 1995; Hildebrand, 1995; Christensen and White, 2000; Hansson and Anton, 2000; Christensen and Hildebrand, 2002). Thus anatomically distinct chemosensory pathways are also functionally distinct in some insect species.

In other organisms, including many vertebrates, the roles of different chemosensory pathways in processing social signals are not always so separate. The noses of many amphibians, reptiles, and mammals also contain multiple anatomically distinct chemosensory

pathways. The largest and most well studied components of the vertebrate chemosensory system are the main olfactory system consisting of the olfactory epithelium and main olfactory bulb, and the vomeronasal system, consisting of the vomeronasal organ and the accessory olfactory bulb (Eisthen, 1997; Christensen and White, 2000; Wyatt, 2003; Baxi *et al.*, 2006; Breer *et al.*, 2006; Spehr *et al.*, 2006b). Additional presumptive chemosensory areas occur on the nasal septum, but the function of these regions is not well understood (Breer *et al.*, 2006; Spehr *et al.*, 2006b; Storan and Key, 2006). Traditionally, the vomeronasal system and the main olfactory system were believed to be functionally distinct, with the vomeronasal system specialized for detection of intraspecific signals or pheromones, and the main olfactory system fulfilling a more general role in processing heterospecific signals and general odorants such as food odors. However, several studies have demonstrated non-traditional roles for each of these pathways, indicating that functional divisions between these odor processing subsystems are not universal. The main olfactory system in some species mediates the response to pheromones (Hudson and Distel, 1986; Dorries *et al.*, 1997; Johnston, 1998; Johnston, 2000; Restrepo *et al.*, 2004; Lin *et al.*, 2005; Baxi *et al.*, 2006; Spehr *et al.*, 2006b), and the vomeronasal system in other species mediates the response to prey odors and heterospecific signals (Halpern *et al.*, 1997; Johnston, 1998; Miller and Gutzke, 1999; Johnston, 2000; Ptacyk and Graves, 2002; Halpern and Martinez-Marcos, 2003; Baxi *et al.*, 2006; Spehr *et al.*, 2006b). Further obscuring functional divisions between the pathways is the finding that in some cases both pathways function together to mediate the response to a particular odorant (Johnston, 1998; Johnston, 2000; Restrepo *et al.*, 2004; Spehr *et al.*, 2006b). The roles of each of the vertebrate chemosensory pathways in odor driven behaviors are thus not always clearly defined nor are functional differences observed

in one organism generalizable across species. Vertebrate chemosensory pathways have both complementary and overlapping roles in different odor mediated behaviors (Johnston, 1998; Johnston, 2000; Restrepo *et al.*, 2004; Spehr *et al.*, 2006b).

Similar to vertebrates and insects, the noses of decapod crustaceans such as the Caribbean spiny lobster (*Panulirus argus*) also contain multiple anatomically distinct neuronal pathways for transmitting peripheral chemosensory input to the brain (Schmidt and Ache, 1992, 1996b; Schachtner *et al.*, 2005). Two of these pathways are on the antennules and are called the aesthetasc / olfactory lobe pathway and the non-aesthetasc / lateral antennular neuropil pathway. The pathways originate in different populations of sensilla located on the antennular flagella (Fig. 4-1A) and project to different neuropils in the brain.

The aesthetasc / olfactory lobe pathway originates in the prominent aesthetasc sensilla that are located in the distal region of the lateral flagella (Laverack, 1964; Cate and Derby, 2001). Aesthetascs are exclusively chemosensitive structures, and each aesthetasc is innervated by the dendrites of approximately 300 olfactory receptor neurons whose cell bodies are located in a cluster beneath the sensillum (Laverack and Ardill, 1965; Grunert and Ache, 1988; Steullet *et al.*, 2000). The axons of these olfactory receptor neurons target the paired olfactory lobes (Schmidt and Ache, 1992, 1996b; Schachtner *et al.*, 2005). The olfactory lobes show the characteristic glomerular organization that typifies the first order olfactory processing centers of many organisms including vertebrates and insects (Hildebrand and Shepherd, 1997; Eisthen, 2002; Ache and Young, 2005).

In addition to the aesthetasc/olfactory lobe pathway, lobsters also possess a non-olfactory antennular pathway – the non-aesthetasc/ lateral antennular neuropil pathway – that regulates many chemically mediated behaviors. This pathway originates in the various types

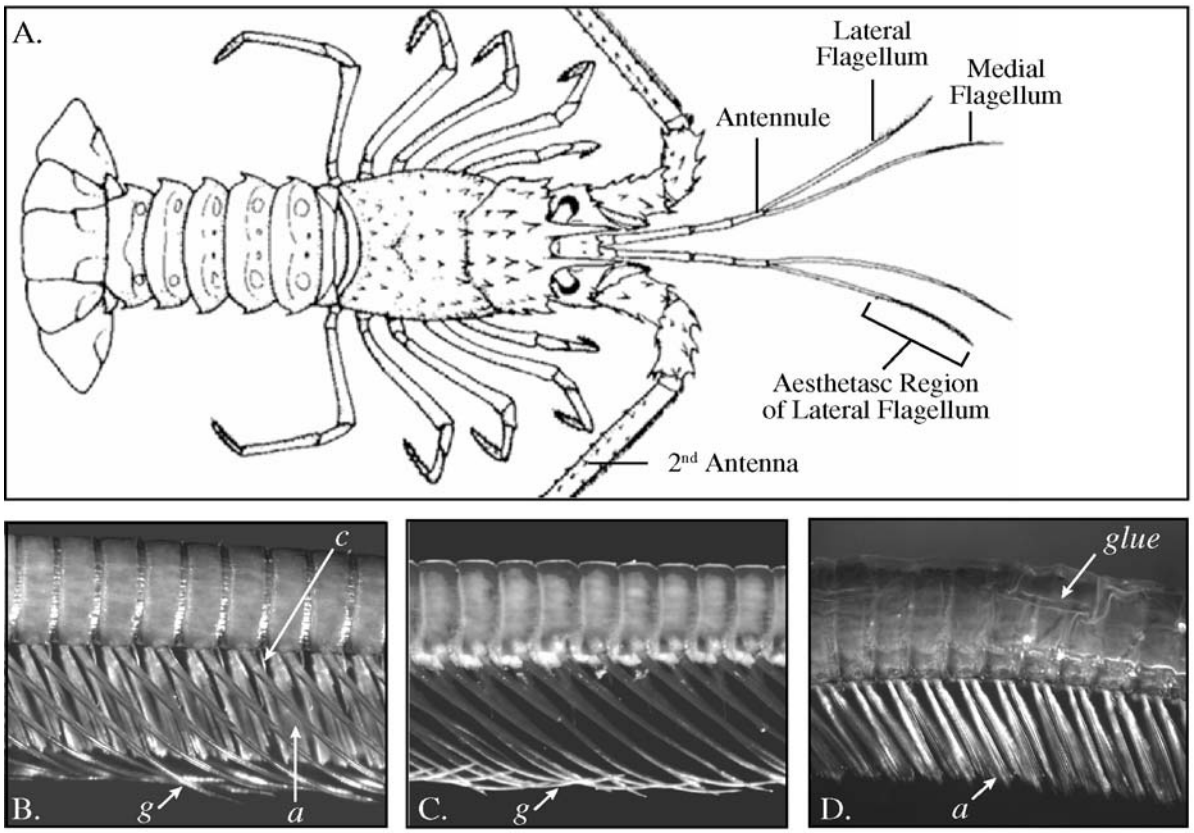

Figure 4-1. Spiny lobster and antennular sensilla. A. Diagram of *P. argus* showing the major components of the chemosensory system. B. Light micrograph of a portion of the aesthetasc tuft region of the lateral flagellum from a control lobster. Aesthetasc (a), guard (g) and companion (c) sensilla are visible. C. Light micrograph of a portion of the aesthetasc tuft region from an aesthetasc-ablated lobster. Guard and companion hairs are intact, but aesthetascs have been removed. D. Light micrograph of a portion of the aesthetasc tuft region of a non-aesthetascs ablated lobster. Aesthetascs (a) are intact, but non-aesthetascs have been removed. The cyanoacrylate glue coating is also visible.

of non-aesthetasc sensilla that are widely distributed on both the lateral and medial antennular flagella (Cate and Derby, 2001, 2002a). Nine morphologically distinct types of non-aesthetascs exist on the antennular flagella of *P. argus*, and most if not all of these appear to be bimodal, innervated by distinct populations of presumptive chemosensory neurons and mechanosensory neurons (Cate and Derby, 2001; Schmidt and Derby, 2005). In contrast to the densely innervated aesthetasc, each non-aesthetasc is innervated by only 2-3 mechanosensory neurons and fewer than 20 chemosensory neurons (Cate and Derby, 2001, 2002a). The axons of non-aesthetasc sensory neurons on the antennular flagella target the paired lateral antennular neuropils (Schmidt *et al.*, 1992; Schmidt and Ache, 1996a). The lateral antennular neuropils lack glomeruli and instead have a stratified organization (Schmidt *et al.*, 1992; Schmidt and Ache, 1996a). In addition to receiving input from antennular chemo- and mechanosensory neurons, the lateral antennular neuropils also contain the major arborizations of antennular motoneurons (Schmidt and Ache, 1993). Consequently, these neuropils function as antennular sensory-motor integration centers.

Although these two crustacean antennular chemosensory pathways are anatomically distinct, functional differences between them have not been easy to demonstrate. Several studies showed overlapping roles for the pathways in food odor mediated behaviors (Derby *et al.*, 2001; Steullet *et al.*, 2001; Steullet *et al.*, 2002; Horner *et al.*, 2004). However, unique roles for each pathway have emerged in different behavioral contexts. The non-aesthetasc pathway in spiny lobsters mediates antennular grooming behavior in response to stimulation with glutamate (Schmidt and Derby, 2005). In other crustacean species, the aesthetasc pathway mediates social interactions including courtship displays (Gleeson, 1982, 1991) and individual recognition (Johnson and Atema, 2005). The latter studies suggest that the

aesthetasc pathway plays a prominent role in intraspecific communication, at least in the context of mating and recognition, but the importance of this pathway in mediating the response to other types of social signals has not been investigated.

Caribbean spiny lobsters are highly social animals that shelter gregariously during the day (Herrnkind *et al.*, 1975; Atema and Cobb, 1980; Kanciruk, 1980; Childress and Herrnkind, 1997). Urine is known to mediate shelter selection (Horner *et al.*, 2006); however, the chemosensory pathways involved in detecting this urine signal are unknown. The goal of this study is to evaluate the role of the aesthetasc and non-aesthetasc chemosensory pathways in urine-mediated shelter selection in order to understand better how social signals are processed in decapod crustaceans.

Methods

Animals

Caribbean spiny lobsters, *Panulirus argus* (Latreille, 1804), with carapace length of 59.0 ± 1.2 mm (mean ± S.E.M., n= 53) were collected in the Florida Keys, shipped to Georgia State University, and held in 800-L aquaria containing aerated, recirculated, filtered artificial seawater (Instant Ocean®, Aquarium Systems, Mentor, OH, USA). Animals were maintained on a 12h: 12h light: dark cycle and fed shrimp or squid 3 times a week. Intermolt lobsters were randomly selected for use in the experimental trials, and at least two days before being tested in the behavioral assay, experimental animals were transported to holding aquaria (0.90 m long x 0.58 m wide x 0.67 m tall) at Georgia Institute of Technology, where they were maintained throughout the course of the experiments.

Ablations

We conducted two sets of experiments to examine separately the importance of the aesthetasc or non-aesthetasc chemosensory pathway for mediating shelter selection in spiny lobsters. Logistical issues prevented us from conducting the two experiments simultaneously, and thus the two experiments were designed from the outset to be conducted and analyzed independently. The aesthetasc ablation experiment was conducted between April and October 2004, and the non-aesthetasc ablation study was conducted between March and September 2005. The two experiments used different batches of lobsters, different handling procedures, and were conducted under slightly different conditions at the test facility.

The ablation procedures described below have been used previously, and their effectiveness has been confirmed through both morphological and electrophysiological investigations (Steullet *et al.*, 2001; Steullet *et al.*, 2002). All ablations were performed on non-anesthetized spiny lobsters immobilized on a plastic restraining device within a shallow container of artificial seawater. Surgical ablations were performed several days in advance of the start of experimental trials, and chemical ablations (using deionized water) were performed within 12 hr of the start of each trial.

Aesthetasc Ablation

All aesthetasc sensilla on both lateral flagella were surgically removed at the base using a hand-tooled narrow blade of 0.2-mm width (Fig 4-1B; Steullet *et al.*, 2001). Removal of aesthetascs in this manner eliminates the outer dendrites of the olfactory sensory neurons, which results first in unresponsiveness to odors, and ultimately in the death and degeneration of the sensory neurons (Harrison *et al.*, 2004). Ablated antennules were excised at the

conclusion of each series of experimental trials, and the efficacy of ablation was evaluated by using light microscopy to count the number of sensilla that remained intact on each antennule. This analysis confirmed that shaving was a highly reliable method for removing sensilla. Shaving removed greater than 99.9% of aesthetascs on the antennules. Some non-aesthetasc sensilla, particularly asymmetric sensilla, guard sensilla, and companion sensilla, were unintentionally removed during the aesthetasc shaving procedure. However, at least 30% of the asymmetric sensilla, 99% of guard sensilla, and 97% of companion sensilla were still present on the antennules after the aesthetasc ablation procedure.

We also tested a set of control (unablated) lobsters to confirm that any behavioral deficits observed with the aesthetasc ablated lobsters were the result of the ablations and not of other confounding factors. Control lobsters for the aesthetasc ablated group were immobilized once in the plastic restraining device in the same manner and for the same duration as the aesthetasc ablated animals, but no sensilla were removed or inactivated. We showed previously that intact (unablated) spiny lobsters associate preferentially with shelters emanating conspecific urine (Horner *et al.*, 2006). Thus, we expected the control lobsters in this study to behave similarly.

Non-Aesthetasc Chemoreceptor Ablation

All visible non-aesthetasc sensilla located on annuli in the aesthetasc tuft region of both lateral flagella were surgically removed with a sharp scalpel blade. This shaved region was then coated with a thin layer of cyanoacrylate glue (Super Glue Corp., Rancho Cucamonga, CA) to prevent stimulus access to any remaining non-aesthetasc sensilla (Fig. 4-1C). Care was taken to insure that the aesthetascs were not affected by the gluing procedure.

Covering the antennules with cyanoacrylate glue effectively prevents stimulation of non-aesthetasc chemosensory neurons, and also prevents stimulation of mechanosensory neurons that are responsive to hydrodynamic and some tactile stimuli (Derby and Atema, 1982). The unglued portions of the antennules (medial flagella and proximal regions of the lateral flagella) were then immersed in deionized water for 15 min to ablate non-aesthetasc chemosensory neurons in these regions. The glued portion of the antennule was maintained in artificial seawater during the deionized water ablation to prevent damage to the aesthetasc sensilla. Deionized water functionally inactivates the chemosensory neurons of marine crustaceans by disrupting the osmotic balance of the outer dendrites (Derby and Atema, 1982; Gleeson *et al.*, 1997). This ablation is temporary and reversible, lasting only about a day before the neurons once again respond to chemical stimuli (Derby and Atema, 1982; Steullet *et al.*, 2001). Thus, distilled water ablations were performed within 12 hr of each experimental trial.

Although shaving and gluing inactivated mechanosensory neurons within the aesthetasc region, at least some of the mechanosensors along the medial flagellum and proximal portion of the lateral flagellum likely remained intact and functional. Some mechanosensory neurons with dendrites projecting up the length of the sensillum may have been exposed to, and inactivated by, the deionized water environment (Derby and Atema, 1982; Garm *et al.*, 2003), but mechanosensory neurons lacking this morphology were probably not affected by the treatment. At the conclusion of the experimental trials, the antennules were excised to examine the efficacy of the non-aesthetasc ablation in the aesthetasc tuft region and to evaluate the condition of the aesthetascs. Overall the aesthetascs remained in excellent condition, and very few non-aesthetasc sensilla remained in this region.

On average, shaving successfully removed all guard sensilla, approximately 97% of asymmetric sensilla, and 96% of companion sensilla from the aesthetasc tuft region.

Control animals for the non-aesthetasc ablated group were immobilized twice, but no sensilla were removed or inactivated. They were immobilized initially to control for handling during the surgical removal of non-aesthetascs in the aesthetasc tuft region. They were then immobilized a second time with their antennules placed in tubes containing artificial seawater to control for handling during the deionized water ablation. Based on previous studies, we also expected these control animals to show a significant preference for the shelter emanating conspecific urine (Horner *et al.*, 2006).

Odor Stimuli

Control stimulus consisted of artificial seawater (Instant Ocean®) taken directly from the test facility before the start of the trials. Experimental odor stimulus consisted of a pooled sample of conspecific urine collected from eight catheterized lobsters (4 males and 4 females). Details of the catheterization and urine collection procedure are described elsewhere (Horner *et al.*, 2006). The urine stimulus was diluted 1:100 in artificial seawater taken directly from the flume at the start of the trials.

Bioassay

The shelter choice assay was conducted in a 5000 liter seawater flume located at Georgia Institute of Technology. The flume measures 12 m long x 0.75 m wide x 0.35 m high with a downstream working section measuring 2 m long x 0.75 m wide x 0.35 m high. All trials were conducted with a background flow rate of approximately 5 cm/sec. Details on

the flow dynamics of this flume and its use in other behavioral experiments are described elsewhere (Webster and Weissburg, 2001; Keller *et al.*, 2003; Weissburg *et al.*, 2003; Horner *et al.*, 2004; Horner *et al.*, 2006). The flume water was filtered through biological, particulate, activated carbon, and UV filters between trial days.

