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Antenna contact and agonism in the male lobster cockroach, *Nauphoeta cinerea*

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Abstract

On any given day, about 35% of 80- to 85-day-old socially naïve male (SNM) lobster cockroaches (*Nauphoeta cinerea*) spontaneously adopted an aggressive posture (AP) without encountering another male [spontaneous AP (SAP)]. Although SAP SNMs showed significantly higher release of the pheromone 3-hydroxy-2-butanone (3H-2B) than non-SAP SNMs, there was no significant difference in hemolymph juvenile hormone (JH) III titer. When different body parts were tested for induction of the attack behavior, the antenna was found to be the most effective. After 1 min of contact with an antenna from another SAP SNM, attack behavior was induced in 100% of SAP and 76.2% of non-SAP SNMs, and the JH III titer was significantly increased in all responders. Among the non-SAP SNMs, the JH III titer before antenna contact was significantly lower in the non-responders than in the responders, and, although the JH III increase induced by 1 min antenna contact was similar between responders and non-responders, the final JH III titer of the non-responders was significantly lower. A similar attack response, JH III titer change, and 3H-2B release were seen when the individual's own antenna was used. After 5 min of contact with an antenna from another SAP SNM, attack behavior was induced in 100% of SAP and 82% of non-SAP SNMs; in the former, 3H-2B release was similar before and after antenna contact, but the JH III titer was significantly increased after antenna contact, while, in the latter, both 3H-2B release and JH III titer were significantly increased after antenna contact. Among the non-SAP SNMs, JH III titer in the non-responders was not elevated after 5 min antenna contact, and was significantly lower than that in the responders. A pentane-washed antenna did not induce attack behavior or increase the hemolymph JH III titer, and a pentane-washed antenna coated with 3H-2B also failed to induce attack behavior. These results indicate that *N. cinerea* male–male agonistic interactions, to which the vertebrate challenge hypothesis can be applied, are due to contact pheromone on the antenna, resulting in the concomitant expression of attack behavior and an increase in 3H-2B release and JH III titer.

Keywords: Aggressive posture; Attack behavior; Antenna contact; Challenge hypothesis; 3-Hydroxy-2-butanone; Juvenile hormone; Spontaneous aggressive posture

Introduction

The lobster cockroach, *Nauphoeta cinerea* (Dictyoptera: Blaberidae), is well known for its male conspecific agonistic behavior (Kramer, 1964; Ewing, 1967). During fights, the dominant male assumes the aggressive posture (AP) (Fig. 1A), characterized by an elongated, upturned abdomen which is usually pumped up and down, and a stilted gait. This male usually chases and vigorously bites the subordinates, which then keep away from the dominant and adopt the submissive posture (SP) (Fig. 1B), in which the animal lies still, with its limbs tucked under its body, its head under the shield of its pronotum, and its abdominal tip lowered (Ewing, 1967). The outcome of these interactions is the formation of unstable dominant-subordinate relationships, in which changes in rank order are common after a male has been dominant for several weeks (Ewing, 1972; Bell and Gorton, 1978).

In terms of factors involved in status discrimination, volatile olfactory cues were first demonstrated by Smith and Breed (1982). Investigations of the relationship between pheromone components and social status were carried out after the identification of sex pheromones (Sréng, 1990; Moore et al., 1995;

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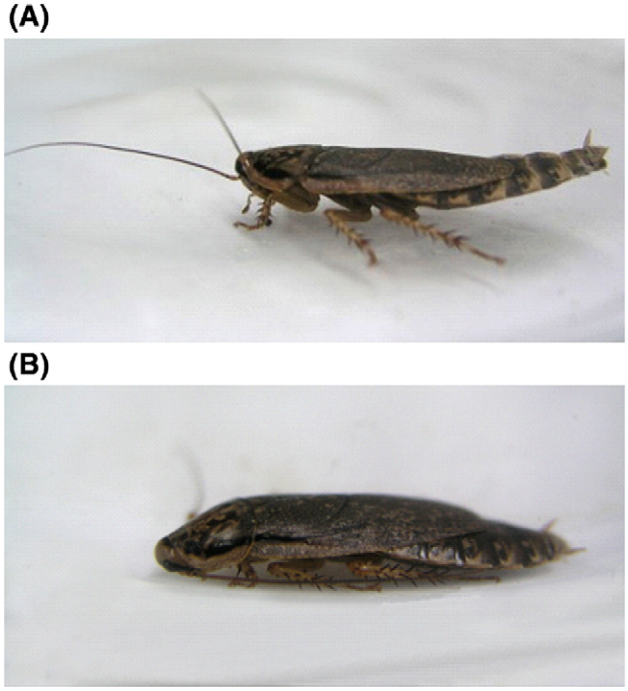

Fig. 1. Aggressive posture of the dominant and submissive posture of the subordinate after rank formation. (A) Aggressive posture; (B) submissive posture.

