Perception of the steroid hormone 20-hydroxyecdysone modulates agonistic interactions in *Homarus americanus*

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The American lobster, *Homarus americanus*, becomes more aggressive just before moulting. This increased aggression is accompanied by an increase in the blood and urine titres of the moulting hormone, 20-hydroxyecdysone (20-HE). During combats with conspecifics, lobsters urinate upon each other to signal their aggressive and/or sexual status. We videotaped staged combats between large intermoult nongravid female lobsters exposed to 20-HE, α-ecdysone or artificial sea water (ASW) and small nongravid female lobsters that could not smell. The nephropores of both combatants were plugged to prevent urine-release. Aggressive, defensive and avoidance behaviours were ranked according to aggressiveness in a rank of aggression hierarchy, which included opponent-directed and (nonopponent) redirected behaviours. Exposure to 20-HE increased the state of arousal of large lobsters: they performed more highly aggressive behaviours, more defensive and more avoidance behaviours than either α-ecdysone-exposed or ASW-exposed lobsters. The small opponents of large 20-HE-exposed lobsters performed more aggressive behaviours. The summed aggressive intensity of all behaviours of both lobsters in a fight was greater in 20-HE fights than in ASW or α-ecdysone fights. The difference in aggressive intensity of all behaviours between the 20-HE-exposed lobsters and their opponents was significantly smaller in 20-HE fights than in α-ecdysone or ASW fights. Our results indicate that 20-HE exposure alters the agonistic behaviour of a lobster, which then evokes an increase in the aggressive behaviour of an opponent lobster. The findings suggest that 20-HE acts as a pheromone modulating aggressive interactions in lobsters.

Keywords: 20-Hydroxyecdysone; aesthetasc; aggressive behaviour; chemoreceptor; American lobster; *Homarus americanus*; moulting hormone; pheromone; steroid

Agonistic encounters are common amongst crustaceans and are important in establishing social hierarchies. In the American lobster, *Homarus americanus*, a commonly used model for the study of aggressive behaviour, many factors affect aggression, such as size (Vye et al. 1997), health (O’Neill & Cobb 1979), territory (Hyatt & Salmon 1978), moult stage (Tamm & Cobb 1978) and memory (Scrivener 1971; Karavanich & Atema 1991; Huber & Kravitz 1995; Rutishauser et al. 2004). Interactions between two lobsters often begin with visual displays and chemical signalling from both lobsters, and can, in extreme cases, escalate into acts of physical injury. Usually the larger lobster can induce its smaller opponent to retreat, thus becoming dominant over the small lobster (Scrivener 1971; Livingstone et al. 1980; Vye et al. 1997). The interactive behaviours are stereotypic and fairly easy to recognize; they have been catalogued and evaluated by a number of different ethograms (Tamm & Cobb 1978; Huber et al. 1997; Mello et al. 1999; Bolingbroke & Kass-Simon 2001; reviewed in Kravitz 2000).

Remarkable changes in behaviour take place over the course of a lobster’s moult cycle (Herrick 1909; Tamm & Cobb 1978; Cromarty et al. 1991, 2000). Just prior to moulting, lobsters become very aggressive (Tamm & Cobb 1978). Tamm & Cobb observed that premoult lobsters win in combats against either postmoult or intermoult lobsters. The physiological basis of moult-related
differences in behaviour has been addressed in a number of studies. Snyder & Chang (1991a,b) found increased titres of the moulting hormone, 20-ecdysone, and its active principle, 20-hydroxyecdysone (20-HE) in both haemolymph and urine of premoult (D stage) lobsters. Premoult haemolymph, when perfused over the dactyl opener muscle of the claw (used in aggressive displays) of intermoult lobsters increased the excitatory junctional potential (EJP) amplitude and decreased inhibitory junctional potential (IJP) amplitude at the neuromuscular junction (Schwanke et al. 1990). 20-HE also produced increased EJP amplitude in the opener muscle of the lobster claw, while decreasing EJP amplitude at the phasic flexor muscle of the tail, which is used in escape swimming (Cromarty & Kass-Simon 1998). When 20-HE was injected into intermoult lobsters it caused them to become more aggressive than noninjected control lobsters during experimentally staged confrontations (Bolingbroke & Kass-Simon 2001). In crayfish, Cooper & Ruffner (1998) report that 20-HE decreased neurotransmitter release in walking legs. Cooper et al. (2003) later observed that a mixture of 20-HE and serotonin was more effective in increasing neuron firing frequency in the slow-adapting muscle receptor organ (MRO) of the abdomen than was either neuromodulator alone. Together, this work suggests that 20-HE acts as a hormonal modulator, or a hormone, that alters the internal physiology and therefore the behaviour of crustaceans.

