

Role of the olfactory pathway in agonistic behavior of crayfish, *Procambarus clarkii*

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Abstract Crayfish establish social dominance hierarchies through agonistic interactions, and these hierarchies are maintained through assessment of social status. Chemical signals influence several aspects of fighting behavior, but the specific chemosensory sensilla involved in detecting these signals in crayfish are unknown. The goal of our study was to examine the importance of aesthetasc sensilla—olfactory sensors on the antennules of decapod crustaceans—in regulating changes in fighting behavior in crayfish, *Procambarus clarkii*, over the course of repeated pairings. We selectively ablated aesthetascs from pairs of crayfish after the first day of trials and compared the behavior of these ablated animals to that of pairs of intact controls. Results show that unablated crayfish significantly decreased the number and duration of fights over repeated pairings, whereas crayfish lacking aesthetascs continued to engage in similar amounts of fighting across all three trial days. This difference shows that aesthetascs regulate fighting behavior in *P. clarkii*.

Keywords Aesthetasc · Antennule · Chemical senses · Crustacea · Fighting · Social behavior

Introduction

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. 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; Zulantz 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, 2000; Karavanich and Atema 1998a, b; Zulantz Schneider et al. 1999, 2001; Breithaupt and Eger 2002). Urine signals affect both the duration and intensity of agonistic interactions in *Orconectes rusticus* (Zulantz 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, b) and social status recognition in *O. rusticus* (Zulantz Schneider et al. 2001).

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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.

Crayfish and other crustaceans have chemosensory structures on most body surfaces, but they are most concentrated on the appendages (Fig. 1a) (Holmes and Homuth 1910; Ache and Macmillan 1980; Derby 1982; Schmidt and Gnatzy 1984; Schmidt 1989; Cate and Derby 2001, 2002a; Corotto and O'Brien 2002; Garm et al. 2003; Belanger and Moore 2006). The antennules are primary structures involved in many crustacean chemosensory behaviors, including agonistic behavior and recognition (Rutherford et al. 1996; Karavanich and Atema 1998a, b; Bergman et al. 2003; Johnson and Atema 2005). Each antennule is composed of three basal segments and two distal flagella, the lateral and medial flagella, which bear a complement of sensilla.

Chemosensory information is transmitted from the antennular sensilla to the brain in two anatomically distinct neuronal pathways called the aesthetasc/olfactory lobe pathway and the non-aesthetasc/lateral antennular neuropil pathway (Schmidt et al. 1992; Schmidt and Ache 1992, 1996a, b). 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. 1b). Aesthetascs are a common feature of crustacean chemosensory systems and are exclusively chemosensory sensilla (Holmes and Homuth 1910; Grünert and Ache 1988; Hallberg et al. 1992, 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). Aesthetasc olfactory receptor neurons target the paired olfactory lobes in the brain, which show the typical glomerular organization that characterizes the first order olfactory processing centers of a variety of organisms (Mellon et al. 1989; Mellon and Munger 1990; Sandeman et al. 1992; Schmidt and Ache 1992; Hildebrand and Shepherd 1997; Eisthen 2002; Ache and Young 2005). The non-aesthetasc/lateral antennular neuropil pathway originates in the various types of non-aesthetascs that are distributed throughout the antennular flagella (Schmidt et al. 1992; Schmidt and Ache 1996a). The antennules of *P. clarkii* and other crayfish species contain three main types of non-aesthetasc sensilla, variously referred to 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 respond to mechanosensory stimuli (Chichibu et al. 1978). However, at least a subset, including those in the medial flagellum, also respond to chemical stimuli (Holmes and Homuth 1910; Dunham et al. 1997; Giri and Dunham 1999, 2000; Mellon 2005). Chemical sensitivity of the medial flagellum is 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