Two concrete blocks served as shelters for the lobsters. The block dimensions were 39.5 cm tall x 19.5 cm wide x 19 cm deep, and the opening size was 14 cm tall x 13 cm wide x 19 cm deep. The blocks were placed at the upstream end of the working section 5 cm from the wall of the flume and 26 cm apart. The area between each block and the sidewall of the flume was filled with a small section of plastic grating (1 cm x 1 cm) to prevent the lobsters from sheltering in this area. A larger piece of plastic grating spanning the width of the flume was placed behind the blocks to prevent the lobsters from escaping the working section. Odor stimuli were introduced into the flow by a dual channel peristaltic pump (Masterflex, Cole Parmer Instrument Company, Vernon Hills, IL) that pumped the diluted conspecific urine through one shelter while simultaneously pumping seawater through the other shelter as a control. We randomly chose which shelter would release which odorant, and switched the site of odorant release between trials. Control and experimental stimuli were pumped into the flume at approximately 15 ml/hr. Thus with a 1:100 dilution, only about 150 µl of urine was released into the flow over the course of 1 hr. Previous experiments have shown this rate and dilution of conspecific urine to be sufficient to elicit sheltering behavior (Horner *et al.*, 2006).

All trials were conducted with the room lights on and with a 60W light mounted above the downstream end of the flume to provide constant illumination in the working section. Lobsters typically search for shelter in the early morning in their natural

environment; however, previous research has shown that spiny lobsters will shelter in the presence of aggregation signal regardless of where they are in their circadian cycle (Ratchford and Eggleston, 2000; Horner *et al*., 2006). Therefore we did not make special efforts to run the trials at specific points in the lobster's natural light-dark cycle.

Trials began when a lobster was placed in a Plexiglas and plastic grate cage (30.5 cm long x 21 cm wide x 20.5 cm high) 1.5 m downstream from the face of the concrete blocks for a 5-min acclimation period. Odor stimuli were pumped into the flume during this acclimation period. After 5 min, the cage was lifted and completely removed from the flume, thus allowing the lobster to move freely around the working section. Each trial lasted for 1 hr, and the movements of the lobsters were recorded by a video camera mounted above the flume.

Video Analysis

A lobster had to explore both sides of the flume and walk more than halfway upstream to be included in the data set. These criteria were established to ensure that lobsters had the opportunity to sample odors eluting from both shelters, and that they were healthy and motivated to explore the flume and shelters. In addition, a majority of the control animals (more than 50% on each trial day) had to behave as previously described for an entire day of trials (including both control and ablation trials) to be included in the data set. We showed previously that healthy, intact animals shelter preferentially with the conspecific urine stimulus (Horner *et al.*, 2006).Thus we established the second criterion to ensure that any deficits in the behavior of ablated animals resulted from our ablations and not from subtle, daily differences in flume conditions. In all cases, a majority of animals met the criteria for

inclusion in the data set. In the aesthetasc ablation experiment, 75 % of control lobsters and 80 % of ablated lobsters met the criteria for inclusion in the data set. In the non-aesthetasc ablation experiment, 64 % of control lobsters and 67 % of ablated lobsters met the criteria for inclusion in the data set.

The sheltering behavior of the lobsters was quantified by recording the number of times each shelter was entered and by measuring the total amount of time spent inside (all body parts except antennae completely within the shelter) or within 30 cm (approximately the equivalent of 2 body lengths of the smallest lobsters used in the trials) of each shelter. Sheltering time was defined to include the time spent within two body lengths of each shelter because animals sometimes were outside of the shelter but behaved as if they were sheltered inside the block. For instance, the lobster approached the shelter head on in several trials, turned around, and then backed into the corner formed by the plastic grate and sidewall of the flume immediately adjacent to the shelter. In this position, the lobster's abdomen was partially protected on two sides, while its antennules were still able to sample water passing though the shelter. Although these animals were not inside the shelter, they still derived protection from the block. Each trial thus produced two values for total sheltering time: one for time spent in or around the control shelter, and the second for time spent in or around the experimental odor shelter. Each trial also produced two values describing the number of entries into each shelter.

Statistical Analysis

In both the aesthetasc and non-aesthetasc ablation experiments, we used a Wilcoxon matched pairs test to determine if there were statistically significant differences in either the

amount of time that the ablated animals spent inside and within two body lengths of the shelter emanating urine versus the control shelter, or in the number of times ablated animals entered each shelter. We used the same analysis to examine potential differences in the responses of the control groups to the shelter emanating urine versus the control shelter. Because previous experiments demonstrated that lobsters with intact antennules significantly prefer shelters emanating conspecific urine (Horner *et al.*, 2006), we analyzed the control data with a one-tailed Wilcoxon matched-pairs test. We analyzed data from ablated animals using a two-tailed Wilcoxon matched-pairs test since we did not have any precedent for the behavior of these groups.

A final analysis determined whether the ablations had a general effect on a lobster's overall tendency to seek shelter, as opposed to a specific effect on shelter preference. We calculated the total amount of time spent sheltering and the total number of entries into both shelters. We then used a Mann-Whitney U test to determine if there were statistically significant differences in overall sheltering time or entry number between control and ablated lobsters.

Results

Aesthetasc Ablation Experiment

Control lobsters generally spent the first part of the trial exploring the working section of the flume and the shelters. After this initial exploratory period (which varied in duration between individual lobsters), most animals began to show more interest in the shelter emanating conspecific urine over the control shelter. Overall, control lobsters showed a statistically significant preference for the shelter emanating conspecific urine over the

control shelter. These animals spent significantly more time in or around this shelter than the control shelter (Fig. 4-2A, Wilcoxon matched pairs test, one tailed, N=8, Z=2.240, p=0.013). Control lobsters entered the shelter emanating urine significantly more often than the shelter emanating seawater (Fig. 4-2B, Wilcoxon matched pairs test, one tailed, N=8, Z=2.201, p=0.014).

Aesthetasc ablated lobsters were generally quite active after being released from the cage, and spent the first part of the trial exploring the flume and shelters. Their behavior was indistinguishable from the behavior of the control lobsters in this respect. However, unlike the control lobsters, aesthetasc ablated lobsters as a group did not show a statistically significant preference for either shelter. Approximately half of the animals showed more interest in the control shelter than the shelter emanating urine whereas the other half showed the opposite pattern of behavior (data not shown). Overall, aesthetasc ablated animals spent approximately equal amounts of time in or within two body lengths of both shelters (Fig. 4-2C, Wilcoxon matched pairs test, two-tailed, N=10, Z=1.070, p=0.285) and entered both shelters with similar frequency (Fig. 4-2D, Wilcoxon matched pairs test, two-tailed, N=10, Z=0.135, p=0.893).

No statistically significant differences emerged when the overall sheltering behavior of control and aesthetasc ablated animals was compared. There were no significant differences in either the total time spent sheltering (Fig. 4-3A, Mann-Whitney U, N=18, U=39, p=0.929) or in the total number of entries into the shelters (Fig. 4-3B, Mann-Whitney U, N=18, U=35, p=0.650) between control and aesthetasc ablated animals.

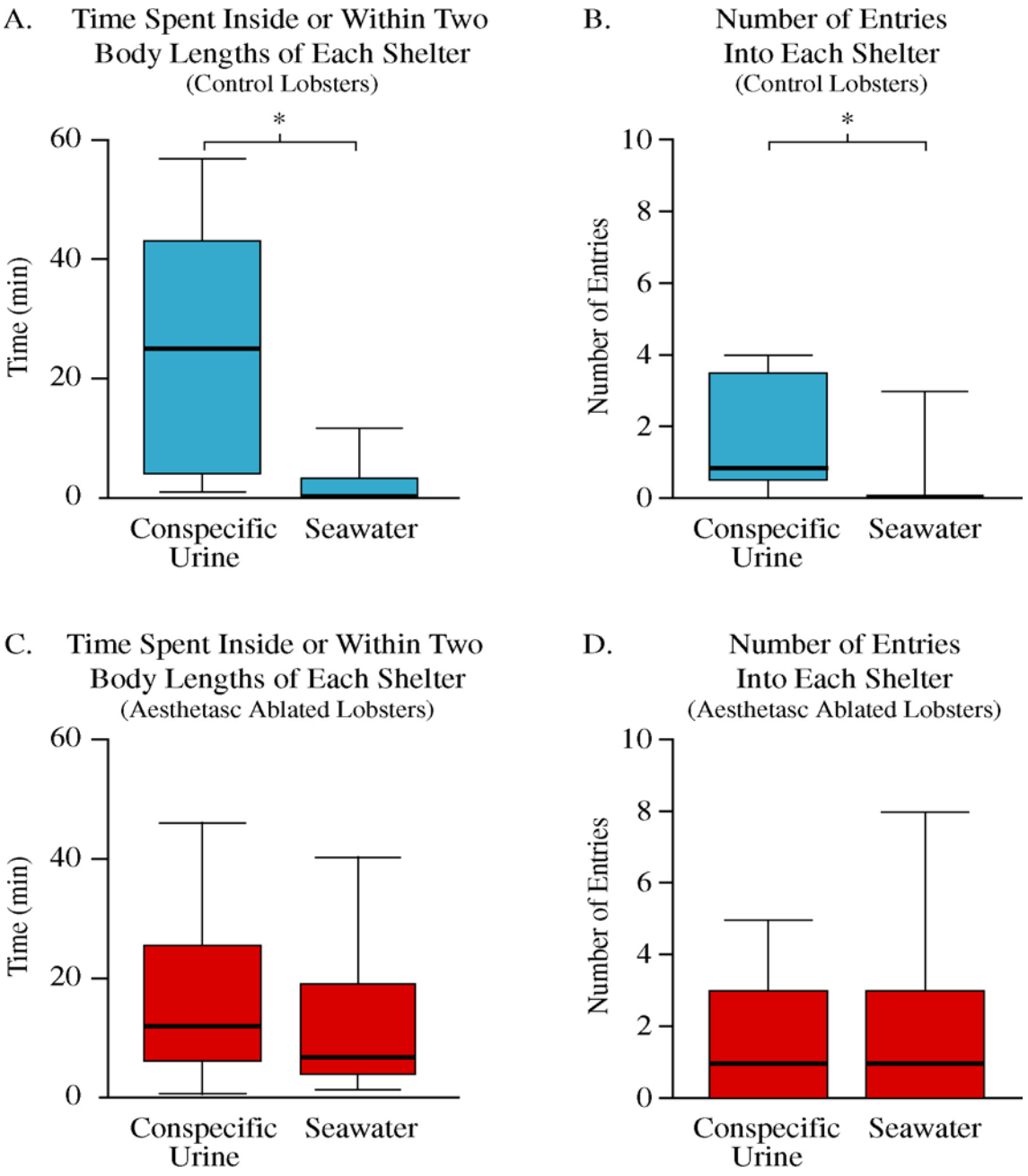

Figure 4-2. Sheltering behavior of control (A and B, N= 8) and aesthetasc ablated lobsters (C and D, N= 10) in response to dilute conspecific urine. Box plots show median (solid black line), interquartile range (box length) and minimum and maximum values (error bars) for time spent inside or within two body lengths of each shelter (A and C) or number of entries into each shelter (B and D). Statistically significant results are indicated by "*". (Wilcoxon matched-pairs test, $P<0.05$.)

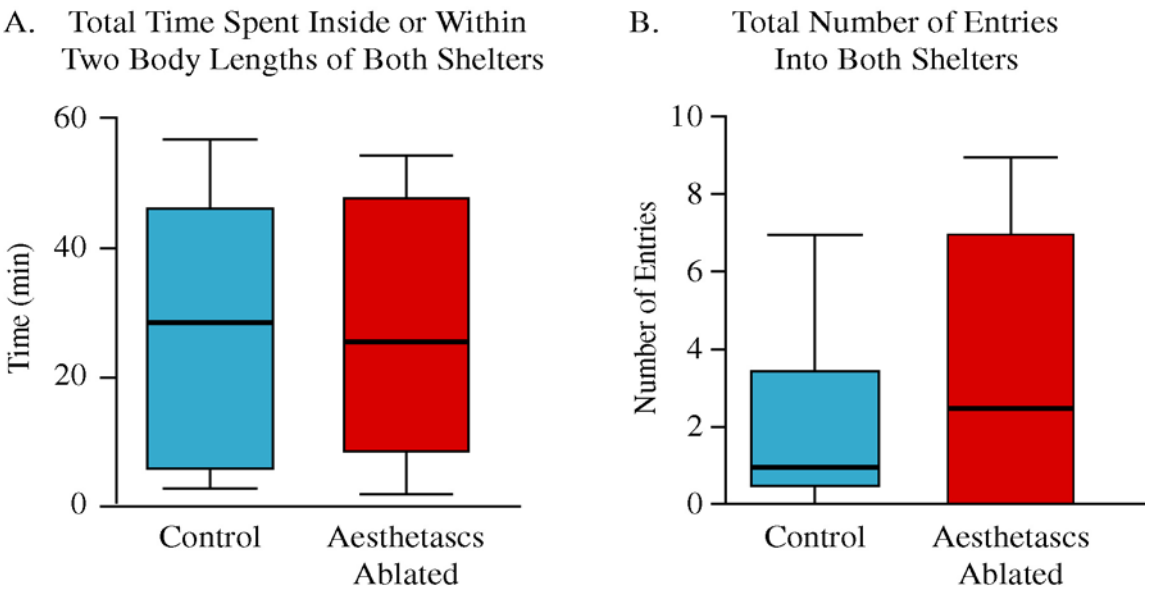

Figure 4-3. Comparison of overall sheltering behavior between control (N= 8) and aesthetasc ablated (N= 10) lobsters. Box plots show median (solid black line), interquartile range (box length), and maximum and minimum values (error bars) for total time spent inside or within two body lengths of both shelters (A) and total number of entries into both shelters (B). No statistically significant differences were observed. Mann-Whitney U Test, P> 0.05.

Non-aesthetasc Ablation Experiment

Control lobsters in this experiment behaved similarly to control lobsters in the aesthetasc ablation experiment. They spent significantly more time in and around the shelter emanating conspecific urine than the control shelter (Fig. 4-4A, Wilcoxon matched pairs test, one-tailed, N=9, Z=1.82, p=0.034) and they also entered the urine-emanating shelter more frequently than the control shelter (Fig. 4-4B, Wilcoxon matched pairs test, one tailed, N=9, Z=2.03, p=0.021).

Non-aesthetasc ablated lobsters behaved similarly to control lobsters in some respects. They spent significantly more time in and around the shelter emanating conspecific urine than the control shelter (Fig. 4-4C, Wilcoxon matched pairs test, two-tailed, N=8,

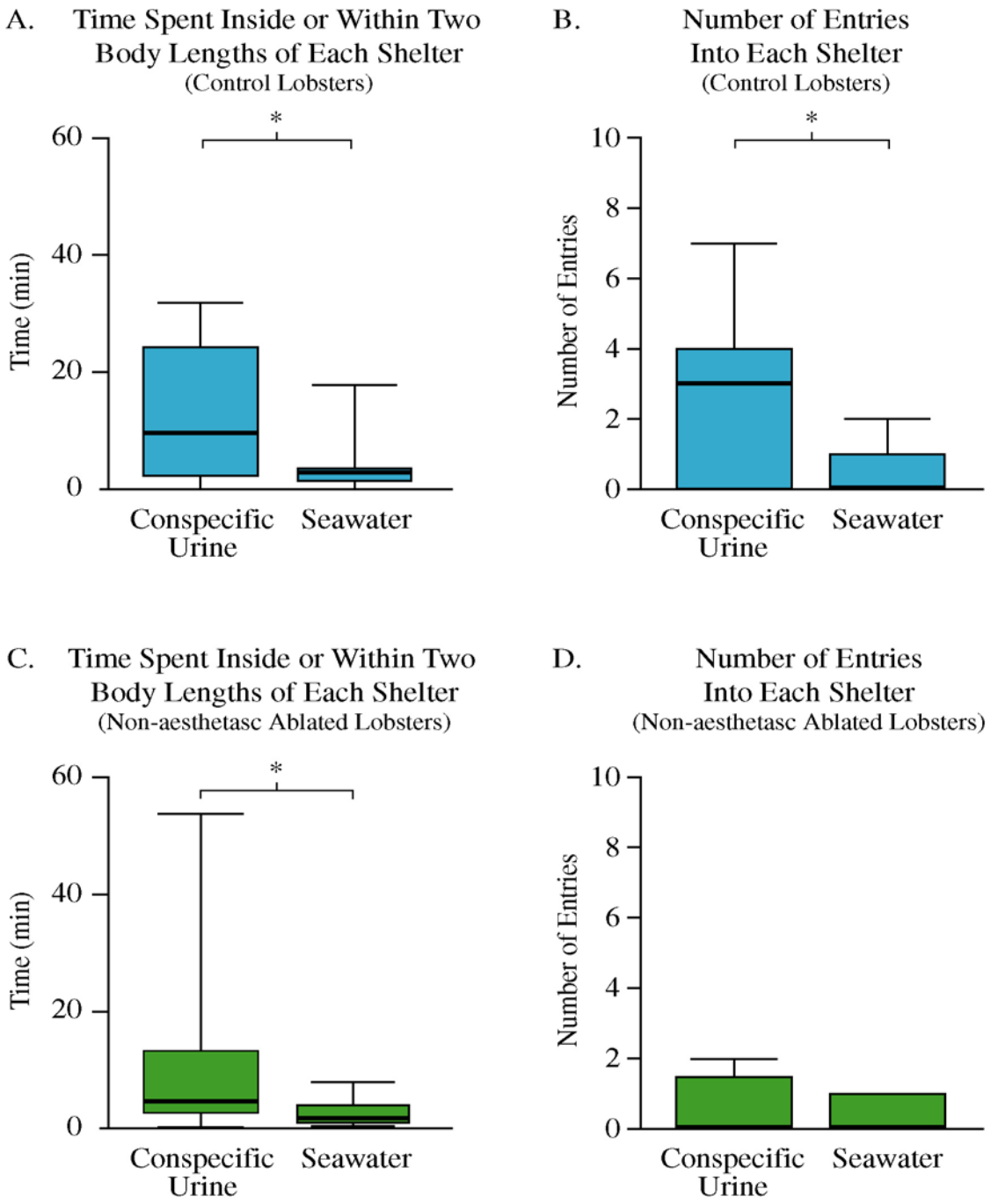

Figure 4-4. Sheltering behavior of control (A and B, N= 9) and non-aesthetasc ablated lobsters (C and D, N= 8) in response to dilute conspecific urine. Box plots show median (solid black line), interquartile range (box length) and minimum and maximum values (error bars) for time spent inside or within two body lengths of each shelter (A and C) or number of entries into each shelter (B and D). Statistically significant results are indicated by "*". Wilcoxon matched-pairs test, $P<0.05$.