Work by Atema and colleagues have provided convincing evidence indicating the presence of a pheromone in the urine of crustaceans that regulates agonistic behaviour (Atema & Voigt 1995). At the onset of an agonistic encounter between two lobsters, both lobsters excrete urine from nephropores located at the basal segments of their antennae; lobsters urinate more often during fights than during nonsocial activities or inactivity (Breithaupt & Atema 1993, 2000). The urine is fanned towards the opponent where it is perceived and processed. Eventual winners of fights are more likely to release more urine than eventual losers, and the probability of urine release increases with increasing levels of aggression. Breithaupt & Atema (2000) showed a direct correlation between the timing and amount of urine released and the level of aggressive behaviour of a lobster. Previously established dominance relationships in lobsters and crayfish are abolished when urine excretion is prevented (Breithaupt et al. 1999; Breithaupt & Atema 2000; Breithaupt & Eger 2002). Breithaupt et al. suggest that therefore individual/status recognition requires a urine signal, since lobsters that can urinate freely are able to recognize previously fought opponents over a period of days (Karavanich & Atema 1998; Rutishauser et al. 2004). Thus, urine, most likely perceived by the lateral aesthetascs, can carry information about the lobster, including its identity, sex, health and level of aggression (Breithaupt & Atema 1993; Atema 1996; Karavanich & Atema 1998). The major receptors for urine recognition appear to be in the lateral antennule filaments, since lobsters whose lateral antennular filaments are destroyed fight twice as long with known opponents (Karavanich & Atema 1998), and lobsters whose aesthetascs on the lateral flagellum are shaved off fail to recognize recently fought opponents (Johnson & Atema 2005). Similar studies in crayfish point to the importance of olfaction in agonistic encounters. In crayfish, urine is used to signal the behavioural state of a stressed individual and to affect its role in social interactions (Zulandt Schneider & Moore 2000; Zulandt Schneider et al. 2001; Bergman et al. 2005; Bergman & Moore 2005). Bergman et al. (2003) found that crayfish were significantly more likely to initiate a fight against an anosmic opponent, which, they suggest, could be a response to the reluctance of the anosmic opponent to initiate a fight. Crayfish, fighting against an olfactory-impaired opponent were slower to escalate the intensity of a fight compared to those who fought nonimpaired crayfish. Although the accumulated evidence for a urine-borne chemical signal modulating agonistic encounters in lobsters and crayfish is strong, the specific chemicals and receptors involved have yet to be identified.

Recent evidence in other systems (e.g. Drosophila: Freeman et al. 1999; fish: Sorensen & Scott 1994; reviewed in: Robison et al. 1998; Poling et al. 2001) has implicated the use of steroid hormones as pheromonal signals in social interactions. Most aquatic pheromone studies, including work done on brachyuran crabs (Eales 1974; Christofferson 1978; Gleeson 1980), and macruran crayfish and lobsters (Atema & Engstrom 1971; Ameyaw-Akufo & Hazlett 1975; Tierney & Dunham 1984; Atema & Cowan 1986), have focused on signals exchanged during mating and courtship. Thus, Kitteredge et al. (1971) provided evidence that the moulting hormone functions as a sex pheromone in male lined shore crab, Pachygrapsus crassipes, brown rock crab, Cancer antennarius and Yellow rock crab, Cancer anthonyi; however, later experiments by Atema & Gagosian (1973) on male American lobsters did not confirm their hypothesis. Electrophysiological studies on male and female spiny lobsters, Panulirus interruptus (Spencer & Case 1984) showed that chemoreceptors in the lateral antennule of the male respond to both 20-ecdysone and 20-HE.