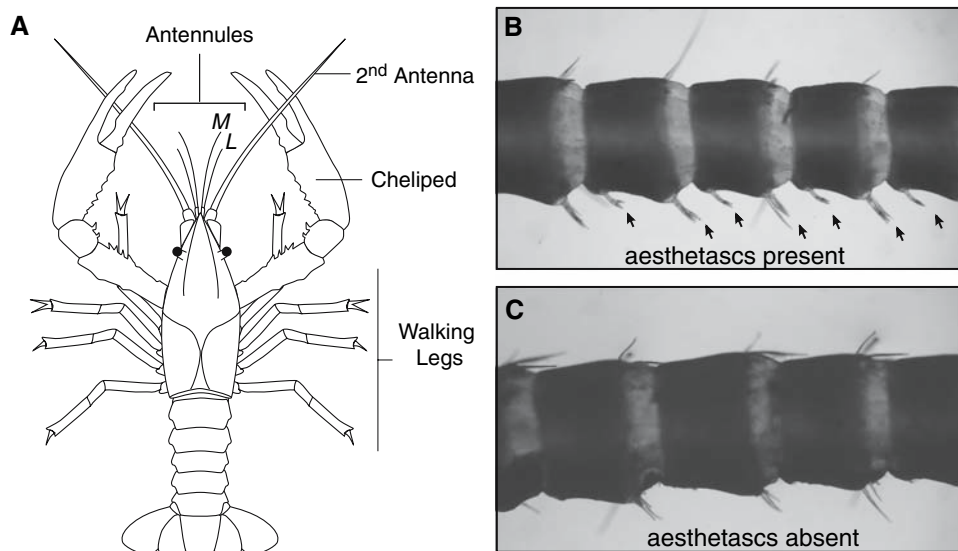


Fig. 1 Red swamp crayfish, *Procambarus clarkii*, and antennular sensilla. **a** Diagram of *P. clarkii* showing the major components of the chemosensory system. The antennular lateral and medial flagella are indicated by *L* and *M*, respectively. **b** Light micrograph of the aesthetasc region of the antennular lateral flagellum of control

crayfish showing both aesthetasc (indicated by *black arrows*) and non-aesthetasc sensilla. **c** Light micrograph of the aesthetasc region of an aesthetasc-ablated crayfish, showing that aesthetasc sensilla have been removed, leaving only non-aesthetasc sensilla

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, which have a stratified organization and function as antennular sensory-motor integration centers and regulate chemo-mechanosensory controlled behaviors such as antennular grooming (Sandeman et al. 1992; Schmidt et al. 1992; Schmidt and Ache 1993, 1996a; Schachtner et al. 2005; 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 was 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.

Materials and methods

Animals

Male Form I crayfish (*Procambarus clarkii*) ranging in total length from 84–98 mm (mean \pm SEM = 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 that was written on their cephalothorax 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 \times 15-cm wide \times 17-cm high) for at least two weeks. 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. Aquarium water was changed twice weekly and an airstone was used to provide constant aeration of water in the aquarium. Animals were fed every other day and maintained on a 12:12 h 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.

To ablate aesthetascs, 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, a process that took about 1 h.

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 \pm 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.

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.

General fight protocol

To examine the effects of aesthetasc ablation on fight dynamics, pairs of crayfish were fought for 1 h periods on three consecutive days. All fights took place within a 38-l aquarium (51-cm long \times 25-cm wide \times 30-cm high) that was lined with white gravel. A grate covered with mesh separated the combatants in between fighting trials. The construction of the divider allowed chemosensory contact 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, and interactions between the crayfish were recorded for 1 h by a video camera mounted in front of the fighting aquarium. The crayfish were separated after 1 h and the barrier replaced to prevent any subsequent interactions before the next observation period. Two hours after conclusion of the first observation period, both crayfish were removed from the aquarium 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 three times (for 1 h each day on three consecutive days). All of the trials were conducted between 0900 and 1400 hours, 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 aquarium on day 1. They remained in the fighting aquarium between pairing days 2 and 3.

Data analysis

The videotapes for all three 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 and 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 s 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 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.

Table 1 Ethogram codes used to score fight intensity levels (see Methods for description; 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

Statistical analysis

We used Friedman analysis of variance, a non-parametric test, to determine whether there were significant differences in behaviors displayed by the crayfish pairs over the three 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.

Results

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 an aquarium. Once established, the dominant-subordinate relationship was generally maintained over the course of repeated pairings. However, the amount of fighting gradually decreased (Fig. 2). Initial pairings were characterized by numerous long episodes of intense agonistic interactions that established the dominant or subordinate status of each combatant. In subsequent pairings, the overall percentage of the trial spent fighting (Fig. 2a), the number of encounters (Fig. 2b), and the maximum encounter duration (Fig. 2c) decreased from initial levels.

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. Thus, a decrease in the intensity of fighting behavior does not always accompany the establishment of stable dominance relationships.