Z=2.10, p=0.036). However, non-aesthetasc ablated lobsters entered both shelters very infrequently, and as a result there were no statistically significant differences in the number of entries into the control and urine-emanating shelters (Fig. 4-4D, Wilcoxon matched pairs test, two-tailed, N=8, Z=0.802, p=0.423). No obvious differences in other aspects of behavior were observed between control and non-aesthetasc ablated lobsters, so it is not clear why non-aesthetasc ablated lobsters entered the shelters so infrequently.

The analysis of general sheltering preferences of control and non-aesthetasc ablated lobsters revealed no significant differences in either the total time spent sheltering (Fig. 4-5A, Mann-Whitney U, N=17, U=29, p=0.501) or in the total number of entries into the shelters (Fig. 4-5B, Mann-Whitney U, N=17, U=18, p=0.075).

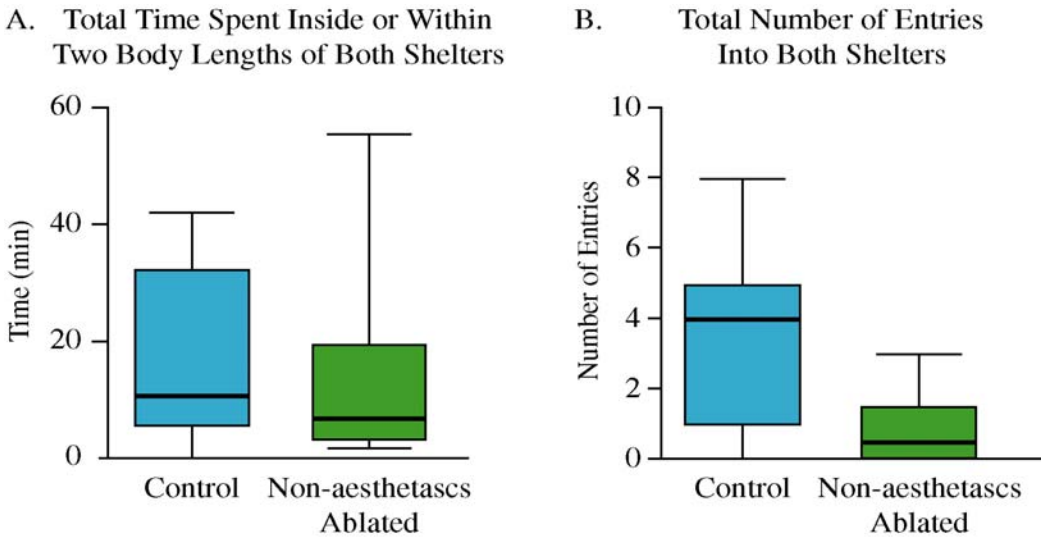

Figure 4-5. Comparison of overall sheltering behavior between control (N= 10) and non-aesthetasc ablated (N= 8) lobsters. Box plots show median (solid black line), interquartile range (box length), and maximum and minimum values (error bars) for total time spent inside or within two body lengths of both shelters (A) and total number of entries into both shelters (B). No statistically significant differences were observed. Mann-Whitney U Test, P> 0.05

Discussion

The purpose of this study was to investigate the roles of the dual antennular chemosensory pathways in mediating the sheltering response to urine-borne aggregation signals. Our results show that the aesthetasc/ olfactory lobe pathway is necessary to mediate shelter selection in our bioassay. Spiny lobsters with intact antennules sheltered preferentially in shelters emanating urine, whereas lobsters with ablated aesthetasc sensilla showed no preference for either shelter. In contrast, the non-aesthetasc/ lateral antennular neuropil pathway does not appear to play a critical role in shelter selection since at least some behavioral measures indicated that lobsters with ablated non-aesthetascs were still capable of discriminating between control and urine emanating shelters. The results of this study demonstrate a difference in the roles of these two antennular chemosensory pathways for processing aggregation signals, with the aesthetasc/ olfactory lobe pathway playing a major role.

Lobsters Seek Shelters with Conspecific Urine

The behavior of control lobsters in the current study replicates previous results showing that Caribbean spiny lobsters with intact antennules associate preferentially with shelters emanating conspecific urine over control shelters (Horner *et al.*, 2006). In the current study, control (unablated) lobsters in both the aesthetasc ablation experiment and the non-aesthetasc ablation experiment showed a significant overall preference for the shelter emanating dilute conspecific urine over the control shelter. They spent significantly more time inside and around the shelter emanating conspecific urine than the control shelter, and they also entered the urine-emanating shelter significantly more often than the control

shelter. Thus, both our previous and current work shows that spiny lobsters with intact antennules prefer shelters emanating conspecific urine.

Aesthetasc Sensilla are Necessary for Urine-evoked Sheltering

Aesthetasc ablated spiny lobsters showed a completely different pattern of behavior from control lobsters. Although the total number of entries into both shelters and total amount of time that aesthetasc ablated spiny lobsters spent sheltering was comparable to that of control animals, their shelter preference was not biased towards the shelter emanating urine. Instead, these animals sheltered randomly, spending approximately equal amounts of time inside and around both shelters and entering both shelters with similar frequency. Removal of the aesthetascs did not affect the overall tendency to shelter, but instead changed shelter choice. Aesthetasc ablated spiny lobsters did not distinguish between the urine emanating shelter and the control shelter.

The behavioral deficit observed in the aesthetasc ablated lobsters cannot be attributed simply to handling of the animals since control animals still distinguished between the shelters. Nor can it be attributed to a non-specific effect of surgery or ablation since non-aesthetasc ablated animals still retained some ability to distinguish between the shelters despite being ablated.

The same lack of shelter preference displayed by the aesthetasc ablated lobsters was observed in previous studies investigating the shelter preference of intact lobsters tested with seawater or other non-urine chemical stimuli (Horner *et al.*, 2006). In these studies, intact spiny lobsters showed a significant preference for the shelter emanating urine, but they sheltered randomly in response to all other odorants examined (Horner *et al.*, 2006). Removal

of the aesthetascs elicited the same pattern of behavior as removal of the urine stimulus, suggesting that the aesthetasc chemosensory pathway is necessary to mediate shelter selection in response to urine borne aggregation signals.

Non-Aesthetasc Sensilla are not Necessary or Sufficient for Urine-Evoked Sheltering

As described in the previous section, removal of aesthetascs resulted in dramatic changes in shelter preference. Although the non-aesthetasc chemosensory pathway was functional in those experiments, its presence failed to rescue the behavior. Thus the non-aesthetasc pathway alone is not sufficient to drive shelter selection in response to conspecific urine signals.

The non-aesthetasc chemosensory pathway also does not seem to play a necessary role in shelter selection. In general, the behavior of non-aesthetasc ablated spiny lobsters was similar to that of control lobsters, but their overall responses were not as robust. The total amount of time that non-aesthetasc ablated spiny lobsters spent sheltering was comparable to the amount of time that control animals spent sheltering. A similar pattern occurred for the total number of entries into each shelter. Like control lobsters, non-aesthetasc ablated lobsters showed an overall preference for the shelter emanating conspecific by spending significantly more time inside and around this shelter than the control shelter. However, unlike control lobsters, non-aesthetasc ablated lobsters did not enter the urine emanating shelter significantly more often than the control shelter.

It is not clear why the ablated lobsters entered the urine emanating shelter so infrequently, as no other striking qualitative differences were noted in the behavior of non-aesthetasc ablated animals compared to control animals. The difference in our two measures

of shelter preference for this treatment group suggests that the non-aesthetasc ablation may have had some effects on the sheltering behavior of the animals in response to conspecific urine signals. However, the difference in sheltering time suggests that ablated lobsters still retained some ability to distinguish between the two shelters despite the lack of non-aesthetasc chemosensory input. The aesthetasc pathway, which remained intact and functional in this treatment group, seems to be sufficient to mediate shelter selection at least in this measure of shelter preference. Although the non-aesthetasc pathway does not play a critical role in sheltering behavior, it may play a supporting role in shelter selection in the natural environment by enhancing or otherwise complementing the response to urine signals by the aesthetasc chemosensory pathway.

The Aesthetasc / Olfactory Lobe Pathway Functions in Crustacean Social Behaviors

The results of this study demonstrate that the aesthetasc pathway plays a unique role in mediating the response to urine-born aggregation signals in the Caribbean spiny lobster. Several previous studies showed that the aesthetasc chemosensory pathway plays a critical role in mediating the response to intraspecific urine signals in other species of decapod crustaceans. Male blue crabs (*Callinectes sapidus*) respond to sex pheromones released from pubertal females with characteristic courtship displays (Gleeson, 1980, 1982, 1991). When the aesthetascs were removed from the antennules of the male crabs, they no longer responded to female signals, demonstrating that the aesthetasc pathway plays a critical role in mediating the response to sex pheromones in this species. More recent studies showed that the aesthetasc chemosensory pathway also plays a critical role in individual recognition in the clawed lobster *Homarus americanus* (Johnson and Atema, 2005). The emerging evidence

from these and the current study suggests that the aesthetasc chemosensory pathway plays an important and unique role in processing complex social signals in decapod crustaceans. However, the aesthetasc chemosensory pathway is not simply a pheromone processing system, since it also functions in several food odor mediated behaviors (Steullet *et al.,* 2001; Steullet *et al.*, 2002; Horner *et al.*, 2004).

Similar to other organisms with multiple chemosensory pathways, the dual antennular chemosensory pathways in the Caribbean spiny lobster have both complementary and overlapping roles in odor mediated behaviors. The aesthetasc and non-aesthetasc chemosensory pathways are both capable of driving food-odor mediated behaviors including activation of searching behavior, odor discrimination and learning, and orientation to distant food odor sources (Derby *et al.*, 2001; Steullet *et al.*, 2001; Steullet *et al.*, 2002; Horner *et al.*, 2004). However, each pathway also has specialized functions in other odor-mediated behaviors. Social signals seem to be primarily processed in the aesthetasc/ olfactory lobe pathway (this study), whereas reflexive chemo-mechano coupled behaviors such as grooming are mediated through the non-aesthetasc/ lateral antennular neuropil pathway (Schmidt and Derby, 2005). Depending on the odor signal and the behavioral context of the signaling, either one or both of the chemosensory pathways may be employed. Thus, dual chemosensory pathways in animals as diverse as crustaceans and mammals can have both unique and overlapping functions.

Chapter 5 - The Role of the olfactory pathway in agonistic behavior of crayfish (*Procambarus clarkii*).

Introduction

Several species of decapod crustaceans including crayfish, clawed lobsters, and hermit crabs form linear social dominance hierarchies. Dominant-subordinate relationships are established through agonistic interactions that usually begin with simple approaches and threat displays, escalate through a series of increasingly intense aggressive behaviors, and end when one animal disengages either by retreating or tail flipping away (Bruski and Dunham, 1987; Huber and Kravitz, 1995). The retreating animal is considered the loser of the fight and becomes the subordinate, whereas the winner of the encounter becomes the new dominant. In the natural environment, the dominant animal gains access to the best resources including shelter, food, and mates (Bergman and Moore, 2003). However, crayfish will also engage in agonistic behavior and form dominant-subordinate relationships in simplified laboratory settings devoid of tangible resources (Bovbjerg, 1953; Lowe, 1956; Guiasu and Dunham, 1999; Issa *et al.*, 1999).

Once established, the social hierarchy remains relatively stable over time. Dominant animals continue to initiate and win a majority of the subsequent encounters, and subordinate animals tend to retreat more readily and otherwise avoid engaging the dominant (Copp, 1986; Issa *et al.*, 1999; Goessmann *et al.*, 2000). The overall amount of fighting as well as the intensity of fighting generally decrease over time, presumably because the crayfish are able

to recognize the social status of potential opponents and avoid energetically costly and potentially injurious interactions (Issa *et al.*, 1999; Goessmann *et al.*, 2000; Zulandt-Schneider *et al.*, 2001).

Chemical signals in general, and urine signals in particular, play an important role in social communication in decapod crustaceans. Chemical signals mediate several aspects of agonistic behavior in American lobsters (*Homarus americanus*) and several crayfish species (Breithaupt and Atema, 1993; Karavanich and Atema, 1998a; Zulandt-Schneider *et al.*, 1999; Breithaupt and Atema, 2000; Zulandt-Schneider *et al.*, 2001; Breithaupt and Eger, 2002). Urine signals affect both the duration and intensity of agonistic interactions in *Orconectes rusticus* (Zulandt-Schneider *et al.*, 2001) and play an important role in reducing the aggression level of opponents in *Astacus leptodactylus* (Breithaupt and Eger, 2002). Urine cues also are important for individual recognition in *H. americanus* (Karavanich and Atema, 1998a) and social status recognition in *O. rusticus* (Zulandt-Schneider *et al.*, 2001). Although chemical signals play an important role in agonistic interactions between decapod crustaceans, it is not clear which parts of the chemosensory system are important for processing these signals.

Like other crustaceans, crayfish have chemosensory structures on most body surfaces, but they are most concentrated on the appendages including the antennules, 2nd antennae, mouthparts, walking legs, and chelipeds (Fig. 5-1A(Holmes and Homuth, ; Ache and Macmillan, 1980; Derby, 1982; Schmidt and Gnatzy, 1984; Schmidt, 1989; Cate and Derby, 2001; Corotto and O'Brien, 2002; Cate and Derby, 2002a; Garm *et al.*, 2003; Belanger and Moore, 2006). The antennules in particular are considered to be the primary structures involved in crustacean chemosensory behaviors, and several studies have shown that the

antennules play an important role in agonistic behavior and recognition (Rutherford *et al.*, 1996; Karavanich and Atema, 1998a; Bergman *et al.*, 2003; Johnson and Atema, 2005).

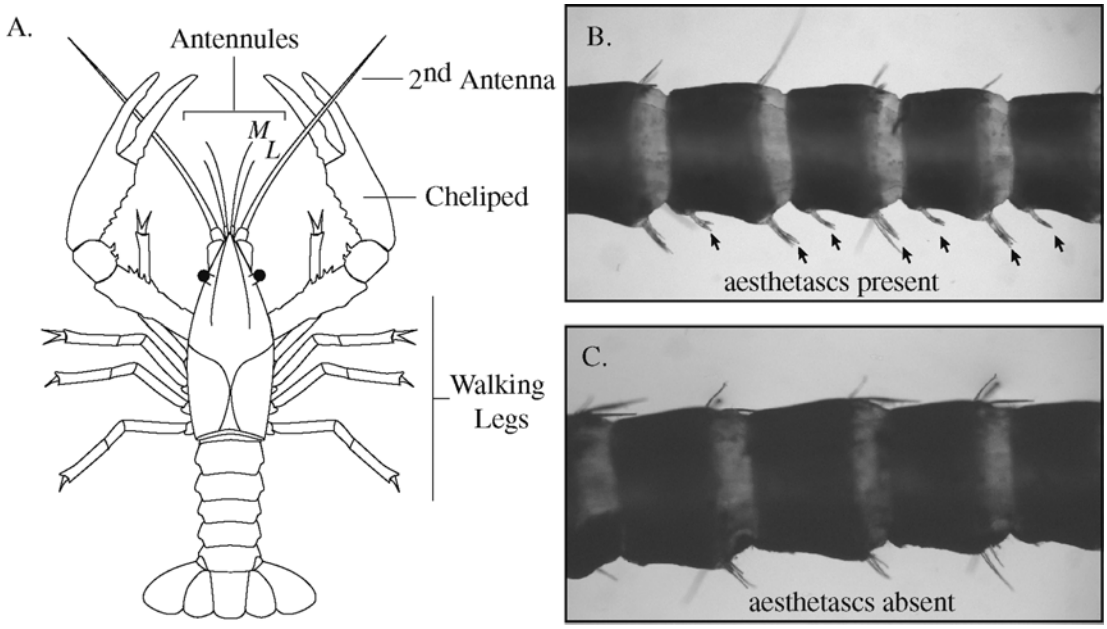

Figure 5-1. Red swamp crayfish (*Procambarus clarkii*) and antennular sensilla. (A) Diagram of *P. clarkii* showing the major components of the chemosensory system. The lateral and medial flagella are indicated by L and M respectively. (B) Light micrograph of the aesthetasc region of a control crayfish antennule showing both aesthetasc (indicated by black arrows) and non-aesthetasc sensilla. (C) Light micrograph of the aesthetasc region of an aesthetasc-ablated crayfish antennule. Aesthetasc sensilla have been removed, leaving only non-aesthetasc sensilla in this region.