In light of the findings that urine acts as a chemical signal during confrontations, and that 20-HE is present in high titres in the urine of premoult aggressive lobsters, we have undertaken a series of studies to investigate the possibility that 20-HE is a pheromone affecting aggressive behaviour. Our current electrophysiological studies indicate that 20-HE is perceived by the aesthetasc sensilla in nongravid female Homarus americanus (S. I. Cromarty, D. Coglianese, M. Sipala, L. Martin & G. Kass-Simon, unpublished data): dose-dependent responses of broadly tuned olfactory receptor neurons (ORNs) to 20-HE are comparable to the ORN responses to food odorants. To determine whether exposure to 20-HE (at levels found in premoult urine) alters the behaviours of the combatants in agonistic encounters, we staged combats between smaller, olfaction-blocked lobsters and larger, olfaction-nonblocked lobsters whose antennules were exposed to 20-HE, 20-ecdysone or artificial sea water (ASW). Our confrontations were staged between nongravid, intermoult female lobsters in a tank filled only with sea water so that neither environmental cues (for food or shelter), nor reproductive cues were present. Similar confrontations between intermoult males are currently in progress. Our
ethogram was designed to avoid the concept of dominance in order to remove this variable from the straightforward analysis of aggressiveness.

MATERIALS AND METHODS

To permit comparisons with our earlier studies on gravid and nongravid female lobsters, the experimental conditions, animals and ethogram are essentially those used in our previous experiments with slight modifications (Mello et al. 1999; Bolingbroke & Kass-Simon 2001).

Lobster Procurement and Maintenance

All lobsters were procured from the Rhode Island Department of Fish and Wildlife and were caught in Narragansett Bay, Rhode Island, U.S.A. Lobsters used in combats were nongravid, sub-lega-sized females in intermollus, stage C (Aiken 1973, 1980), with a carapace length of less than 85 mm. Upon collection, the lobsters were weighed and measured for carapace length and length of cutter claw. They were divided into two categories based on carapace length: small lobsters (carapace length < 76 mm) and large lobsters (carapace length > 82 mm). To prevent injury and to keep lobsters from establishing dominance hierarchies, the lobsters were placed into individual compartments in storage tanks. All storage tanks were supplied with filtered circulating sea water from Narragansett Bay. The temperature of the water ranged from 12°C to 18°C and with a salinity range of 28–33 ppt. The lobsters were maintained under a 12 h light:dark cycle and fed twice weekly with scraps of fish and shellfish supplied by the local fish market. All lobsters were returned to holding tanks immediately after an experiment and released into Narragansett Bay after a recovery period of 1–2 days.

Experimental Conditions and Prefight Protocol

Experimental lobsters were not fed 48 h prior to a fight. No lobsters were kept longer than 2 weeks before a fight. Each lobster was used only once. Only lobsters in perfect condition were used (all claws, pleopods and periopods intact, no shell disease, complete intact antennae and antennules). A round tank 78 cm in diameter was used as the experimental ‘fighting’ arena. A Panasonic WV-CD20 video camera was positioned directly above the tank to record combats for later analysis. Combats were recorded for 30 consecutive minutes.

Each fight consisted of one small and one large lobster in order to bias the fight in favour of the larger combatant, since it has been shown that large lobsters win over small ones in combat (Scrivener 1971). There was a minimum of 10% difference in carapace length and 5% difference in cutter claw length (Scrivener 1971) between combatants. One hour before the fight, both lobsters were catheterized using a modified method of Breithaupt et al. (1999). Simple nephropore ‘plugs’, large enough to contain more than the maximum amount of urine released by a fighting lobster over a 30 min time span (Breithaupt & Atema 2000), were constructed by cutting small pieces of tubing and sealing off one end with waterproof cyanoacrylate glue (Zap-A-Gap, Super Glue Corp., Rancho Cucamonga, CA, U.S.A.). These plugs were then glued around each nephropore with the same waterproof glue, thereby effectively sealing the nephropores and preventing urine release into the water. The nephropore-blocking technique was tested during pilot studies by injecting lobsters with methylene blue after blocking nephropores and checking that no blue dye leaked out of the plugs when the lobsters urinated.

Before each fight, the smaller opponent’s antennules were dipped into distilled water for 15 min to disable olfactory receptors in their antennules. This method immediately blocks chemoreceptors in the walking legs of Homarus americanus, and the effect lasts from 1 day to 1 week (Derby & Atema 1982b). To ensure that the larger opponent was subjected to the same physical stress, the larger opponent’s antennules were dipped into sea water for 15 min, which does not affect their olfactory ability. Although it has been shown that lobsters that are anosmic fight longer (Johnson & Atema 2005), this finding does not significantly affect our experimental results, because all small opponents of treated lobsters (20-HE-, z-ecdysone- and ASW-exposed) were anosmic.