Everaerts et al., 1997; Moore et al., 1997). In this species, sex pheromone is only produced by males and is composed of 3 major components, 2-methylthiazolidine (2-MT), 4-ethyl-2-methoxyphenol (4E-2M), and 3-hydroxy-2-butanone (3H-2B) (Sréng, 1990). This sex pheromone may serve dual functions, acting in both male–male competition and female mate choice (Moore et al., 1997). Recently, we showed that, during a first encounter fight, microgram amounts of 3H-2B are released by the AP-adopting dominants (2-MT and 4E-2M being detected in only a few cases and in small quantities within the 1 h sampling period). We found that there was a linear relationship between the amount of 3H-2B released and attack duration, and the rate of 3H-2B emission was significantly higher the stronger the attacks (Kou et al., 2006). Another factor thought to be involved in status discrimination is the role of cuticular hydrocarbons, since differences in the profiles of the compounds between dominants and subordinates after rank formation have been reported by Everaerts et al. (1997) and Roux et al. (2002).

Regulation of pheromone production was first suggested by Hartman and Suda (1973), who also showed that it did not involve the corpora allata (CA). The insect CA are endocrine glands closely associated with and innervated by axons originating in the central nervous system. CA are the site of synthesis and release of juvenile hormone (JH) which plays a vital role in regulating the processes of metamorphosis, reproduction (Thompson and Tobe, 1990) and agonism (Hartfelder, 2000). Schal and Bell (1983) demonstrated that the CA does not directly influence the ontogeny of agonism. Sréng et al. (1999) showed that allatectomy results in a decrease in sex pheromone levels, which can be restored by administration of JH III, the only form of JH in this species (Baker et al., 1984). Subsequently, a significantly higher JH III *in vitro* release rate was observed in dominants than in subordinates (Chen et al., 2005).

Despite the results described above, how and why agonistic behavior is initiated between two males meeting for the first time is unknown. Fuki and Takahashi (1983) reported that sex discrimination in this species may involve an inter-male recognition pheromone, which inhibits courtship and is found only in the cuticle of males. Moore et al. (1995) reported that social experience alters the amount of pheromone produced. In fact, antenna contact is most likely the first step in social interactions, and the importance of the antenna was demonstrated in a previous study (Schal and Bell, 1983), which showed that agonism failed to occur when the antennae of both males were removed. Results from recent experiments in our laboratory have shown that the hemolymph JH III titer is significantly increased in both AP-adopting dominants and SP-adopting subordinates at 30 min after a first encounter fight, with no significant difference between the two [50.9±3.6 and 45.7±3.5 pg/μl hemolymph for the dominants and subordinates, respectively, compared to 16.2±0.7 for socially naive males (SNMs)]. The fact that the first agonistic encounter significantly activates the endocrine system, fits the vertebrate challenge hypothesis, which explains the species-level and individual-level temporal patterns of variation in plasma testosterone (T), and predicts that T levels will respond to prevailing social conditions (Wingfield et al., 1987, 1990). The challenge hypothesis has been recently extended to an invertebrate species, as high JH levels and aggression associated with periods of high social instability have been observed in burying beetles (Scott, 2006). In *N. cinerea*, on any given day, about 35% of 80- to 85-day-old SNM spontaneously adopted an AP without encountering another male [spontaneous AP (SAP)]. In the present study, we demonstrated that, under conditions of social instability such as the male–male agonistic interaction, attack behavior, concurrent 3H-2B release, and especially an increase in JH III titer, are initiated by antenna contact in both non-SAP and SAP SNMs.