Each antennule is composed of three basal segments and two distal flagella (the lateral and medial flagella) that bear a complement of sensilla. Chemosensory information is transmitted from the antennular sensilla to the brain in two main neuronal pathways called the aesthetasc/ olfactory lobe pathway and the non-aesthetasc / lateral antennular neuropil

pathway (Schmidt and Ache, 1992; Schmidt *et al.*, 1992; Schmidt and Ache, 1996b, 1996a). The pathways originate in different populations of sensilla on the antennular flagella and target different neuropils in the brain.

The aesthetasc-olfactory lobe pathway originates in the prominent aesthetasc sensilla, which are located exclusively on annuli in the distal half of each lateral flagellum (Fig. 5-1B). Aesthetascs are a common feature of crustacean chemosensory systems and are exclusively chemosensory sensilla (Holmes and Homuth, 1910; Grunert and Ache, 1988; Hallberg *et al.*, 1992; Hallberg *et al.*, 1997; Steullet *et al.*, 2000). In the crayfish, *Procambarus clarkii*, each aesthetasc is innervated by the dendrites of approximately 175 olfactory receptor neurons (Mellon *et al.*, 1989; Mellon and Munger, 1990). Tract tracing studies in crayfish and other decapods have shown that aesthetasc olfactory receptor neurons target the paired olfactory lobes in the brain (Mellon *et al.*, 1989; Mellon and Munger, 1990; Sandeman *et al.*, 1992; Schmidt and Ache, 1992). The olfactory lobes show the typical glomerular organization that characterizes the first order olfactory processing centers of a variety of organisms (Sandeman *et al.*, 1992; Hildebrand and Shepherd, 1997; Eisthen, 2002; Ache and Young, 2005).

In addition to the aesthetasc-olfactory lobe pathway, decapod crustaceans also have a secondary antennular chemosensory pathway called the non-aesthetasc lateral antennular neuropil pathway (Schmidt *et al.*, 1992; Schmidt and Ache, 1996a). This pathway originates in the various types of non-aesthetascs that are distributed throughout the antennular flagella. The antennules of *P. clarkii* and other crayfish species contain three main types of non-aesthetascs variously referred to by different authors as large guard hairs or L-type setae, acuminate, companion, small guard hairs or S-type setae, and feather or F-type setae

(Sandeman and Luff, 1974; Chichibu *et al.*, 1978; Tierney *et al.*, 1986; Sandeman and Sandeman, 1996). Several of these setae have been shown to respond to mechanosensory stimuli (Chichibu *et al.*, 1978), however at least a subset must also respond to chemical stimuli. Physiological studies have shown that flicking behavior can be elicited by chemical stimulation of the medial flagellum alone (Mellon, 2005), and several behavioral studies have also demonstrated chemical sensitivity of the medial flagellum (Holmes and Homuth, 1910; Dunham *et al.*, 1997; Giri and Dunham, 1999, 2000). Chemical sensitivity of the medial flagellum must be mediated through the non-aesthetasc chemosensory pathway because aesthetascs do not occur on this flagellum. In other decapod crustaceans, several types of antennular non-aesthetascs are bimodal and innervated by both chemosensory and mechanosensory neurons (Cate and Derby, 2001, 2002b). It is likely that at least some crayfish non-aesthetascs are similarly innervated. Chemosensory and mechanosensory neurons associated with non-aesthetasc antennular sensilla target the paired lateral antennular neuropils (Sandeman *et al.*, 1992; Schmidt *et al.*, 1992; Schmidt and Ache, 1996a; Schachtner *et al.*, 2005). The lateral antennular neuropils have a stratified organization, and in addition to receiving chemosensory and mechanosensory afferents from the antennular flagella, they also contain the major arborizations of antennular motoneurons (Schmidt and Ache, 1993; Schachtner *et al.*, 2005). Consequently, these neuropils function as antennular sensory-motor integration centers, and are involved in chemo-mechano coupled behaviors such as antennular grooming (Maynard, 1966; Schmidt and Ache, 1993; Schmidt and Derby, 2005).

Although chemical signals are known to play an important role in aggressive behavior in crayfish, the specific chemosensory pathways that mediate the response to these signals

are not known. The goal of this study is to examine the importance of the dual chemosensory pathways in regulating the dynamics of agonistic interactions in crayfish and to determine if the aesthetasc pathway plays a critical role in the establishment of dominant-subordinate relationships.

Methods

Animals

Male Form I crayfish (*Procambarus clarkii*) ranging in total length from 84-98 mm (mean ± S.E.M. = 87.5 ± 1.0 mm, N= 22) were obtained from a commercial supplier (Atchafalaya Biological Supply, Raceland, LA) and shipped overnight to Georgia State University. Upon arrival the crayfish were weighed, measured, and assigned a number, which was written on their dorsal and lateral carapace with a silver marker (Sharpie ®). Only intermolt animals with intact antennules were used in this study. Crayfish were isolated in individual aquaria (23 cm long x 15 cm wide x 17 cm high) for at least 2 weeks to remove any memory of previous social encounters (Karavanich and Atema, 1998b). The isolation aquaria were lined with gravel and contained a single shelter constructed of either a short length of PVC pipe or a terra cotta flower pot. Tank water was changed twice weekly and an airstone was used to provide constant aeration of the tank. Animals were fed every other day and maintained on a 12 hr: 12 hr light: dark cycle. Pairs of animals were size matched by total body length and chelae length. Pairs of crayfish were size matched within 3% of total body length and 6% chelae length.

Ablations

To examine the importance of the aesthetasc chemosensory pathway in agonistic behavior, we selectively ablated all of the aesthetasc sensilla on both lateral flagella from one group of crayfish and compared their behavior to that of intact control animals. Both members of the crayfish pair were treated the same way and were either ablated or control animals. Five control pairs and six ablated pairs of crayfish were used in this study.

Control pairs were restrained in the same manner and for the same amount of time as ablated crayfish. The antennules of the control animals were periodically brushed with a cotton swab to simulate manipulation of the antennular sensilla experienced by the ablated crayfish.

Aesthetasc-ablated crayfish were restrained on their dorsal carapace on a perforated plastic platform. The antennules were extended across the surface of a microscope slide coated with a thin layer of silicon (Sylgard), and held in place using small staples. The entire platform was placed into a container of fresh water just deep enough to cover the animal. Aesthetasc sensilla on both lateral flagella were physically removed by shaving with a handmade narrow blade (Fig. 5-1C). Ablation of the aesthetascs on both lateral flagella typically took about 1 hr to complete. Although ablation of the aesthetascs by shaving is a somewhat severe treatment, no overt differences in the general (i.e. non-fighting) behavior of control and ablated animals were observed following the ablation procedure. Removal of the aesthetascs in this way does not appear to have non-specific effects on the overall behavior of the animals.

The efficacy of the aesthetasc ablation was evaluated with light microscopy at the conclusion of the experimental trials. The ablations were very efficient, and left very few

intact aesthetascs on the antennules (mean ± SEM = 1.1 ± 0.46 total aesthetascs remaining on each crayfish, n= 12 crayfish). Although some non-aesthetascs were inadvertently removed during the shaving process, many remained intact following the ablation.

General Fight Protocol

To examine the effects of aesthetasc ablation on fight dynamics, pairs of crayfish were fought for 1 hr periods on three consecutive days. All fights took place within a 38-l aquarium (51 cm long x 25 cm wide x 30 cm high) that was lined with white gravel and devoid of shelters or other objects. A mesh and plastic grate divider was used to separate the combatants in between fighting trials. The construction of the divider allowed for chemosensory and obscured visual contact between the animals, but the small mesh size precluded any physical contact.

On the first day of trials, the two members of the pair were placed on opposite sides of the divider at roughly the same time. After a 10 min acclimation period, the divider was removed, allowing the animals to interact. All interactions between the crayfish were recorded for 1 hr by a video camera mounted in front of the fighting tank. The crayfish were separated after 1 hr and the barrier replaced to prevent any subsequent interactions before the next observation period. Approximately 2 hr after the conclusion of the first observation period, both crayfish were removed from the tank and were either ablated or sham ablated (control animals). The animals were then returned to the fighting arena where they remained separated by the divider until the next observation period. Animals were paired a total of 3 times (for 1 hr each day on three consecutive days). All of the trials were conducted between 0900 and 1400 hr, but each set of three trials was conducted at the same time on each of the

three trial days. Animals were only removed from the fighting tank on day 1. They remained in the fighting tank between pairing days 2 and 3.

Data Analysis

The videotapes for all 3 trial days were later analyzed by an individual unaware of the pair's ablation status. For each trial, we recorded the start and end time of each encounter as well as the types of behaviors displayed by each animal during the encounter. The start of an encounter was considered the time that an approach or threat display began. The encounter was considered to be over 5 sec after fighting had ceased and the movements of the animals were no longer correlated. We noted the identity of the animal that initiated and won each encounter as well as the types of behaviors displayed by each of the crayfish during the encounter. A modified version of the ethogram of Bergman *et al.*, (2003) was used to assign intensity values to each of the behaviors observed during an encounter (Table 5-1). The collected data were used to calculate the number of encounters that occurred during each observation period, the percentage of each trial that the animals spent engaged in encounters, the maximum encounter duration, the highest intensity levels reached for each encounter, and the percentage of encounters initiated and won by the overall dominant animal.

Statistical Analysis

We used a Friedman analysis of variance to determine whether there were significant differences in behaviors displayed by the crayfish pairs over the 3 trial days. When significant differences were identified, a multiple comparisons post hoc test (Siegel and Castellan, 1988) was used to determine whether observed significant differences were the

result of changes between the values on day 1 and day 2 or between the values on day 1 and day 3. We chose a priori to examine day 1 versus day 2 and day 3 because we expected to see changes in behavior following the ablation or control restraining period.

Table 5-1. Ethogram codes used to score fight intensity levels. (Modified from Bergman *et al*., 2003)

Intensity Level	Description of Behavior
-2	Tailflip away from opponent or fast retreat
-1	Retreat by slowly backing away from opponent
0	Visually ignore opponent with no response or threat display
1	Approach without a threat display
2	Approach with meral spread
3	Initial contact and claw use by boxing, pushing or touching with closed claws
4	Active claw use by grabbing and/or holding opponent
5	Unrestrained fighting by pulling at opponent's claws or body parts

Results

Control Crayfish Pairs

Control crayfish pairs displayed statistically significant decreases in the total number of encounters, the overall percentage of the trial spent in encounters, and the maximum encounter duration ($p<0.05$, Friedman analysis of variance) over the course of the three trial days. The overall percentage of the trial spent in an encounter decreased significantly from day one levels on both subsequent trial days (Fig. 5-2A; $p<0.05$, multiple comparisons post hoc test, (Siegel and Castellan, 1988). Both the number of encounters (Fig. 5-2B) and the

maximum encounter duration (Fig. 5-2C) decreased over the three trial days with statistically significant decreases observed between trial days one and three ($p<0.05$, multiple comparisons post hoc test, (Siegel and Castellan, 1988). There were no changes in the maximum intensity values reached for each day of trials (data not shown), nor were there changes in the dominant-subordinate status of the crayfish as measured by the percentage of trials initiated and won by the dominant animal. The eventual dominant initiated and won a majority of the encounters on all three trial days (median % trials initiated = 88%, 93%, 78% for trial days 1, 2, and 3 respectively; and median % trials won = 89%, 79%, 89% for trial days 1, 2, and 3 respectively).

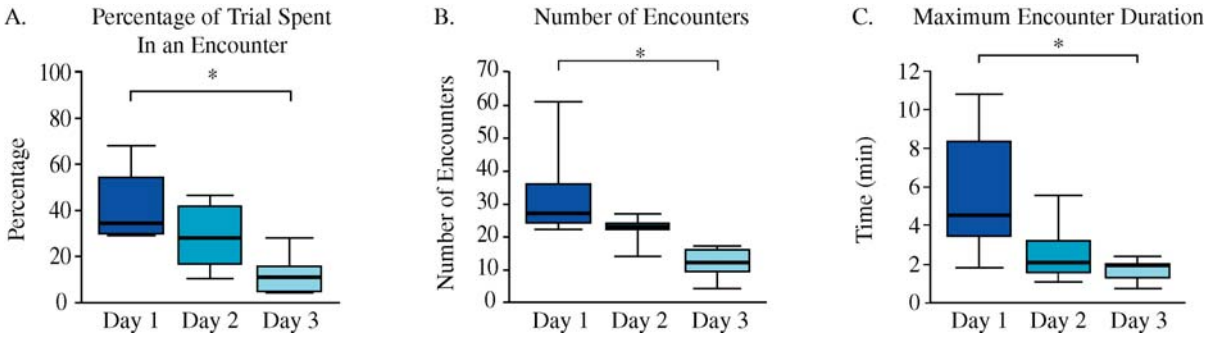

Figure 5-2. Fight dynamics of control crayfish pairs. Box plots show median (solid black line), interquartile range (box length), and minimum and maximum values (error bars) for the five pairs of control crayfish on each trial day. Overall significant differences were found in all three paramters (Friedman analysis of variance, $p <0.05$). Statistically significant pairwise comparisons as determined by a multiple comparisons post-hoc test are indicated by an "*" ($p<0.05$)

Ablated Crayfish Pairs

No statistically significant changes were observed in any of the fight parameters examined with aesthetasc ablated crayfish. The percentage of the overall trial spent in an encounter (Fig. 5-3A), the total number of encounters (Fig. 5-3B), and the maximum encounter duration (Fig. 5-3C) did not change significantly over the course of the three trial days ($p>0.05$, Friedman analysis of variance). As was the case with control animals, there were also no changes in either the maximum intensity values reached for each trial ($p> 0.05$, Friedman analysis of variance, data not shown) or in the dominant-subordinate status of the crayfish as measured by the percentage of trials initiated and won by the overall dominant animal ($p>0.05$, Friedman analysis of variance, data not shown).

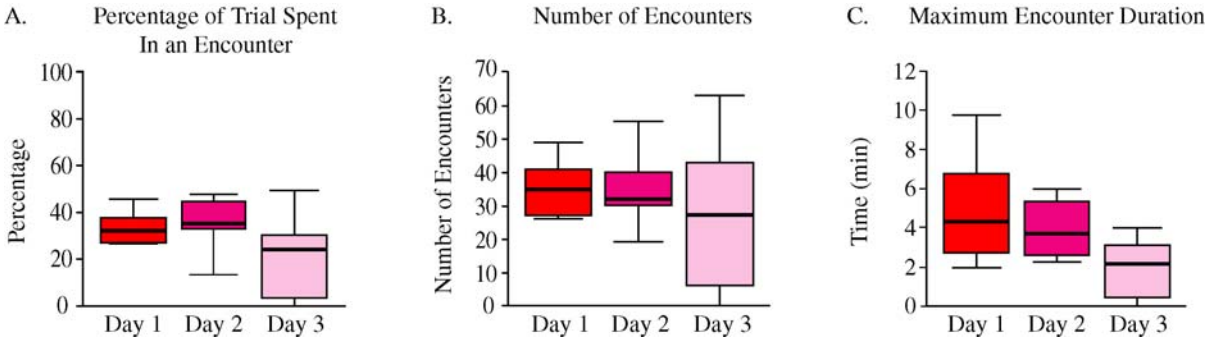

Figure 5-3. Fight dynamics of aesthetasc ablated crayfish pairs. Box plots show median (solid black line), interquartile range (box length), and minimum and maximum values (error bars) for the five pairs of control crayfish on each trial day. No statistically significant differences were observed (Friedman analysis of variance, $p <0.05$).

Discussion

The goal of this study was to determine whether the aesthetasc / olfactory lobe chemosensory pathway plays a role in regulating the fighting behavior of crayfish. Our results show that the amount of fighting between control pairs gradually decreased over the course of three daily pairings, whereas the amount of fighting between aesthetasc ablated pairs remained at similar levels over all the three days. This finding suggests that the aesthetasc / olfactory lobe chemosensory pathway has some role in crayfish agonism and further supports the emerging view that this pathway is essential for intraspecific communication in decapod crustaceans.

Crayfish with intact chemosensory systems show declines in fighting upon repeated pairings

Sized-matched male crayfish with intact antennules readily engaged in agonistic encounters and quickly established stable dominant-subordinate relationships when placed together in a single aquarium. Once established, the dominant-subordinate relationship was generally maintained over the course of repeated pairings. As has been observed in other crayfish species, the amount of fighting observed in these control pairs gradually decreased over the course of the three trial days (Copp, 1986; Issa *et al.*, 1999; Goessmann *et al.*, 2000; Zulandt-Schneider *et al.*, 2001). Initial pairings were characterized by numerous long episodes of intense agonistic interactions which established the dominant or subordinate status of each combatant. In subsequent pairings, the overall percentage of the trial spent fighting decreased significantly from initial levels on both trial days. This significant decrease reflected a decline in both the number of encounters and the maximum encounter duration, which were statistically different from initial values by the third trial day.