A thin plastic tube (inside diameter 1.02 mm, outside diameter 2.16 mm) was glued to the carapace just behind the rostrum of each lobster. The tube for each lobster led to the outside of the tank where it was connected to a 10 ml syringe with 18-gauge needle that could be manipulated by the experimenter. In this way, an artificial signal could be puffed onto a lobster without the experimenter disturbing the encounter.

Fights

The experimental protocol was as follows. One small and one large lobster were paired for each combat. Large lobsters, whose antennules were not blocked, were puffed with one of the three test substances during fights. The test substances were puffed directly onto the larger lobster to ensure that the substances reached the larger lobster’s antennules, and also to ensure that all larger lobsters received the same puffing treatment, independent of proximity to the smaller lobster (which would have been a factor had we attempted to recreate urine fanning from the small lobster to the large lobster). The nephropores of both small and large lobsters were blocked to prevent urine release. Over the course of the 30 min confrontation, either ASW, z-ecdysone dissolved in ASW or 20-HE dissolved in ASW was puffed onto the antennules of the larger opponent in 1 ml aliquots (z-ecdysone and 20-HE purchased from Sigma, St Louis, MO, U.S.A.). A total of seven puffs of 1 ml each, one every 4 min, was administered during each fight, with the first puff being given as soon as the lobsters came within one body length of one another. Although none of the small lobsters (opponents of 20-HE-, z-ecdysone- or ASW-exposed) could smell, sea
water was puffed onto the antennules of the smaller opponent in all fights to ensure that responses were not due to the mechanical disturbance of puffing. Preliminary tests with methylene blue-dyed water showed that the artificial signal flows over the antennules of the puffed lobster when treated in this fashion. Snyder & Chang (1991a,c) found that mid-D stage lobsters had a 20-HE haemolymph concentration of 600 ng/ml. They also found that 95% (or 570 ng/ml) of this ecdysteroid concentration was excreted in the urine. To achieve the correct ecdysonic urine concentration of the small lobsters, 20-HE was dissolved in ASW to a concentration of 570 ng/ml, while z-ecdysone was dissolved in ASW to 23 ng/ml, also to 95% of its haemolymph concentration (Snyder & Chang 1991c). The maximum possible volume of haemolymph in a smaller lobster was determined by correlating the average wet-weight of small lobsters to a standard volume given on a previously established linear curve that plotted measured haemolymph volumes against measured weights (Bolingbroke & Kass-Simon 2001). This curve was then used to estimate the appropriate concentration and volume of 20-HE or z-ecdysone to puff over the larger lobster.

A total of 30 interactions were carried out: 10 with 20-HE puffed over the large lobster, 10 with z-ecdysone and 10 with ASW. A total of 60 lobsters (30 small, 30 large) were used. There was no significant difference in the weight, carapace length or claw length among the 20-HE-exposed, used. There was no significant difference in the weight, carapace volume of 20-HE or puffed over the large lobster, 10 with z-ecdysone-exposed or ASW-exposed lobsters, or among the opponents of the 20-HE-exposed, z-ecdysone-exposed or ASW-exposed lobsters (weight: \( F_{2,26} = 0.75, P = 0.48 \); carapace length: \( F_{2,26} = 2.39, P = 0.11 \); claw length: \( F_{2,26} = 0.09, P = 0.91 \)).