The dominant-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 3 days. The fifth pair of crayfish experienced a hierarchy reversal between trial days 1 and 2. In this instance, the

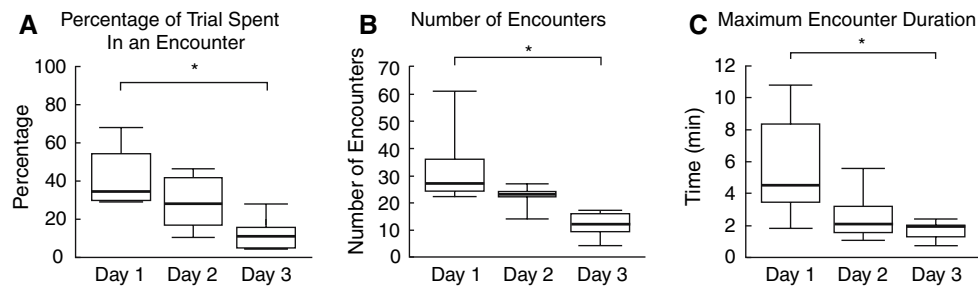


Fig. 2 Fight dynamics of control crayfish pairs. Box plots show median (solid black line), interquartile range (box length), and minimum and maximum values (error bars) for the five pairs of

control crayfish on each trial day. Statistically significant differences ($P < 0.05$, Friedman analysis of variance, with multiple comparisons post hoc tests) are indicated by asterisk

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. Overall, the eventual dominant initiated and won a majority of the encounters on all three trial days: the median % trials initiated was 88, 93, and 78% for trial days 1, 2, and 3 respectively; and median % trials won was 89, 79, and 89% for trial days 1, 2, and 3, respectively. Thus, *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 reducing 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 (Fig. 3). Ablated crayfish pairs spent similar overall percentages of each trial day engaged in encounters (Fig. 3a), and continued to engage in similar numbers of fights (Fig. 3b) with similar maximum durations (Fig. 3c) on all three trial days. 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 the first trial day 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 necessary for other aspects of dominance hierarchy formation in *P. clarkii*.

Discussion

Aesthetascs are essential in regulating crayfish aggression

Our results show that the amount of fighting between control pairs of crayfish, *Procambarus clarkii*, gradually decreased over the course of three daily pairings (Fig. 2).

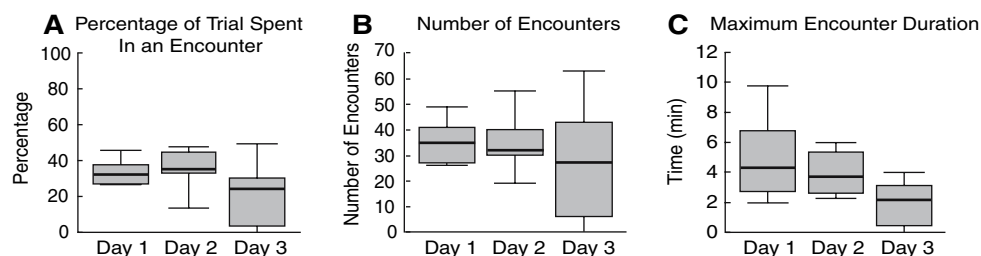


Fig. 3 Fight dynamics of aesthetasc-ablated crayfish pairs. Box plots show median (solid black line), interquartile range (box length), and minimum and maximum values (error bars) for the six pairs of

aesthetasc-ablated crayfish on each trial day. No statistically significant differences were observed ($P > 0.05$, Friedman analysis of variance)

This phenomenon is not unique to *P. clarkii* (Copp 1986; Issa et al. 1999; Goessmann et al. 2000; Zulantz Schneider et al. 2001). In addition, we show that the amount of fighting between aesthetasc-ablated pairs of crayfish remained at similar levels over all the three days of pairing (Fig. 3). We did not observe any overt differences in the general (i.e., non-fighting) behavior of control and ablated animals, and thus we do not believe that the constant amount of fighting of the ablated crayfish is attributed to a general increase in the level of reactivity due to ablation. Thus, we conclude that the aesthetasc/olfactory lobe chemosensory pathway plays an essential role in crayfish agonism.

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, 2000; Karavanich and Atema 1998a, b; Zulantz 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, b). Urine signals are also important for regulating aggression levels in crayfish (Zulantz 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 (Zulantz 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 (Zulantz Schneider et al. 1999, 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 accurately assess 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 three 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 gregarious Caribbean spiny lobsters, *Panulirus argus* (Horner et al. 2008). The aesthetasc/olfactory lobe pathway also plays a necessary role in courtship behavior in blue crabs (*Callinectes sapidus*) (Gleeson 1982, 1991). 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.

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