Although the amount of time spent fighting changed over the course of repeated pairings, other aspects of agonistic behavior did not. Fewer fights occurred on each subsequent trial day, but the overall percentage of fights reaching the highest intensity levels did not differ significantly between trial days. Similar percentages of high intensity fights were observed across all three trial days. A similar pattern of behavior was observed in dominance studies in the crayfish *Orconectes rusticus*, where no significant differences in the intensity of fighting were observed between first and second fights (Zulandt-Schneider *et al.*, 2001). Thus a decrease in the intensity of fighting behavior does not always accompany the establishment of stable dominance relationships.

The dominant and subordinate status of the two members of the crayfish pair was maintained over the course of repeated pairings in most cases. In four of the five pairs, the crayfish that initially emerged as the dominant on day one retained this status over all three trial days. This animal initiated and won a majority of the encounters on all three days. The fifth pair experienced a hierarchy reversal between trial days 1 and 2. In this instance, the initial subordinate became the dominant animal during the second trial day and subsequently initiated and won a majority of the encounters on that day and the third trial day. As is the case in other crayfish species, *P. clarkii* individuals with intact chemosensory systems readily establish stable dominant-subordinate relationships that result in a reduction in both the amount of fighting and the duration of fighting over the course of repeated pairings.

Aesthetascs are important for reductions in fighting behavior over repeated encounters

In contrast to the reduction in fighting behavior observed in the control pairs, aesthetasc ablated crayfish showed no statistically significant changes in the amount of

fighting over the course of repeated pairings. Ablated crayfish pairs continued to engage in similar numbers of fights with similar maximum durations on all three trial days. They also spent similar overall percentages of each trial day engaged in encounters. The difference in behavior between control and aesthetasc-ablated crayfish suggests that the aesthetasc/ olfactory lobe pathway plays an important role in regulating the amount of fighting in *P. clarkii* over the course of repeated interactions.

Although ablation of the aesthetasc pathway affected some aspects of fighting behavior, other aspects of fight dynamics and dominant-subordinate relationships were not affected. Like control crayfish, aesthetasc ablated crayfish showed no differences in the percentages of encounters reaching the highest intensity levels across the three days, and continued to fight aggressively on all trial days. Aesthetasc ablated pairs also established and maintained stable-dominant subordinate relationships. In all six ablated pairs, the animal that emerged as dominant on day 1 remained dominant on the second and third trial days. Overall, the aesthetasc pathway plays an important role in mediating the amount of fighting that occurs over the course of repeated trials, but is not critical for other aspects of dominance hierarchy formation in *P. clarkii*.

Chemical signals and changes in fighting behavior

The changes in fighting behavior that occurred after removal of the aesthetascs were most likely the result of a disruption of chemical signaling between the two combatants. Chemical signals, particularly those contained within the urine, are known to play a significant role in determining the dynamics and eventual outcome of agonistic encounters in the clawed lobster *Homarus americanus* and several crayfish species (Breithaupt and Atema,

1993; Karavanich and Atema, 1998a; Breithaupt and Atema, 2000; Zulandt-Schneider *et al.*, 2001; Breithaupt and Eger, 2002). Urine signals are critical for individual recognition in *H. americanus*, and removal of these signals by catheterization resulted in longer and more intense fights during repeated interactions (Karavanich and Atema, 1998a). Urine signals are also important for regulating aggression levels in crayfish (Zulandt-Schneider *et al.*, 2001; Breithaupt and Eger, 2002). Blocking urine release in the crayfish, *Orconectes rusticus*, resulted in longer and more intense second fights between blocked animals in comparison to control groups (Zulandt-Schneider *et al.*, 2001).

Removal of the aesthetascs affects perception of these chemical signals, since the behavioral deficits observed in the ablated pairs in this study closely match the deficits observed when urine release was altered in previous studies. In crayfish, chemical signals are important for social status recognition between opponents (Zulandt-Schneider *et al.*, 1999; Zulandt-Schneider *et al.*, 2001). Social status recognition is a potentially important mechanism for reducing aggression between crayfish over repeated pairings. Animals that are able to assess the social status of a potential opponent chemically do not need to engage continuously in potentially injurious interactions to assess dominance. On the other hand, a crayfish that is anosmic may not be able to assess accurately its own social status or the social status of an opponent, which would lead to longer and more frequent interactions.

Although chemical signals play an important role in regulating the dynamics of agonistic behavior, they are not the only cues involved. Both visual and tactile information can also provide much information about the aggressive state and dominance level of an animal (Bruski and Dunham, 1987). Both of these cues were readily available in our assay, and ablated animals could have used either or both of these types of signals to assess

dominance in the absence of appropriate chemical stimulation. It is likely that the use of these non-chemical cues allowed for the establishment and maintenance of stable dominant-subordinate relationships in aesthetasc ablated crayfish pairs.

Aesthetascs are important for intraspecific communication in decapod crustaceans

The results of this study closely mirror the results of other studies that show that the aesthetasc-olfactory lobe chemosensory pathway of decapod crustaceans has a specialized function in social behaviors. Aesthetascs play a critical role in mediating shelter selection and aggregation in response to conspecific urine signals in the gregarious Caribbean spiny lobster, *Panulirus argus* (Chapter 3 of this dissertation). The aesthetasc-olfactory lobe pathway also plays a necessary role in courtship behavior in blue crabs (*Callinectes sapidus*) (Gleeson, 1982, 1991) and is suspected to mediate the response of male Japanese helmet crabs (*Telmessus cheiragonus*) to female sex pheromones (Kamio *et al.*, 2005). In the American lobster, *H. americanus,* the aesthetasc chemosensory pathway is necessary for individual recognition by interacting animals (Johnson and Atema, 2005). The results of the current study suggest that the aesthetasc chemosensory pathway plays a similar role in *P. clarkii* by mediating aspects of social status recognition during agonistic encounters. Thus in crayfish as in other species of decapod crustaceans, the aesthetascs play an important role in intraspecific communication.

Chapter 6 - General Discussion

The olfactory systems of many organisms are partitioned into multiple, anatomically distinct neuronal pathways. The functional significance of this organization is not well understood in many organisms including both vertebrates and decapod crustaceans. The work presented in this dissertation examines the roles of the aesthetasc and non-aesthetasc pathways in different odorant and behavioral contexts with the goal of gaining more insight into why the chemosensory systems of crustaceans and other organisms are partitioned into anatomically separate pathways.

Overlapping roles of multiple chemosensory pathways

The main and accessory olfactory pathways of many vertebrates and arthropods show partial overlap in both the functional classes of chemical signals detected and the types of behaviors mediated. Pheromones and general odorants are detected by receptor neurons in both the main and accessory olfactory pathways of vertebrates. For instance, receptor neurons in both the main olfactory epithelium and vomeronasal organ of mice respond to urine pheromones such as 2-heptanone and major histocompatability complex (MHC) peptides (Xu *et al.*, 2005; Brennan and Zufall, 2006; Spehr *et al.*, 2006b; Spehr *et al.*, 2006a). Neurons in both the main and accessory olfactory pathways of turtles respond with similar sensitivities to various general odorants (Shoji and Kurihara, 1991). In addition to displaying overlapping functions in odor detection, the vertebrate main and accessory olfactory pathways also have overlapping roles in regulating odor driven behaviors. For instance, ultrasonic vocalizations by male rats can be evoked by stimulation of either the main or

accessory olfactory pathway with fresh female urine (Sipos *et al.*, 1995). Elimination of both pathways causes a cessation of vocalizations, but if either one of the pathways is left intact then the vocalizations continue (Sipos *et al.*, 1995).

The dual antennular chemosensory pathways in decapod crustaceans also have overlapping roles in several food-odor mediate behaviors. Both the aesthetasc and non-aesthetasc chemosensory pathways can drive food odor discrimination, food odor learning, and activation of searching behavior in small aquaria (Steullet *et al.*, 2001; Steullet *et al.*, 2002). Both pathways can also mediate orientation to a distant food odor stimulus in a large laboratory flume (Chapter 2; Horner *et al.*, 2004). Spiny lobsters lacking both aesthetasc and non-aesthetasc sensillar inputs failed to locate the source of a 2-m distant food odor, demonstrating that antennular sensilla in general are necessary for localization of distant food odors. However, spiny lobsters with at least one functional antennular chemosensory pathway located the source regularly, demonstrating that either the aesthetasc or non-aesthetasc pathway alone is sufficient for food odor localization. Thus there is some redundancy in the functional roles of aesthetasc and non-aesthetasc chemosensory pathways in food-odor mediated behaviors.

Benefits of functional overlap

Functional overlap is an important feature of many sensory systems and can benefit an organism in several important ways (Derby and Steullet, 2001). Possession of multiple similarly tuned chemosensory neurons allows an animal to continue to function normally in the event of loss or damage to a subset of sensors (Derby and Steullet, 2001). A multiplicity

of similarly tuned chemosensory neurons also increases the probability of stimulus detection and enhances the overall sensitivity of the system (Derby and Steullet, 2001).

The odor sensitivity and specificity of an individual chemosensory neuron are determined by the type(s) of odorant receptors that it expresses. The combined activity of many chemosensory neurons with overlapping odor specificities increases the overall sensitivity of an olfactory system through response summation (Derby and Steullet, 2001). Response summation allows for even very weak signals to be effectively detected and amplified by the central processing centers of the olfactory system.

All of the aforementioned benefits of redundancy apply equally well to having many similarly tuned neurons contained within a single chemosensory pathway or distributed across multiple chemosensory pathways. There are additional potential benefits to partitioning neurons with overlapping odor responses into separate pathways with different anatomical organizations and different central nervous system connections. Distribution of similarly tuned neurons into different chemosensory pathways may allow for simultaneous processing of different stimulus attributes. For instance, one pathway may provide information about the quality of an odor signal, while a second pathway may provide information about the location or spatial distribution of the odor signal. Differently organized chemosensory pathways could also mediate different physiological or behavioral responses to the same odor signal. The combined activity of multiple chemosensory pathways in these examples would provide a more complete picture of the odor stimulus and could potentially allow for more complex behavioral responses.

Unique functions of multiple chemosensory pathways

The main and accessory olfactory pathways of vertebrates and arthropods also have unique functions in odor detection and odor mediated behaviors. Different pathways contain different complements of receptor neurons with non-overlapping odor sensitivities and specificities. The presence of a diversity of neurons with different odor response properties increases the dynamic range of an olfactory system by allowing a broader range of chemical stimuli to be detected and discriminated. Packaging neurons with different sensitivities and specificities into differently organized pathways allows for different processing requirements to be met and also allows for a greater range of behavioral responses.

The olfactory systems of moths is one of the best and most elegant examples of a clear functional separation between pathways based on the types of odorants detected and the types of behaviors mediated. The main olfactory pathway mediates the response to general odorants whereas the male specific pathway responds exclusively to female sex pheromones (Hansson, 1995; Hildebrand, 1995; Christensen and White, 2000; Hansson and Anton, 2000; Christensen and Hildebrand, 2002). Organizational features of the male specific pathway (including the high affinity of the pheromone receptors and the massive convergence of the axons of pheromone sensitive neurons onto a few specific glomeruli) make it a highly sensitive and selective pheromone processing system. Packaging pheromone sensitive neurons into a separate pathway allows these signals to be detected and discriminated with much greater sensitivity than general odorants.

Although the vertebrate main and accessory olfactory pathways respond to some of the same odorants, they do not have completely redundant odor sensitivities. For instance

chemical signals involved in trail following, prey consumption, and other behaviors in garter snakes are processed by the vomeronasal system rather than the main olfactory system (Halpern and Martinez-Marcos, 2003). Pheromones mediating suckling behavior in rabbits and standing behavior in female boars are processed by the main olfactory system and not by the vomeronasal system (Hudson and Distel, 1986; Dorries *et al.*, 1997).

The aesthetasc and non-aesthetasc chemosensory pathways in decapod crustaceans also have unique roles in different odor and behavioral contexts. One of the major findings of this dissertation is that the pathways have different roles in mediating the behavioral response to social signals, particularly those contained within urine.

Urine signals mediate social behaviors

Substances contained within urine play an important role in social behaviors in many organisms. Many mammals use urine signals extensively for chemical communication. Urine signals act as both primer and releaser pheromones in mice and other rodents, and provide information about sex, reproductive state, degree of genetic relatedness, territory ownership, and overall health (Beauchamp and Yamazaki, 2003; Wyatt, 2003; Beynon and Hurst, 2004; Hurst and Beynon, 2004). Urine signals provide information about estrous state in female elephants (Rasmussen *et al.*, 2001). Alarm signals contained within the urine of stressed pigs affect the behavior of conspecifics (Vieuille-Thomas and Signoret, 1992; Amory and Pearce, 2000). Substances contained within the urine of fish act as primer and releaser pheromones for sexual behaviors (Yambe *et al.*, 1999; Stacey, 2003; Wyatt, 2003; Yambe *et al.*, 2006). Brief exposure to preovulatory a female urine increases hormone levels

and milt production in male goldfish, and exposure to postovulatory female urine further increases milt production and releases courtship and mating behaviors (Stacey, 2003). Chemicals contained within the urine of mature female masu salmon function as male attracting pheromones (Yambe *et al.*, 1999; Yambe *et al.*, 2006).

Urine signals also regulate diverse social interactions in decapod crustaceans. Urine-signals mediate courtship and mating behaviors in several decapod species (Ryan, 1966; Christofferson, 1978; Gleeson, 1980; Atema and Cowan, 1986; Bamber and Naylor, 1997; Bushmann and Atema, 1997; Bushmann and Atema, 2000; Kamio *et al.*, 2000; Hardege *et al.*, 2002; 2002; Raethke *et al.*, 2004; Ekerholm and Hallberg, 2005) and also play an important role in the determination of social status in crayfish (Zulandt-Schneider *et al.*, 2001; Breithaupt and Eger, 2002) and individual recognition in clawed lobsters (Breithaupt and Atema, 1993; Karavanich and Atema, 1998c; Breithaupt *et al.*, 1999; Breithaupt and Atema, 2000; Johnson and Atema, 2005). Urine signals also mediate shelter selection in *P. argus* (Chapter 3; Horner *et al.*, 2006). Spiny lobsters tested in a shelter choice assay showed a statistically significant preference for shelters emanating conspecific urine over shelters emanating seawater. Preference for one shelter over the other was specific to the urine stimulus and did not occur in response to food or predator odors. There was no sex specificity to the sheltering response, and both male and female lobsters associated preferentially with shelters releasing conspecific urine of either sex. Conspecific urine is thus one source of chemical signals mediating gregarious sheltering by *P. argus* in the natural environment.

Unique functions of the aesthetasc pathway in decapod crustaceans

Urine evoked shelter selection in *P. argus* is mediated primarily by the aesthetasc/ olfactory lobe chemosensory pathway (Chapter 4). Spiny lobsters with intact antennules associated preferentially with shelters emanating conspecific urine over control shelters emanating seawater. Aesthetasc ablated lobsters, however, failed to distinguish between the shelters. The dramatic change in shelter preference resulting from removal of the aesthetascs demonstrates that this chemosensory pathway plays a critical role in urine-mediated shelter selection. Non-aesthetasc ablated spiny lobsters retained the ability to distinguish between the two shelters in some measures, indicating that the non-aesthetasc pathway is not critical for urine evoked shelter selection. However, the non-aesthetasc pathway may play a supporting role in shelter selection in the natural environment by enhancing or otherwise complementing the response of the aesthetasc pathway to urine signals.

In addition to mediating gregarious sheltering in spiny lobsters, the aesthetasc/ olfactory lobe pathway also plays an important role in regulating aggressive behavior in the freshwater crayfish, *Procambarus clarkii* (Chapter 5). The amount of fighting between control pairs of size matched male *P. clarkii* decreased significantly over the course of repeated pairings. The reduction in fighting likely results from the ability of the crayfish to recognize chemically the social status of potential opponents and avoid unnecessary and potentially injurious interactions (Issa *et al.*, 1999; Goessmann *et al.*, 2000; Zulandt-Schneider *et al.*, 2001). In contrast to the decline in fighting behavior observed in control crayfish pairs, aesthetasc ablated crayfish pairs continued to fight at similar levels on all three trial days. The difference in behavior between control and ablated crayfish pairs suggests that

the aesthetasc/ olfactory lobe pathway plays an important role in regulating the amount of fighting in *P. clarkii*, presumably by mediating chemical communication between the combatants.

The aesthetasc/ olfactory lobe pathway also mediates the response to social signals in other decapod crustaceans. Aesthetascs are both necessary and sufficient to mediate characteristic courtship display of male blue crabs (*Callinectes sapidus*) in response to female sex pheromones (Gleeson ,1980, 1982, 1991), and likely mediate aspects of mating behavior in the helmet crab (Kamio *et al.*, 2005). The aesthetasc pathway is also necessary for individual recognition in the clawed lobster, *Homarus americanus* (Johnson and Atema, 2005).

In all of these decapod species there are differences between the dual antennular chemosensory pathways in mediating the response to social signals, with the aesthetasc pathway playing a more critical role. Given that there are differences between the pathways in odor processing, why are social signals detected preferentially by the aesthetasc pathway when food odors are detected by both pathways?