**Data Analysis**

The tapes of the recorded fights were coded so that the two people analysing the fights had no knowledge of the type of fight being analysed. Although the ethogram was used in our previous studies, some readers may be unfamiliar with it, so we reiterate the considerations used in its application. The ethogram was originally developed by Mello et al. (1999) for a study on gravid female lobsters. The ethogram was constructed naively and without regard to previous ethograms in the literature, ‘because, from an anthropomorphic viewpoint, during a combative interaction some behaviours appeared to be clearly aggressive, others appeared to be defensive, and still others appeared to be avoiding in character’ (Mello et al. 1999). Although behavioural sequences during agonistic encounters have been described by others (Scrivener 1971; Atema & Cobb 1980; Huber & Kravitz 1995), our ethogram uniquely permits the quantification of behaviours according to their aggressive content and allows that content to be statistically analysed (Tables 1, 2). At the top of the hierarchy are placed aggressive behaviours in descending order of their apparent intensity. Defensive behaviours are then ranked from the ‘most aggressive’ defensive behaviour to the ‘least aggressive’ defensive behaviour. Avoidance behaviours, placed last, are ranked from least intense avoidance to most intense avoidance because the least intense avoidance behaviour is considered to have the most aggressive content. All behaviours are placed in a ranked continuum and, for convenience, given rank values on an even number scale from 150 to 2. Aggressive behaviours are defined as behaviours designed to cause damage to the opponent (e.g. grabbing, pulling, hitting) or to signal a threat of such behaviour (e.g. meral spread, large or small ready posture). This definition is consonant with that used by Scrivener (1971), Atema & Cobb (1980) and Huber & Kravitz (1995). Defensive behaviours are meant to ward off an attack. Therefore, although certain behaviours are categorized as both aggressive and defensive (e.g. antenna whipping), whether a given behaviour was intended as aggressive or defensive is determined by its context. Avoidance behaviours occur when an animal is trying to exit a confrontation (Mello et al. 1999; Bolingbroke & Kass-Simon 2001). This is also the Scrivener definition of avoidance behaviour. Using this ethogram, the relative aggressiveness of each combatant and the overall aggressive content of a fight can be quantified. Statistical analysis was performed on three parameters (given for each lobster in each fight): (1) frequency of observed behaviours per 30 min bout (\( f \)), (2) rank frequency (\( RF \)) and (3) average rank (\( AR \)). RF values are the number of times that a behaviour occurs (\( f \)) in a 30 min interaction multiplied by its rank (\( R \)) in the ethogram. AR values are calculated as the average of the sum of the RF values divided by the frequency of those behaviours for each lobster; this reflects the degree of aggressiveness of the behaviours of each combatant. The summed RF values of both combatants in a fight (\( RF_{20HE-O20HE} \), \( RF_{E-E} \), \( RF_{ASW-OASW} \)) of all behaviours together reflect the overall ‘aggressive, defensive and avoidance intensity’ of the fight, while the subtracted differences (\( RF_{20HE-O20HE} \), \( RF_{E-E} \), \( RF_{ASW-OASW} \)) reveal the relative aggressiveness of the two combatants in each category of fights. The following abbreviations are used: 20-HE-exposed lobsters (\( E_{20HE} \)), z-ecdysone-exposed lobsters (\( E_{zE} \)) and ASW-exposed lobsters (\( E_{ASW} \)); opponents of 20-HE-exposed lobsters (\( O_{20HE} \)), opponents of z-ecdysone-exposed lobsters (\( O_{zE} \)) and opponents of ASW-exposed lobsters (\( O_{ASW} \)). Data analysis follows that of Mello et al. (1999) and Bolingbroke & Kass-Simon (2001). Single factor analyses of variance (ANOVA) were used to analyse the various planned comparisons between \( E_{20HE} \), \( E_{zE} \) and \( E_{ASW} \) and between \( O_{20HE} \), \( O_{zE} \) and \( O_{ASW} \). ANOVAs were also used to compare the summed RF values of the combatants in a fight (\( RF_{20HE-O20HE} \), \( RF_{E-E} \), \( RF_{ASW-OASW} \)) of all behaviours, and the subtracted differences of the RF values (\( RF_{20HE-O20HE} \), \( RF_{E-E} \) and \( RF_{ASW-OASW} \)) of all behaviours. The statistical tests were performed on Microsoft Excel software. Values were considered significant at \( P < 0.05 \).

Lobsters directed behaviour not only towards their opponents, but also towards the wall that surrounded the arena. These behaviours, which were noted in our previous studies, appear to be either a substitute for actual opponent-directed behaviour, or to act as a signal to opponents. They have been designated ‘wall behaviours’. Wall behaviours, like opponent-directed behaviours, are ranked from most aggressive to least aggressive and are inserted before the opponent-directed defensive behaviours on the rank of aggression scale (Table 1). All analyses...
were done both with and without wall behaviours. Since the analyses with and without wall behaviours revealed no significant differences (with one exception, presented in the Results), only the results that included wall behaviours are presented. Aggressive, defensive and avoidance behaviours were all analysed separately. However, in a post hoc analysis, we compared the total number of wall behaviours for all the lobsters in each treatment group.

**RESULTS**

**All Behaviours**

The overall aggressive intensity (RF) of all behaviours of the combatants in fights with 20-HE-exposed lobsters (RF$_{20HE-O20HE}$) was significantly higher than the aggressive intensity in fights with either z-ecdysone-exposed (RF$_{zE-OzE}$) or ASW-exposed (RF$_{ASW-OASW}$) lobsters, so that RF$_{20HE-O20HE}$ > (RF$_{zE-OzE} = RF_{ASW-OASW}$) ($F_{2,26} = 11.65, P = 0.0002$; Fig. 1a). The subtracted difference of the aggressive intensities of the combatants in fights with 20-HE-exposed lobsters (RF$_{20HE-O20HE}$) was significantly smaller than the subtracted difference in the intensities of the behaviours of the combatants in fights with either z-ecdysone-exposed (RF$_{zE-OzE}$) or ASW-exposed (RF$_{ASW-OASW}$) lobsters, so that RF$_{20HE-O20HE}$ < (RF$_{zE-OzE} = RF_{ASW-OASW}$) ($F_{2,26} = 4.21, P = 0.02$; Fig. 1b).

**Aggressive Behaviours**

There was no significant difference in the frequency (F) of aggressive behaviours among lobsters exposed to 20-HE (F$_{20HE}$), z-ecdysone (F$_{zE}$) and ASW (F$_{ASW}$) either with or without wall behaviours ($F_{2,26} = 0.477, P = 0.625$). However, opponents of 20-HE-exposed lobsters (FO$_{20HE}$) performed more aggressive behaviours than opponents of z-ecdysone-exposed (FO$_{zE}$) or ASW-exposed (FO$_{ASW}$) lobsters, so that FO$_{20HE}$ > (FO$_{zE} = FO_{ASW}$) ($F_{2,26} = 19.91, P = 0.000005$; Fig. 2a).

The average rank (AR), or degree of aggressiveness, of the aggressive behaviours was greater for 20-HE-exposed lobsters than for z-ecdysone-exposed and ASW-exposed lobsters, so that AR$_{20HE}$ > (AR$_{zE} = AR_{ASW}$) ($F_{2,26} = 4.32, P = 0.02$). There were no significant differences in AR values among the opponents of these lobsters ($F_{2,26} = 0.291, P = 0.75$; Fig. 2b).

**Defensive Behaviours**

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<th>Avoidance behaviours</th>
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</table>
Defensive Behaviours

Lobsters exposed to 20-HE performed significantly more defensive behaviours than lobsters exposed to \( \alpha \)-ecdysone or ASW, so that \( F_{20\text{HE}} > F_{\alpha\text{E}} = F_{\text{ASW}} \) \( (F_{2,26} = 9.52, P = 0.0007) \). There were no significant differences in frequency of defensive behaviours among the opponents of lobsters exposed to 20-HE, \( \alpha \) -ecdysone or ASW \( (F_{2,26} = 3.03, P = 0.066; \text{ Fig. 3a}) \).

There was no significant difference in the average rank of defensive behaviours (AR values) between 20-HE-exposed, \( \alpha \)-ecdysone or ASW \( (F_{2,26} = 0.395, P = 0.678) \). When wall behaviours were
excluded (i.e. when only those behaviours directed against exposed lobsters were considered), the average rank of the defensive behaviours of the small opponents of 20-HE-exposed lobsters was greater than that of the small opponents of either α-ecdysone-exposed or ASW-exposed lobsters, so that $\text{AR}_{20\text{HE}} > (\text{AR}_{α\text{E}} = \text{AR}_{\text{ASW}})$ ($F_{2,26} = 4.77, P = 0.02$; Fig. 3b). With wall behaviours, $\text{AR}_{20\text{HE}} = \text{AR}_{α\text{E}} = \text{AR}_{\text{ASW}}$ ($F_{2,26} = 1.66, P = 0.21$; data not shown).

Avoidance Behaviours

20-HE-exposed lobsters performed significantly more avoidance behaviours than either the α-exposed or ASW-exposed lobsters, so that $F_{20\text{HE}} > (F_{α\text{E}} = F_{\text{ASW}})$ ($F_{2,26} = 7.95, P = 0.002$). There were no significant differences in the frequency of avoidance behaviours among the opponents of lobsters exposed to 20-HE, α-ecdysone or ASW ($F_{2,26} = 2.64, P = 0.09$; Fig. 4).

Post Hoc Analysis of Wall Behaviours

A post hoc analysis of wall behaviours revealed that opponents of lobsters exposed to 20-HE performed more wall behaviours than opponents of lobsters exposed to α-ecdysone or ASW, so that $F_{20\text{HE}} > (F_{α\text{E}} = F_{\text{ASW}})$ ($F_{2,26} = 8.92, P = 0.001$; Fig. 5).