The structural organization and central nervous system connections of aesthetasc and non-aesthetasc chemosensory pathways are quite different, suggesting that each pathway has specialized functions in odor processing and odor mediated behaviors. Both the peripheral and central organization of the aesthetasc pathway suggests that it is both a sensitive and highly discriminatory odor processing pathway. Pheromone processing pathways in other organisms are often highly sensitive and selective. If processing crustacean social signals

requires a similarly sensitive and selective system, then such signals are likely to be processed preferentially by the aesthetasc chemosensory pathway.

General properties of pheromone processing systems

Many pheromones and semiochemicals are present in the environment at very low concentrations, and thus require sensitive detection systems. Sex pheromones of some moths species show activity at concentrations of 10^{-18}M (Wyatt, 2003). Pheromones released from larval lamprey are present in the environment in picomolar concentrations (Polkinghorne *et al.*, 2001), and electrophysiological recordings from adult lampreys show that larval pheromones are detected at these low concentrations (Li *et al.*, 1995). Vomeronasal neurons in mice are sensitive to some pheromones at concentrations of 10^{-11}M (Leinders-Zufall *et al.*, 2000). Although the concentration of aggregation signals contained within the urine of spiny lobsters is unknown, the results of the shelter selection experiments demonstrate that only a very small quantity of the signal is necessary to mediate the behavioral response (Chapter 3; Horner *et al.*, 2006). A total of 150 μl of urine was released into the flume over the course of the hour long trial, and thus the amount of aggregation signal present at any given time would have been considerably lower than this.

In addition to being very sensitive, pheromone detecting systems must also be able to discriminate between highly similar odors. Male moths are able to distinguish between the highly similar pheromone blends used by conspecific and sympatric female moths, and only engage in upwind flight in response to conspecific signals (Hansson, 1995; Vickers *et al.*,

1998; Wyatt, 2003). Pregnant female mice are able to distinguish between the scents familiar and unfamiliar males and will abort their pregnancies in response to unfamiliar male scents (Wyatt, 2003). Blue crabs can distinguish the urine of pubertal molt females from the urine of males and females in other reproductive stages (Gleeson, 1980, 1991). American lobsters can distinguish between urine signals of familiar and unfamiliar conspecifics (Karavanich and Atema, 1998a).

The ability of spiny lobsters to discriminate between intra- and interspecific urine signals has not been thoroughly examined. However, spiny lobsters searching for a shelter must at least be able to distinguish between conspecific urine and urine from potential predators inhabiting the same reef environment. Crayfish must also be able to discriminate between potentially similar urine signals. In order for social status recognition to occur, crayfish must be able to distinguish chemically between dominant and subordinate animals (Zulandt-Schneider *et al.*, 1999; Zulandt-Schneider *et al.*, 2001). Crayfish must also be able to assess their own social status in relation to the social status of potential opponents when deciding whether or not to initiate a fight. Behaviorally this can accomplished by using the antennules to sample the urine stream as it is released from the nephropores. Increased flicking and downward movements of the antennules have been observed during periods of spontaneous urine release in crayfish (Breithaupt and Eger, 2002), suggesting that the animals were sampling their own urine signals. Thus the context of urine detection may allow crayfish to distinguish between self and non-self urine signals.

Organization of the aesthetasc pathway suggests an important role in odor discrimination

The peripheral and central organizations of the aesthetasc-olfactory lobe pathway provide the neural substrate for complex odor discriminations and high sensitivity to chemical signals. The aesthetascs of decapod crustaceans are densely innervated sensory structures (Hallberg *et al.*, 1992; Hallberg *et al.*, 1997). An individual aesthetasc can be innervated by the dendrites of up to several hundred olfactory receptor neurons depending on the species examined (Laverack and Ardill, 1965; Grunert and Ache, 1988; Hallberg *et al.*, 1992; Hallberg *et al.*, 1997; Steullet *et al.*, 2000; Derby *et al.*, 2003). Electrophysiological and activity labeling studies have shown that different olfactory receptor neurons have different odor sensitivities and specificities (Derby and Atema, 1988; Steullet and Derby, 1997; Derby, 2000; Steullet *et al.*, 2000). This diversity of receptor neurons allows the aesthetascs to detect a broad range of chemical stimuli including both general odors and intraspecific signals (Gleeson, 1982; Derby and Atema, 1988; Steullet *et al.*, 2000; Johnson and Atema, 2005). Non-aesthetascs, which are innervated by far fewer neurons, may have a more limited capacity for detecting different types of odor stimuli. The non-aesthetascs simply may not contain the cells or receptors necessary to detect the active components of the urine signal. However, the identities of the active components of the urine signal are currently unknown and thus this hypothesis cannot yet be rigorously examined.

Aesthetascs are considered to be repeating functional units, and each aesthetasc contains a similar complement of olfactory receptor neurons (Steullet *et al.*, 2000). As a result of this organization there are many copies of each olfactory receptor neuron type on

the lateral flagella. The presence of multiple copies of each cell type in the periphery can increase the sensitivity of the system through response summation (Derby and Steullet, 2001). Thus the aesthetasc pathway may be able to detect and discriminate social signals and other odorants at very low concentrations.

In both the main and accessory olfactory systems of vertebrates and arthropods, primary sensory neurons expressing the same odorant receptor converge onto one or a few glomeruli in the central nervous target neuropils (Christensen and White, 2000; Eisthen, 2002; Wyatt, 2003; Ache and Young, 2005; Chen and Shepherd, 2005). Glomeruli are considered to be functional units of olfactory coding and play an important role in odor discrimination and signal amplification (Christensen and White, 2000; Eisthen, 2002; Wyatt, 2003; Ache and Young, 2005; Chen and Shepherd, 2005). Odor stimuli are represented by patterns of glomerular activation that reflect patterns of receptor activation in the periphery (Christensen and White, 2000; Eisthen, 2002; Wyatt, 2003; Ache and Young, 2005; Chen and Shepherd, 2005). Different odorants are distinguished by the different patterns of glomerular activation that they elicit. Inhibitory circuitry both within an individual glomerulus and between olfactory glomeruli sharpen the resolution of the system through contrast enhancement, thus allowing even highly similar odorants to be discriminated (Chen and Shepherd, 2005; Cleland and Sethupathy, 2006). Organization of the olfactory lobes into glomeruli suggests that the aesthetasc pathway has an important role in the discrimination of odor quality. Although the non-aesthetasc chemosensory pathway is capable of some complex odor discriminations (Steullet *et al.*, 2002), the glomerular organization of the aesthetasc pathway may allow for finer discriminations of odor stimuli.

Unique functions of the non-aesthetasc chemosensory pathway in decapod crustaceans

Although the aesthetasc pathway shares many organizational features with the vertebrate and insect main and accessory olfactory pathways, the non-aesthetasc pathway of decapod crustaceans is strikingly different. Instead of being a purely chemosensory pathway, the non-aesthetasc pathway carries both chemosensory and mechanosensory information from the periphery to the central nervous system (Schmidt *et al.*, 1992; Schmidt and Ache, 1996a). In vertebrates and other arthropods, the first order processing centers of both the main and accessory olfactory pathways are organized into glomeruli (Christensen and White, 2000; Eisthen, 2002; Wyatt, 2003; Ache and Young, 2005). In contrast, the first order processing centers of the non-aesthetasc pathway in decapod crustaceans (the lateral antennular neuropils) have a stratified rather than a glomerular organization (Sandeman *et al.*, 1992; Schmidt *et al.*, 1992; Schmidt and Ache, 1996a). Although some other organisms such as snails have multiple chemosensory pathways with glomerular and non-glomerular target neuropils, the functions of these different pathways in odor mediated behaviors are not well understood (Chase and Tolloczco, 1993).

The lateral antennular neuropils are considered to be sensory motor integration centers because they receive both the afferents of chemo and mechanosensory neurons on the antennular flagella, and also contain major arborizations of the antennular motoneurons. The non-aesthetasc pathway plays a specialized role in driving different sensory guided movements of the antennules (Maynard, 1966; Schmidt and Ache, 1993). Asymmetric sensilla (a particular type of non-aesthetasc sensilla) drive the stereotyped antennular grooming response to stimulation with L-glutamate in spiny lobsters (Schmidt and Derby,

2005). Evidence suggests that the non-aesthetasc chemosensory pathway also mediates flicking, a reflexive downward deflection of the lateral flagellum. Flicking was observed in response to chemical stimulation of the medial flagellum in crayfish, although the specific types of sensilla driving the behavior were not identified (Mellon, 2005). However, the medial flagellum is devoid of aesthetascs, indicating that flicking is mediated by some component of the non-aesthetasc chemosensory pathway. The proximity of sensory and motor pathways within the lateral antennular neuropils, makes the non-aesthetasc pathway an ideal system for mediating reflexive movements of the antennules. The more direct link between sensory and motor systems in this pathway allows odor driven movements of the antennule to occur without having to pass through the several layers of higher order processing centers that would be required by the layout of the aesthetasc-olfactory lobe pathway.

Although it has not been demonstrated experimentally, the non-aesthetasc pathway may also provide information about the spatial distribution of an odor stimulus. The bi-lobed structure and stratified organization of the lateral antennular neuropils have been hypothesized to represent a topographic map of sensory inputs on the antennules (Schmidt *et al.*, 1992; Schmidt and Ache, 1996a). The presence of a topographic map would provide information about the location of odor stimulation on the antennules. In other organisms such as cockroaches, tract tracing studies suggest that the positional relationships of contact chemoreceptors on the antennae are maintained in the central nervous system (Nishino *et al.*, 2005). This somatotopic arrangement of contact chemosensory afferents is thought to aid in

orientation to odors by providing information about the location of chemical stimulation on the antennae (Nishino *et al.*, 2005).

Anatomically distinct chemosensory pathways in a diversity of organisms show both unique and overlapping functions in different odor and behavioral contexts. The dual antennular chemosensory pathways in decapod crustaceans show overlapping roles in food odor mediated behaviors, but distinct roles in processing social signals and in driving movements of the antennules. Partitioning of the crustacean chemosensory system into multiple pathways with different structural features and central connections allows the animal to processes a broader range of chemical stimuli and respond to odor stimulation with a greater variety of behaviors. The organization of the vertebrate and insect olfactory systems into multiple anatomically separate chemosensory pathways likely reflects a similar need for different odorant processing strategies in these organisms.

Literature Cited

Ache, B. W., Macmillan, D. L. (1980). Neurobiology. *The Biology and Management of Lobsters*. B. F. Phillips. New York, Academic Press. **1:** 165-213.

Ache, B. W., Young, J. M. (2005). Olfaction: diverse species, conserved principles. *Neuron* **48**: 417-430.

Amory, J. R., Pearce, G. P. (2000). Alarm pheromones in urine modify the behaviour of weaner pigs. *Animal Welfare* **9**(2): 167-175.

Atema, J., Cobb, J. S. (1980). Social Behavior. *The Biology and Management of Lobsters*. B. F. Phillips. **1:** 409-450.

Atema, J., Cowan, D. F. (1986). Sex-identifying urine and molt signals in lobster *Homarus americanus*. *Journal of Chemical Ecology* **12**(11): 2065-2080.

Atema, J. (1995). Chemical signals in the marine environment: dispersal, detection, and temporal signal analysis. *Proceedings of the National Academy of Sciences of the United States of America* **92**(1): 62-66.

Bamber, S. D., Naylor, E. (1997). Sites of release of putative sex pheromone and sexual behaviour in female *Carcinus maenas* (Crustacea: Decapoda). *Estuarine, Coastal and Shelf Science* **44**(2): 195-202.

Barbato, J. C., Daniel, P. C. (1997). Chemosensory activation of an antennular grooming behavior in the spiny lobster, *Panulirus argus*, is tuned narrowly to L- glutamate. *Biological Bulletin* **193**(2): 107-115.

Baxi, K. N., Dorries, K. M., Eisthen, H. L. (2006). Is the vomeronasal system really specialized for detecting pheromones? *Trends in Neurosciences* **29**(1): 1-7.

Beauchamp, G. K., Yamazaki, K. (2003). Chemical signalling in mice. *Biochemical Society Transactions* **31**: 147-151.

Beglane, P. F., Grasso, F. W., Basil, J. A., Atema, J. (1997). Far field chemo-orientation in the American Lobster, *Homarus americanus*: effects of unilateral ablation and lesioning of the lateral antennule. *Biological Bulletin* **193**(2): 214-215.

Belanger, R. M., Moore, P. A. (2006). The use of the major chelae by reproductive male crayfish (*Orconectes rusticus*) for discrimination of female odors. *Behaviour* **143**: 713-731.

Berger, D. K., Butler, M. J. (2001). Octopuses influence den selection by juvenile Caribbean spiny lobster. *Marine and Freshwater Research* **52**(8): 1049-1053.

Bergman, D. A., Kozlowski, C. P., McIntyre, J. C., Huber, R., Daws, A. G., Moore, P. A. (2003). Temporal dynamics and communication of winner-effects in the crayfish, *Orconectes rusticus*. *Behaviour* **140**(6): 805-825.

Bergman, D. A., Moore, P. A. (2003). Field observations of intraspecific agonistic behavior of two crayfish species, *Orconectes rusticus* and *Orconectes virilis*, in different habitats. *Biological Bulletin* **205**(1): 26-35.

Berrill, M. (1975). Gregarious behavior of juveniles of the spiny lobster, Panulirus argus (Crustacea: Decapoda). *Bulletin of Marine Science* **25**(4): 515-522.

Bertelsen, R. D., Matthews, T. R. (2001). Fecundity dynamics of female spiny lobster (*Panulirus argus*) in a south Florida fishery and Dry Tortugas National Park lobster sanctuary. *Marine and Freshwater Research* **52**(8): 1559-1565.

Beynon, R. J., Hurst, J. L. (2004). Urinary proteins and the modulation of chemical scents in mice and rats. *Peptides*
M. Altstein **25**(9): 1553-1563.

Bovbjerg, R. V. (1953). Dominance order in the crayfish *Orconectes virilis* (Hagen). *Physiological Zoology* **26**: 173-178.

Breer, H., Fleischer, J., Strotmann, J. (2006). The sense of smell: multiple olfactory subsystems. *Cellular and Molecular Life Sciences* **63**(13): 1465-1475.

Breithaupt, T., Atema, J. (1993). Evidence for the use of urine signals in agonistic interactions of the American lobster. *Biological Bulletin* **185**(2): 318.

Breithaupt, T., Schmitz, B., Tautz, J. (1995). Hydrodynamic orientation of crayfish (*Procambarus clarkii*) to swimming fish prey. *Journal of Comparative Physiology A: Sensory Neural and Behavioral Physiology* **177**(4): 481-491.

Breithaupt, T., Lindstrom, D. P., Atema, J. (1999). Urine release in freely moving catheterised lobsters (*Homarus americanus*) with reference to feeding and social activities. *Journal of Experimental Biology* **202**(7): 837-844.

Breithaupt, T., Atema, J. (2000). The timing of chemical signaling with urine in dominance fights of male lobsters (*Homarus americanus*). *Behavioral Ecology and Sociobiology* **49**(1): 67-78.

Breithaupt, T., Eger, P. (2002). Urine makes the difference: chemical communication in fighting crayfish made visible. *Journal of Experimental Biology* **205**(9): 1221-1231.

Brennan, P. A., Zufall, F. (2006). Pheromonal communication in vertebrates. **444**(7117): 308-315.

Briones-Fourzan, P., Lozano-Alvarez, E. (2005). Seasonal variations in chemical response to conspecific scents in the spotted spiny lobster, *Panulirus guttatus*. *New Zealand Journal of Marine and Freshwater Research* **39**(2): 383-390.

Bruski, C. A., Dunham, D. W. (1987). The importance of vision in agonistic communication of the crayfish *Orconectes rusticus*. I. An analysis of bout dynamics. *Behaviour* **103**: 83-107.

Bushmann, P., Atema, J. (1994). Aggression-reducing courtship signals in the lobster, *Homarus americanus*. *Biological Bulletin* **187**(2): 275-276.

Bushmann, P. J., Atema, J. (1997). Shelter sharing and chemical courtship signals in the lobster *Homarus americanus*. *Canadian Journal of Fisheries and Aquatic Sciences* **54**(3): 647-654.

Bushmann, P. J. (1999). Concurrent signals and behavioral plasticity in blue crab (*Callinectes sapidus* Rathbun) courtship. *Biological Bulletin* **197**(1): 63-71.

Bushmann, P. J., Atema, J. (2000). Chemically mediated mate location and evaluation in the lobster, *Homarus americanus*. *Journal of Chemical Ecology* **26**(4): 883-899.

Carr, W. E. S. (1988). The molecular nature of chemical stimuli in the aquatic environment. *Sensory Biology of Aquatic Animals*. W. N. Tavolga. New York, Springer-Verlag**:** 3-29.

Cate, H. S., Roye, D. B. (1997). Ultrastructure and physiology of the outer row statolith sensilla of the blue crab *Callinectes sapidus*. *Journal of Crustacean Biology* **17**(3): 398-411.

Cate, H. S., Derby, C. D. (2001). Morphology and distribution of setae on the antennules of the Caribbean spiny lobster *Panulirus argus* reveal new types of bimodal chemo-mechanosensilla. *Cell and Tissue Research* **304**(3): 439-454.

Cate, H. S., Derby, C. D. (2002a). Ultrastructure and physiology of the hooded sensillum, a bimodal chemo-mechanosensillum of lobsters. *Journal of Comparative Neurology* **442**(4): 293-307.

Cate, H. S., Derby, C. D. (2002b). Hooded sensilla homologues: Structural variations of a widely distributed bimodal chemo-mechanosensillum. *Journal of Comparative Neurology* **444**(4): 345-357.

Cate, H. S., Derby, C. D. (2002a). Hooded sensilla homologues: Structural variations of a widely distributed bimodal chemo-mechanosensillum. *Journal of Comparative Neurology* **444**(4): 345-357.

Cate, H. S., Derby, C. D. (2002b). Ultrastructure and physiology of the hooded sensillum, a bimodal chemo-mechanosensillum of lobsters. *Journal of Comparative Neurology* **442**(4): 293-307.

Chase, R., Tolloczco, B. (1993). Tracing neural pathways in snail olfaction: from the tip of the tentacles to the brain and beyond. *Microscopy Research and Technique* **24**(3): 214-230.

Chen, W. R., Shepherd, G. M. (2005). The olfactory glomerulus: A cortical module with specific functions. *Journal of Neurocytology* **34**(3-5): 353-360.

Chichibu, S., Wada, T., Komiya, H. (1978). Structure of mechanoreceptive hairs on the crayfish first antenna. *Acta medica Kinki University* **3**(1): 27-39.

Childress, M. J., Herrnkind, W. F. (1997). Den sharing by juvenile Caribbean spiny lobsters (*Panulirus argus*) in nursery habitat: cooperation or coincidence? *Marine and Freshwater Research* **48**(8): 751-758.

Childress, M. J., Herrnkind, W. F. (2001a). Influence of conspecifics on the ontogenetic habitat shift of juvenile Caribbean spiny lobsters. *Marine and Freshwater Research* **52**(8): 1077-1084.

Childress, M. J., Herrnkind, W. F. (2001b). The guide effect influence on the gregariousness of juvenile Caribbean spiny lobsters. *Animal Behaviour* **62**: 465-472.

Christensen, T. A., White, J. (2000). Representation of olfactory information in the brain. *The Neurobiology of Taste and Smell*. D. Restrepo. New York, Wiley-Liss**:** 201-232.

Christensen, T. A., Hildebrand, J. G. (2002). Pheromonal and host-odor processing in the insect antennal lobe: how different? *Current Opinion in Neurobiology* **12**: 393-399.

Christofferson, J. P. (1978). Evidence for the controlled release of a crustacean sex pheromone. *Journal of Chemical Ecology* **4**(6): 633-639.

Cleland, T. A., Sethupathy, P. (2006). Non-topographical contrast enhancement in the olfactory bulb. *BMC Neuroscience* **7**.

Copp, N. H. (1986). Dominance hierarchies in the crayfish *Procambarus clarkii* (Girard, 1852) and the question of learned individual recognition (Decapoda, Astacidea). *Crustaceana* **51**: 9-23.

Corotto, F. S., O'Brien, M. R. (2002). Chemosensory stimuli for the walking legs of the crayfish *Procambarus clarkii*. *Journal of Chemical Ecology* **28**: 1117-1130.

Daniel, P. C., Shineman, M., Fischetti, M. (2001). Comparison of chemosensory activation of antennular grooming behaviour in five species of decapods. *Marine and Freshwater Research* **52**(8): 1333-1337.

Derby, C. D. (1982). Structure and function of cuticular sensilla of the lobster *Homarus americanus*. *Journal of Crustacean Biology* **2**: 1-21.

Derby, C. D., Atema, J. (1982). The function of chemoreceptors and mechanoreceptors in lobster *Homarus americanus* feeding behavior. *Journal of Experimental Biology* **98**: 317-328.

Derby, C. D., Atema, J. (1988). Chemoreceptor cells in aquatic invertebrates: peripheral mechanisms of chemical signal processing in decapod crustaceans. *Sensory Biology of Aquatic Animals*. W. N. Tavolga. New York, Springer Verlag**:** 365-385.

Derby, C. D. (2000). Learning from spiny lobsters about chemosensory coding of mixtures. *Physiology & Behavior* **69**(1-2): 203-209.

Derby, C. D., Steullet, P. (2001). Why do animals have so many receptors? The role of multiple chemosensors in animal perception. *Biological Bulletin* **200**(2): 211-215.

Derby, C. D., Steullet, P., Horner, A. J., Cate, H. S. (2001). The sensory basis of feeding behaviour in the Caribbean spiny lobster, *Panulirus argus*. *Marine and Freshwater Research* **52**(8): 1339-1350.

Derby, C. D., Cate, H. S., Steullet, P., Harrison, P. J. H. (2003). Comparison of turnover in the olfactory organ of early juvenile stage and adult Caribbean spiny lobsters. *Arthropod Structure & Development* **31**(4): 297-311.

Devine, D. V., Atema, J. (1982). Function of chemoreceptor organs in spatial orientation of the lobster, *Homarus americanus*: differences and overlap. *Biological Bulletin* **163**: 144-153.

Dorries, K. M., Adkins-Regan, E., Halpern, B. P. (1997). Sensitivity and behavioral responses to the pheromone androstenone are not mediated by the vomeronasal organ in domestic pigs. *Brain Behavior and Evolution* **49**(1): 53-62.

Dunham, D. W., Ciruna, K. A., Harvey, H. H. (1997). Chemosensory role of antennules in the behavioral integration of feeding by the crayfish *Cambarus bartonii*. *Journal of Crustacean Biology* **17**(1): 27-32.

Eggleston, D. B., Lipcius, R. N. (1992). Shelter selection by spiny lobster under variable predation risk, social conditions, and shelter size. *Ecology* **73**(3): 992-1011.

Eisthen, H. L. (1997). Evolution of vertebrate olfactory systems. *Brain Behavior and Evolution* **50**(4): 222-233.

Eisthen, H. L. (2002). Why are olfactory systems of different animals so similar? *Brain Behavior and Evolution* **59**(5-6): 273-293.

Ekerholm, M., Hallberg, E. (2005). Primer and short-range releaser pheromone properties of premolt female urine from the shore crab (*Carcinus maenas*). *Journal of Chemical Ecology* **31**(8): 1845-1864.

Fuzessery, Z. M. (1978). Quantitative stimulation of antennular chemoreceptors of the spiny lobster *Panulirus argus*. *Comparative Biochemistry and Physiology A* **60**(3): 303-308.

Garm, A., Hallberg, E., Hoeg, J. T. (2003). Role of maxilla 2 and its setae during feeding in the shrimp *Palaemon adspersus* (Crustacea: Decapoda). *Biological Bulletin* **204**(2): 126-137.

Giri, T., Dunham, D. W. (1999). Use of the inner antennule ramus in the localisation of distant food odours by *Procambarus clarkii* (Girard, 1852) (Decapoda, Cambaridae). *Crustaceana* **72**: 123-127.

Giri, T., Dunham, D. W. (2000). Female crayfish (*Procambarus clarkii* (Girard, 1852)) use both antennular rami in the localization of male odour. *Crustaceana* **73**(4): 447-458.

Gleeson, R., Wheatly, M., Reiber, C. (1997). Perireceptor mechanisms sustaining olfaction at low salinities: insight from the euryhaline blue crab *Callinectes sapidus*. *Journal of Experimental Biology* **200**(3): 445-456.

Gleeson, R. A. (1980). Pheromone communication in the reproductive behavior of the blue crab, *Callinectes sapidus*. *Marine Behavior and Physiology* **7**: 119-134.

Gleeson, R. A. (1982). Morphological and behavioral identification of the sensory structures mediating pheromone reception in the blue crab *Callinectes sapidus*. *Biological Bulletin* **163**(1): 162-171.

Gleeson, R. A. (1991). Intrinsic factors mediating pheromone communication in the blue crab, *Callinectes sapidus*. *Crustacean Sexual Biology*. J. W. Martin. New York, Columbia University Press**:** 17-32.

Gleeson, R. A., Carr, W. E. S., Trapido-Rosenthal, H. G. (1993). Morphological characteristics facilitating stimulus access and removal in the olfactory organ of the spiny lobster, *Panulirus argus*: insight from the design. *Chemical Senses* **18**(1): 67-75.

Goessmann, C., Hemelrijk, C., Huber, R. (2000). The formation and maintenance of crayfish hierarchies: behavioral and self-structuring properties. *Behavioral Ecology and Sociobiology* **48**(6): 418-428.

Grunert, U., Ache, B. W. (1988). Ultrastructure of the aesthetasc (olfactory) sensilla of the spiny lobster, *Panulirus argus*. *Cell and Tissue Research* **251**: 95-103.

Guiasu, R. C., Dunham, D. W. (1999). Agonistic contests in male form I *Cambarus bartonii bartonii* (Fabricus, 1798) (Decapoda, Cambaridae) crayfish and a comparison with contests of the same type in *Cambarus robustus* (Girard, 1852). *Crustaceana* **72**(9): 1079-1091.

Hallberg, E., Johansson, K. U. I., Elofsson, R. (1992). The aesthetasc concept: structural variations of putative olfactory receptor cell complexes in Crustacea. *Microscopy Research and Technique* **22**(4): 325-335.

Hallberg, E., Johansson, K. U. I., Wallen, R. (1997). Olfactory sensilla in crustaceans: morphology, sexual dimorphism, and distribution patterns. *International Journal of Insect Morphology & Embryology* **26**(3-4): 173-180.

Halpern, M., Halpern, J., Erichsen, E., Borghjid, S. (1997). The role of nasal chemical senses in garter snake response to airborne odor cues from prey. *Journal of Comparative Psychology* **111**(3): 251-260.

Halpern, M., Martinez-Marcos, A. (2003). Structure and function of the vomeronasal system: an update. *Progress in Neurobiology* **70**: 245-318.

Halpern, M., Daniels, Y., Zuri, I. (2005). The role of the vomeronasal system in food preferences of the gray short-tailed opossum, *Monodelphis domestica*. *Nutrition and Metabolism* **2**: 6.

Hansson, B. S. (1995). Olfaction in lepidoptera. *Experientia* **51**: 1003-1027.

Hansson, B. S., Anton, S. (2000). Function and morphology of the antennal lobe: new developments. *Annual Review of Entomology* **45**: 203-231.

Hardege, J. D., Jennings, A., Hayden, D., Muller, C. T., Pascoe, D., Bentley, M. G., Clare, A. S. (2002). Novel behavioural assay and partial purification of a female-derived sex pheromone in *Carcinus maenas*. *Marine Ecology Progress Series* **244**: 179-189.

Harrison, P. J. H., Cate, H. S., Steullet, P., Derby, C. D. (2001a). Structural plasticity in the olfactory system of adult spiny lobsters: postembryonic development permits life-long growth, turnover, and regeneration. *Marine and Freshwater Research* **52**(8): 1357-1365.

Harrison, P. J. H., Cate, H. S., Derby, C. D. (2004). Localized ablation of olfactory receptor neurons induces both localized regeneration and widespread replacement of neurons in spiny lobsters. *Journal of Comparative Neurology* **471**(1): 72-84.

Hazlett, B. A. (1971a). Antennule chemosensivitivy in marine decapod Crustacea. *Journal of Animal Morphology and Physiology* **10**: 1-10.

Hazlett, B. A. (1971b). Chemical and chemotactic stimulation of feeding behavior in the hermit crab *Petrochirus diogenes*. *Comparative Biochemistry and Physiology A* **39**: 665-670.

Herrnkind, W. F. (1969). Queuing Behavior of Spiny Lobsters. *Science* **164**: 1425-1427.

Herrnkind, W. F. (1970). Migration of the Spiny Lobster. *Natural History* **70**(5): 36-43.

Herrnkind, W. F., Vanderwalker, J. A., Barr, L. (1975). Population dynamics, ecology and behavior of spiny lobsters, *Panulirus argus*, of St. John, U.S.V.I.: (IV) Habitation, patterns of movement and general behavior. *Results of the Tektite Program* **2**: 31-45.

Herrnkind, W. F. (1980). Spiny lobsters: Patterns of movement. *The Biology and Management of Lobsters*. B. F. Phillips. New York, Academic Press. **1:** 349-407.

Herrnkind, W. F., Childress, M. J., Lavalli, K. L. (2001). Cooperative defence and other benefits among exposed spiny lobsters: inferences from group size and behaviour. *Marine and Freshwater Research* **52**(8): 1113-1124.

Hildebrand, J. G. (1995). Analysis of chemical signals by nervous systems. *Proceedings of the National Academy of Sciences of the United States of America* **92**(1): 67-74.

Hildebrand, J. G., Shepherd, G. M. (1997). Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. *Annual Review of Neuroscience* **20**(1): 595-631.

Holmes, S. J., Homuth, E. S. (1910). The seat of smell in the crayfish. *Biological Bulletin* **18**: 155-160.

Horner, A. J., Weissburg, M. J., Derby, C. D. (2004). Dual antennular chemosensory pathways can mediate orientation by Caribbean spiny lobsters in naturalistic flow conditions. *Journal of Experimental Biology* **207**(21): 3785-3796.

Horner, A. J., Nickles, S. P., Weissburg, M. J., Derby, C. D. (2006). Source and specificity of chemical cues mediating shelter preference of Caribbean spiny lobsters (*Panulirus argus*). *Biological Bulletin* **211**(2): 128-139.

Huber, R., Kravitz, E. A. (1995). A quantitative analysis of agonistic behavior in juvenile American lobsters (*Homarus americanus* L.). *Brain Behavior and Evolution* **46**: 72-83.

Hudson, R., Distel, H. (1986). Pheromonal release of suckling in rabbits does not depend on the vomeronasal organ. *Physiology & Behavior* **37**(1): 123-128.

Hurst, J. L., Beynon, R. J. (2004). Scent wars: the chemobiology of competitive signalling in mice. *BioEssays* **26**(12): 1288-1298.

Issa, F. A., Adamson, D. J., Edwards, D. H. (1999). Dominance hierarchy formation in juvenile crayfish *Procambarus clarkii*. *Journal of Experimental Biology* **202**(24): 3497-3506.

Johnson, M. E., Atema, J. (2005). The olfactory pathway for individual recognition in the American lobster *Homarus americanus*. *Journal of Experimental Biology* **208**(15): 2865-2872.

Johnston, R. E. (1998). Pheromones, the vomeronasal system, and communication: from hormonal responses to individual recognition. *Olfaction and Taste Xii*. **855:** 333-348.

Johnston, R. E. (2000). Chemical communication and pheromones: the types of chemical signals and the role of the vomeronasal system. *The Neurobiology of Taste and Smell*. D. Restrepo. New York, Wiley-Liss**:** 101-127.

Kamio, M., Matsunaga, S., Fusetani, N. (2000). Studies on sex pheromones of the helmet crab, *Telmessus cheiragonus* 1. An assay based on precopulatory mate-guarding. *Zoological Science* **17**(6): 731-733.

Kamio, M., Matsunaga, S., Fusetani, N. (2002). Copulation pheromone in the crab *Telmessus cheiragonus* (Brachyura : Decapoda). *Marine Ecology Progress Series* **234**: 183-190.

Kamio, M., Araki, M., Nagayama, T., Matsunaga, S., Fusetani, N. (2005). Behavioral and electrophysiological experiments suggest that the antennular outer flagellum is the site of pheromone reception in the male helmet crab *Telmessus cheiragonus*. *Biological Bulletin* **208**: 12-19.

Kanciruk, P. (1980). Ecology of juvenile and adult Palinuridae (Spiny lobsters). *The Biology and Management of Lobsters*. B. F. Phillips. New York, Academic Press. **2:** 59-96.

Karavanich, C., Atema, J. (1998a). Olfactory recognition of urine signals in dominance fights between male lobsters, *Homarus americanus*. *Behaviour* **135**: 719-730.

Karavanich, C., Atema, J. (1998b). Individual recognition and memory in lobster dominance. *Animal Behaviour* **56**: 1553-1560.

Karavanich, C., Atema, J. (1998c). Olfactory recognition of urine signals in dominance fights between male lobster, *Homarus americanus*. *Behaviour* **135**: 719-730.

Keller, T. A., Powell, I., Weissburg, M. J. (2003). Role of olfactory appendages in chemically mediated orientation of blue crabs. *Marine Ecology Progress Series* **261**: 217-231.

Kozlowski, C., Yopak, K., Voigt, R., Atema, J. (2001). An initial study on the effects of signal intermittency on the odor plume tracking behavior of the American lobster, *Homarus americanus*. *Biological Bulletin* **201**(2): 274-276.

Kraus-Epley, K. E., Moore, P. A. (2002). Bilateral and unilateral antennal lesions alter orientation abilities of the crayfish, *Orconectes rusticus*. *Chemical Senses* **27**(1): 49-55.

Laverack, M. S. (1964). The antennular sense organs of *Panulirus argus*. *Comparative Biochemistry and Physiology A* **13**: 301-321.

Laverack, M. S., Ardill, D. J. (1965). The innervation of the aesthetasc hairs of *Panulirus argus*. *Quarterly Journal of Microscopical Science* **106**(45-60).

Leinders-Zufall, T., Lane, A. P., Puche, A. C., Ma, W., Novotny, M. V., Shipley, M. T., Zufall, F. (2000). Ultrasensitive pheromone detection by mammalian vomeronasal neurons. **405**(6788): 792-796.

Li, W., Sorensen, P. W., Gallaher, D. D. (1995). The olfactory system of migratory adult sea lamprey is specifically and acutely sensitive to unique bile acids released by conspecific larvae. *Journal of General Physiology* **105**: 569-587.

Lin, D. Y., Zhang, S. Z., Block, E., Katz, L. C. (2005). Encoding social signals in the mouse main olfactory bulb. *Nature* **434**: 470-477.