DISCUSSION

In this study we have shown that in a staged confrontation between a large and small female lobster, puffing 20-HE onto the antennules of the larger combatant modifies the behaviour of both lobsters. The overall intensity of behaviours, that is, the rank frequency (RF factor) of the summed behaviours of both lobsters, was significantly greater in 20-HE fights than the summed intensity of the two combatants in either α-ecdysone or ASW fights. At the same time, the differences in intensity of the behaviours (the subtracted RF factors) of the two opponents in these fights were significantly less than the differences in α-ecdysone or ASW fights. This suggests that 20-HE exposure to one of the combatants is sufficient to remodel the dynamics of an agonistic interaction. Lobsters exposed to 20-HE became more aroused than did their nonexposed counterparts: they performed more highly aggressive behaviours (increased AR), more defensive behaviours (increased F) and more avoidance behaviours (increased F) than those exposed to either α-ecdysone or ASW. The small opponents of exposed animals became more aggressive (increased F), which we interpret to be a response to the behaviour of the larger lobster. Together, these changes resulted in an increase in the overall intensity of the interaction. Our findings present the first evidence that 20-HE, at levels found in the urine of premoult lobsters, acts as a pheromone modulating the agonistic interactions of crustaceans.

As discussed in the Introduction, perception of 20-HE by the antennules is concordant with the many studies pointing to the importance of urine signalling in crustacean social interactions. By creating artificial signals, consisting of only 20-HE, or only α-ecdysone, or only ASW, our results show that exposing the antennules to 20-HE alone (but not α-ecdysone alone) is sufficient to modify the behavioural responses in combatant lobsters. Although it is possible that other chemoreceptors (i.e. those located on the walking legs, maxillipeds, antennae...
or medial antennules) might have perceived the steroid signals (Derby & Atema 1982a), our experiments were designed to focus only on the lateral filaments because of the strong physiological evidence in the literature that the primary olfactory chemoreceptors (for arousal and plume tracking) are on the lateral filament of the antennules (Derby & Atema 1982b; Spencer & Case 1984; Cromarty et al., unpublished data). If nonantennular perception of the steroid signal had played a significant role, one would expect that the small opponent of the large 20-HE-exposed lobster would have displayed behaviours that mimicked those of the exposed lobster, since the puffed 20-HE must eventually have flowed to the smaller lobster.

In our experiments, exposure to 20-HE resulted in an increased state of arousal in the large lobster (compared to the large lobsters exposed to \( \alpha \)-ecdysone or ASW), whereas the smaller unexposed opponent of the 20-HE-exposed lobster became more aggressive (compared to its smaller counterparts in the fights with \( \alpha \)-ecdysone or ASW). Previous studies have reported other situations in which the smaller animal displays increased aggression. Mello et al. (1999) found that, in agonistic encounters, small gravid females are more aggressive than their small nongravid counterparts, and similar to our studies, the large opponent of the small gravid animals displayed increased arousal. In contrast, a study by Bolingbroke & Kass-Simon (2001) showed that while smaller lobsters, injected with 20-HE, performed more aggressive behaviours than their non-20-HE-injected counterparts (when paired with larger noninjected lobsters), the larger opponents in these same combats were less aggressive (decreased F) than their counterparts (larger opponents of non-20-HE-injected animals). Thus, although the results of the Bolingbroke & Kass-Simon study might have led us to hypothesize that our large 20-HE-exposed lobsters would also have become less aggressive (because in the Bolingbroke & Kass-Simon study there would have been a high concentration of 20-HE in the injected animal’s urine), our study cannot be compared with either the Bolingbroke & Kass-Simon or the Mello et al. (1999) study. This is because, unlike the previous studies, in our study the animals were deprived of all other signalling molecules possibly contained in the urine that might have given other information to the receiver including information on sex, health and social status (cf. Karavanich & Atema 1998; Behringer et al. 2006). Therefore, because

![Figure 2](attachment:figure2.png)

**Figure 2.** (a) Frequencies and (b) average rank values of aggressive behaviours in fights in which the large combatant was exposed to 20-HE, \( \alpha \)-ecdysone or ASW. Values are means ± SD. \( N = 10 \) fights/treatment (20 lobsters/treatment). Note scale differences in the Y axes. Asterisks indicate significant differences in adjacent columns. Abbreviations as given in Fig. 1.
in our study all the small animals were anosmic and all the large animals were exposed to a single substance (20-HE, \(\alpha\)-ecdysone or ASW), the differences that we observed in the individual behavioural responses and in the interactions between the small and the large combatants in the three types of fights can only be due to exposure of the large animals to the test substances.