Lindstrom, D. P. (1991). Crustacean sex pheromones: a new technique for collection of urine from the American lobster, *Homarus americanus*. Boston University Marine Program. Boston, Boston University.

Lowe, M. E. (1956). Dominance-subordinance relationships in the crayfish *Cambarellus shufeldtii*. *Tulane Studies in Zoology* **4**: 139-170.

Lyle, W. G., MacDonald, C. D. (1983). Molt stage determination in the Hawaiian spiny lobster *Panulirus marginatus*. *Journal of Crustacean Biology* **3**(2): 208-216.

Maynard, D. M. (1966). Integration in crustacean ganglia. *Symposium of the Society Experimental Biology* **20**: 111-149.

McLeese, D. W. (1973). Orientation of lobsters (*Homarus americanus*) to odor. *Journal Fisheries Research Board of Canada* **30**(6): 838-840.

Mellon, D., Tuten, H. R., Redick, J. (1989). Distribution of radioactive leucine following uptake by olfactory sensory neurons in normal and heteromorphic crayfish antennules. *The Journal of Comparative Neurology* **280**: 645-662.

Mellon, D., Munger, S. D. (1990). Nontopographic projection of olfactory sensory neurons in the crayfish brain. *The Journal of Comparative Neurology* **296**: 253-262.

Mellon, D., Alones, V. (1994). Identification of three classes of multiglomerular, broad spectrum neurons in the crayfish olfactory midbrain by correlated patterns of electrical activity and dendritic arborization. *Journal of Comparative Neurology* **177**: 55-71.

Mellon, D., Jr. (2005). Integration of hydrodynamic and odorant inputs by local interneurons of the crayfish deutocerebrum. *Journal of Experimental Biology* **208**(19): 3711-3720.

Miller, L. R., Gutzke, W. H. N. (1999). The role of the vomeronasal organ of crotalines (Reptilia: Serpentes: Viperidae) in predator detection. *Animal Behaviour* **58**(1): 53-57.

Moore, P. A., Scholz, N., Atema, J. (1991). Chemical orientation of lobsters *Homarus americanus* in turbulent odor plumes. *Journal of Chemical Ecology* **17**(7): 1293-1308.

Moore, P. A., Grills, J. L. (1999). Chemical orientation to food by the crayfish *Orconectes rusticus*: influence of hydrodynamics. *Animal Behaviour* **58**: 953-963.

Nevitt, G., Pentcheff, N., Lohmann, K., Zimmer-Faust, R. (1995). Evidence for hydrodynamic orientation by spiny lobsters in a patch reef environment. *Journal of Experimental Biology* **198**(10): 2049-2054.

Nevitt, G., Pentcheff, N. D., Lohmann, K. J., Zimmer, R. K. (2000). Den selection by the spiny lobster *Panulirus argus*: testing attraction to conspecific odors in the field. *Marine Ecology Progress Series* **203**: 225-231.

Nishino, H., Nishikawa, M., Yokohari, F., Mizunami, M. (2005). Dual, multilayered somatosensory maps formed by antennal tactile and contact chemosensory afferents in an insect brain. *Journal of Comparative Neurology* **493**(2): 291-308.

Polkinghorne, C. N., Olson, J. M., Gallaher, D. G., Sorensen, P. W. (2001). Larval sea lamprey release two unique bile acids** to the water at a rate sufficient to produce detectable riverine pheromone plumes. *Fish Physiology and Biochemistry* **V24**(1): 15-30.

Ptacyk, J. S., Graves, B. M. (2002). Prey detection by vomeronasal chemoreception in a plethodontid salamander. *Journal of Chemical Ecology* **28**(5): 1017-1036.

Raethke, N., MacDiarmid, A. B., Montgomery, J. C. (2004). The role of olfaction during mating in the southern temperate spiny lobster *Jasus edwardsii*. *Hormones and Behavior* **46**(3): 311-318.

Rasmussen, L. E. L., Lazar, J., Greenwood, D. R. (2001). Olfactory adventures of elephantine pheromones. *Biochemical Society Transactions* **31**: 137-141.

Ratchford, S. G., Eggleston, D. B. (1998). Size- and scale-dependent chemical attraction contribute to an ontogenetic shift in sociality. *Animal Behaviour* **56**(4): 1027-1034.

Ratchford, S. G. (1999). The influence of chemical communication on shelter selection, shelter sharing, and aggregation among spiny lobsters, *Panulirus argus*., North Carolina State University.

Ratchford, S. G., Eggleston, D. B. (2000). Temporal shift in the presence of a chemical cue contributes to a diel shift in sociality. *Animal Behaviour* **59**(4): 793-799.

Reeder, P. B., Ache, B. W. (1980). Chemotaxis in the Florida lobster, *Panulirus argus*. *Animal Behaviour* **28**(3): 831-839.

Restrepo, D., Arellano, J., Oliva, A. M., Schaefer, M. L., Lin, W. (2004). Emerging views on the distinct but related roles of the main and accessory olfactory systems in responsiveness to chemosensory signals in mice. *Hormones and Behavior* **46**: 247-256.

Rutherford, P. L., Dunham, D. W., Allison, V. (1996). Antennule use and agonistic success in the crayfish *Orconectes rusticus*. *Crustaceana* **69**: 117-122.

Ryan, E. P. (1966). Pheromones: Evidence in a decapod crustacean. *Science* **151**.

Sam, M., Vora, S., Malnic, B., Ma, W., Novotny, M. V., Buck, L. B. (2001). Odorants may arouse instinctive behaviours. **412**(6843): 142.

Sandeman, D., Sandeman, R., Derby, C., Schmidt, M. (1992). Morphology of the brain of crayfish, crabs, and spiny lobsters - a common nomenclature for homologous structures. *Biological Bulletin* **183**(2): 304-326.

Sandeman, D., Mellon, D., Jr. (2002). Olfactory centers in the brain of freshwater crayfish. *The Crustacean Nervous System*. K. Wiese. Berlin, Springer**:** 386-404.

Sandeman, D. C., Luff, S. E. (1974). Regeneration of the antennules in the Australian freshwater crayfish, *Cherax destructor*. *Journal of Neurobiology* **5**(6): 475-488.

Sandeman, D. C., Denburg, J. L. (1976). The central projections of chemoreceptor axons in the crayfish revealed by axoplasmic transport. *Brain Research* **115**: 492-496.

Sandeman, R., Sandeman, D. (1996). Pre- and postembryonic development, growth and turnover of olfactory receptor neurones in crayfish antennules. *Journal of Experimental Biology* **199**(11): 2409-2418.

Schachtner, J., Schmidt, M., Homberg, U. (2005). Organization and evolutionary trends of primary olfactory brain centers in Tetraconata (Crustacea+Hexapoda). *Arthropod Structure & Development* **34**(3): 257-299.

Schmidt, M., Gnatzy, W. (1984). Are the funnel-canal organs the campaniform sensilla of the shore crab, *Carcinus maenas* (Decapoda, crustacea) II. Ultrastructure. *Cell and Tissue Research* **237**(1): 81-94.

Schmidt, M. (1989). The hair-peg organs of the shore crab, *Carcinus maenas* (Crustacea, decapoda): ultrastructure and functional properties of sensilla sensitive to changes in seawater concentration. *Cell and Tissue Research* **257**(3): 609-622.

Schmidt, M., Ache, B. W. (1992). Antennular projections to the midbrain of the spiny lobster. II. Sensory innervation of the olfactory lobe. *Journal of Comparative Neurology* **318**(3): 291-303.

Schmidt, M., Vanekeris, L., Ache, B. W. (1992). Antennular projections to the midbrain of the spiny lobster. I. Sensory Innervation of the lateral and median antennular neuropils. *Journal of Comparative Neurology* **318**(3): 277-290.

Schmidt, M., Ache, B. W. (1993). Antennular projections to the midbrain of the spiny lobster. III. Central arborizations of motoneurons. *Journal of Comparative Neurology* **336**(4): 583-594.

Schmidt, M., Ache, B. W. (1996a). Processing of antennular input in the brain of the spiny lobster, *Panulirus argus*. I. Non-olfactory chemosensory and mechanosensory pathway of the lateral and median antennular neuropils. *Journal of Comparative Physiology A: Sensory, Neural and Behavioral Physiology* **178**(5): 579-604.

Schmidt, M., Ache, B. W. (1996b). Processing of antennular input in the brain of the spiny lobster, *Panulirus argus*. II. The olfactory pathway. *Journal of Comparative Physiology A: Sensory, Neural and Behavioral Physiology* **178**(5): 605-628.

Schmidt, M., Ache, B. W. (1996a). Processing of antennular input in the brain of the spiny lobster, *Panulirus argus* .1. Non-olfactory chemosensory and mechanosensory pathway of the lateral and median antennular neuropils. *Journal of Comparative Physiology a-Sensory Neural and Behavioral Physiology* **178**(5): 579-604.

Schmidt, M., Grünert, U., Derby, C. D. (2003). *Non-olfactory sensilla mediate chemically induced antennular grooming in the spiny lobster, Panulirus argus*. Society for Neuroscience.

Schmidt, M., Derby, C. D. (2005). Non-olfactory chemoreceptors in asymmetric setae activate antennular grooming behavior in the Caribbean spiny lobster *Panulirus argus*. *Journal of Experimental Biology* **208**(2): 233-248.

Schneider, R. A. Z., Huber, R., Moore, P. A. (2001). Individual and status recognition in the crayfish, Orconectes rusticus: The effects of urine release on fight dynamics. *Behaviour* **138**: 137-153.

Shabani, S., Kamio, M., Derby, C. D. (2006). Chemicals released by injured or disturbed conspecifics mediate defensive behaviors via the aesthetasc pathway in the spiny lobster *Panulirus argus*. *Chemical Senses* **31**: abstract #285.

Shoji, T., Kurihara, K. (1991). Sensitivity and transduction mechanisms of responses to general odorants in turtle vomeronasal system 10.1085/jgp.98.5.909. *J. Gen. Physiol.* **98**(5): 909-919.

Siegel, S., Castellan, N. J. J. (1988). *Nonparametric statistics for the behavioral sciences*. New York, McGraw-Hill.

Sipos, M. L., Wysocki, C. J., Nyby, J. G., Wysocki, L., Nemura, T. A. (1995). An ephemeral pheromone of female house mice: Perception via the main and accessory olfactory systems. *Physiology & Behavior* **58**(3): 529-534.

Spehr, M., Kelliher, K. R., Li, X.-H., Boehm, T., Leinders-Zufall, T., Zufall, F. (2006a). Essential role of the main olfactory system in social recognition of major histocompatibility complex peptide ligands. *Journal of Neuroscience* **26**(7): 1961-1970.

Spehr, M., Spehr, J., Ukhanov, K., Kelliher, K. R., Leinders-Zufall, T., Zufall, F. (2006b). Parallel processing of social signals by the mammalian main and accessory olfactory systems. *Cellular and Molecular Life Sciences* **63**(13): 1476-1484.

Spencer, M. (1986). The innervation and chemical sensitivity of single aesthetasc hairs. *Journal of Comparative Physiology A: Sensory Neural and Behavioral Physiology* **158**: 59-68.

Stacey, N. (2003). Hormones, pheromones and reproductive behavior. *Fish Physiology and Biochemistry* **28**(229-235).

Steullet, P., Derby, C. D. (1997). Coding of blend ratios of binary mixtures by olfactory neurons in the Florida spiny lobster, *Panulirus argus*. *Journal of Comparative Physiology A: Sensory, Neural and Behavioral Physiology* **180**(2): 123-135.

Steullet, P., Cate, H. S., Michel, W. C., Derby, C. D. (2000). Functional units of a compound nose: aesthetasc sensilla house similar populations of olfactory receptor neurons on the crustacean antennule. *Journal of Comparative Neurology* **418**(3): 270-280.

Steullet, P., Dudar, O., Flavus, T., Zhou, M., Derby, C. D. (2001). Selective ablation of antennular sensilla on the Caribbean spiny lobster *Panulirus argus* suggests that dual

antennular chemosensory pathways mediate odorant activation of searching and localization of food. *Journal of Experimental Biology* **204**(24): 4259-4269.

Steullet, P., Krutzfeldt, D. R., Hamidani, G., Flavus, T., Ngo, V., Derby, C. D. (2002). Dual antennular chemosensory pathways mediate odor-associative learning and odor discrimination in the Caribbean spiny lobster *Panulirus argus*. *Journal of Experimental Biology* **205**(6): 851-867.

Storan, M. J., Key, B. (2006). Septal organ of Grüneberg is part of the olfactory system. *Journal of Comparative Neurology* **494**(5): 834-844.

Sullivan, J. M., Beltz, B. S. (2001). Development and connectivity of olfactory pathways in the brain of the lobster *Homarus americanus*. *Journal of Comparative Neurology* **441**(1): 23-43.

Thompson, H., Ache, B. W. (1980). Threshold determination for olfactory receptors of the spiny lobster. *Marine Behavior and Physiology* **7**: 249-260.

Tierney, A. J., Thompson, C. S., Dunham, D. W. (1986). Fine structure of aesthetasc chemoreceptors in the crayfish *Orconectes propinquus*. *Canadian Journal of Zoology* **64**(2): 392-399.

Vickers, N. J., Christensen, T. A., Hildebrand, J. G. (1998). Combinatorial odor discrimination in the brain: attractive and antagonist odor blends are represented in distinct combinations of uniquely identifiable glomeruli. *Journal of Comparative Neurology* **400**(1): 35-56.

Vieuille-Thomas, C., Signoret, J. P. (1992). Pheromonal transmission of an aversive experience in domestic pig. *Journal of Chemical Ecology* **18**(9): 1551-1557.

Webster, D. R., Weissburg, M. J. (2001). Chemosensory guidance cues in a turbulent chemical odor plume. *Limnology and Oceanography* **46**(5): 1034-1047.

Weissburg, M. (2000). The fluid dynamical context of chemosensory behavior. *Biological Bulletin* **198**(2): 188-202.

Weissburg, M. J., Zimmer-Faust, R. K. (1993). Life and death in moving fluids: hydrodynamic effects on chemosensory-mediated predation. *Ecology* **74**(5): 1428-1443.

Weissburg, M. J., Zimmer-Faust, R. K. (1994). Odor plumes and how blue crabs use them in finding prey. *Journal of Experimental Biology* **197**: 349-375.

Weissburg, M. J., Dusenbery, D. B. (2002). Behavioral observations and computer simulations of blue crab movement to a chemical source in a controlled turbulent flow. *Journal of Experimental Biology* **205**(21): 3387-3398.

Weissburg, M. J., Ferner, M. C., Pisut, D. P., Smee, D. L. (2002). Ecological consequences of chemically mediated prey perception. *Journal of Chemical Ecology* **28**(10): 1953-1970.

Weissburg, M. J., James, C. P., Smee, D. L., Webster, D. R. (2003). Fluid mechanics produces conflicting constraints during olfactory navigation of blue crabs, *Callinectes sapidus*. *Journal of Experimental Biology* **206**(1): 171-180.

Wilkens, L. A., Schmitz, B., Herrnkind, W. F. (1996). Antennal responses to hydrodynamic and tactile stimuli in the spiny lobster *Panulirus argus*. *Biological Bulletin* **191**(2): 187-198.

Wroblewska, J., Whalley, S., Fischetti, M., Daniel, P. C. (2002). Identification of chemosensory sensilla activating antennular grooming behavior in the Caribbean spiny lobster, *Panulirus argus*. *Chemical Senses* **27**(9): 769-778.

Wyatt, T. D. (2003). *Pheromones and animal behaviour: communication by smell and taste*. Cambridge, Cambridge University Press.

Xu, F., Schaefer, M., Kida, I., Schafer, J., Liu, N., Rothman, D. L., Hyder, F., Restrepo, D., Shepherd, G. M. (2005). Simultaneous activation of mouse main and accessory olfactory bulbs by odors or pheromones. *The Journal of Comparative Neurology* **489**(4): 491-500.

Yambe, H., Shindo, M., Yamazaki, F. (1999). A releaser pheromone that attracts males in the urine of mature female masu salmon. *Journal of Fisheries Biology* **55**: 158-171.

Yambe, H., Kitamura, S., Kamio, M., Yamada, M., Matsunaga, S., Fusetani, N., Yamazaki, F. (2006). L-Kynurenine, an amino acid identified as a sex pheromone in the urine of ovulated female masu salmon. *Proceedings of the National Academy of Sciences of the United States of America* **103**(42): 15370-15374.

Zimmer-Faust, R. K., Tyre, J. E., Case, J. F. (1985). Chemical attraction causing aggregation in the spiny lobster, *Panulirus interruptus* (Randall), and its probable ecological significance. *Biological Bulletin* **169**: 106-118.

Zimmer-Faust, R. K., Spanier, E. (1987). Gregariousness and sociality in spiny lobsters: implications for den habitation. *Journal of Experimental Marine Biology and Ecology* **105**: 57-71.

Zulandt-Schneider, R. A., Schneider, R. W. S., Moore, P. A. (1999). Recognition of dominance status by chemoreception in the red swamp crayfish, *Procambarus clarkii*. *Journal of Chemical Ecology* **25**(4): 781-794.

Zulandt-Schneider, R. A., Huber, R., Moore, P. A. (2001). Individual and status recognition in the crayfish, *Orconectes rusticus*: The effects of urine release on fight dynamics. *Behaviour* **138**: 137-153.