**Redirected Wall Behaviours**

Our ethogram included behaviours that were directed towards an opponent and behaviours that were redirected away from an opponent towards the wall of the fighting arena. Although redirected behaviours have been observed in other animals (e.g. gulls) (Tinbergen 1959; Hinde 1966), their purpose is not known. As we have previously pointed out (Bolingbroke & Kass-Simon 2001), it is possible that redirected wall behaviours could be used as a visual or acoustic signalling device towards another lobster; the latter hypothesis is currently being explored in lobsters by Henninger & Watson (2005). Another hypothesis is that behaviours directed away from the opponent represent ‘sublimated aggression’. In our experiments, small opponents of the larger

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**Figure 3.** (a) Frequencies and (b) average rank values of defensive behaviours in fights in which one of the combatants was exposed to 20-HE, \(\alpha\)-ecdysone or ASW. Values are means ± SD. \(N = 10\) fights/treatment (20 lobsters/treatment). Note scale differences in the Y axes. Asterisks indicate significant differences in adjacent columns. Abbreviations as given in Fig. 1.

**Figure 4.** Frequencies of avoidance behaviours in fights in which one of the combatants was exposed to 20-HE, \(\alpha\)-ecdysone or ASW. Values are means ± SD. \(N = 10\) fights/treatment (20 lobsters/treatment). Note scale differences in the Y axes. Asterisks indicate significant differences in adjacent columns. Abbreviations as given in Fig. 1.
Avoidance Behaviours

The behavioural hierarchy used in our experiments also included behaviours that were intended to remove a lobster from a confrontation. These behaviours, known as avoidance behaviours, have the lowest aggressiveness ranking. Our results indicate that large lobsters exposed to 20-HE performed more avoidance behaviours (increased F) than large zE-exposed or ASW-exposed lobsters, while their smaller opponents did not perform more avoidance behaviours than the smaller opponents of zE-exposed or ASW-exposed lobsters. This suggests that if 20-HE signals aggressiveness in an opponent, it evokes a variety of self-protective behaviours: increased aggressiveness, defensiveness and avoidance.

In summary, 20-HE, the moulting hormone that is present in high titres in premoult lobsters, appears to function not only internally as a humoral modulator, but also externally, to signal increased aggressiveness to potential conspecific competitors and thus preempt a confrontation during the critical time at ec dysis when the lobster is vulnerable to predation.

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References


Figure 5. Frequencies of aggressive wall behaviours of opponents in fights in which one of the combatants was exposed to 20-HE, zE-ecdysone or ASW. Values are means ± SD. N = 10 fights/treatment (20 lobsters/treatment). Asterisks indicate significant differences in adjacent columns. Abbreviations as given in Fig. 1.

20-HE-exposed lobsters performed significantly more aggressive behaviours (increased F) towards the wall than did opponents of zE-ecdysone-exposed or ASW-exposed lobsters. This result may indicate that the smaller opponent lobster was receiving conflicting signals from the larger lobster. Although the smaller lobster may have been able to visually assess that its opponent was larger, it could also see that the larger lobster was more defensive and more timid than expected (larger lobsters in 20-HE fights performed more defensive and avoidance behaviours than their counterparts in zE-ecdysone or ASW fights). These factors, coupled with the fact that the small lobster was receiving no chemical signal from its opponent, may have led the small lobster to become more aggressive, but at the same time more wary than it would have if the chemical and visual signals it was receiving matched. Bergman et al. (2003) suggested that anosmic combatants are reluctant to fight. All lobsters in all treatment groups performed some wall behaviours, so it is not inconceivable that these behaviours provided some sort of visual or acoustic signal; however, the fact that the small opponents of 20-HE-exposed lobsters performed more wall behaviours than zE-ecdysone-exposed or ASW-exposed lobsters supports the idea that these behaviours may have functioned as sublimated aggression.


that these cues evolved as a result of chemical spying rather than signal specialization. *Acta Physiologica Scandinavica*, **152**, 191–205.