Methods

Cockroaches

Mass rearing was according to Kou et al. (2006). Each male was isolated within the 24 h period following the imaginal moult to control for the effects of learning (Manning and Johnstone, 1970); these animals are referred hereafter as SNMs. The day of emergence was adopted as day 1. Because of the readiness with which they initiate agonistic behavior, 80- to 85-day-old SNMs were used, as described previously (Kou et al., 2006).

Experiment 1: ability of different body parts to induce attack behavior

During the early scotophase of the test day (1–3 h into scotophase under a Light:Dark=16:8 photoperiod), all the body parts to be tested (antenna, wing, thorax tergum, and the last 2–3 abdominal tergites) were removed from a randomly chosen SAP SNM (since adoption of the AP indicated a status of readiness for fighting) and attached to a wooden rod (2 mm diameter×10 cm long) with a small piece of transparent adhesive tape. Non-SAP SNMs were housed individually in a glass aquarium (12×12×12 cm) the day before the test. The antenna of each test male was touched lightly with each body part; each body part was tested on only one male, and a total of 30 individuals were tested with each type of body part. We observed that the lag time from contact to response usually was no more than 50 s (after having waited up to 2 min). So any

test individual showing no positive response (i.e., no lunging and biting toward the test body part) within 50 s was scored as non-responsive. To test the mechanosensory effect, a wooden rod with tape is used to touch the antenna of the tested insects ($n=30$). To test other sensory effects (such as olfaction, vision and sound/vibration), the body part is presented in front of the tested insect, with a vibration range of about 3–4 mm, but without actually touching the antenna of the tested insects ($n=20$ for each kind of body part).

Since the antenna was found to be the most effective body part in initiating agonistic behavior, attack behavior induced in individual SNMs by their own antenna and those of other individuals was also compared. Briefly, one antenna was removed from each non-SAP individual, then the removed antenna was used to touch the remaining antenna of the same individual or another individual. The sample size was 30 for each group.

Experiment 2: effect of antenna contact on 3H-2B release and hemolymph JH-III titer

In this experiment, SAP and non-SAP SNMs were used. To determine how quickly 3H-2B release and hemolymph JH III titer were changed by antenna contact, periods of 1 min or 5 min of antenna contact were used.

(I) 1 min of antenna contact:
  (a) Before antenna contact, a pheromone air sample was collected for 1 min from each SAP ($n=20$) or non-SAP SNM ($n=42$), then a hemolymph sample was collected for background data. Twenty minutes later, each individual was subjected to 1 min of contact with an antenna taken from another SAP SNM of the same age. A pheromone air sample was collected during this 1 min, while a hemolymph sample was collected immediately after this period, regardless of whether the animal was responsive or not.
  (b) To examine the physiological response to the insect's own antenna, an experiment similar to (a) was performed by using non-SAP SNMs, except that the antenna used was taken from the same insect, as described in Experiment 1. The sample size was 30.
  (c) To eliminate the possibility that the hemolymph JH III titer change was due to the cut made to collect the hemolymph sample before antenna contact, hemolymph samples were taken from a separate set of SAP individuals immediately after and at 20 min after the cut was made, in the absence of antenna contact. The sample size was 25. Our pretest also showed that handling of the animal to obtain the hemolymph samples did not affect the animal's response to the antennae.

(II) 5 min of antenna contact:
  (a) All procedures were as in (Ia) except antenna contact was for 5 min and the pheromone air sample was collected over these 5 min (for comparison, the pheromone air sample was also collected for 5 min before antenna contact). The sample size was 21 and 50 for SAP and non-SAP SNMs, respectively.

(III) Pheromone sampling, analysis, and quantification
  (a) Sampling of air for pheromone: Air samples were taken using adsorbent sample tubes, each packed with activated charcoal granules (# 226-01, 20–40 mesh, 150 mg, SKC Co., PA, USA), connected to an air sampling pump (model 210-1000, SKC Co., PA, USA) operating at 200 ml/min. During pheromone sampling, the opening of the glass aquarium containing a single male was covered with a piece of aluminum foil to prevent dissipation of the emitted pheromone, then the sample tube was inserted through the foil to a point 2–3 cm above the male, which was subjected to antenna contact for 1 min or 5 min as described above. At the end of the sampling session, the collector tube was eluted with 4 ml of dichloromethane and 2 μg of 2-phenylethanol added to the eluate as the internal standard. The eluates were stored in glass vials with Teflon-lined screw caps at −20 °C and were analyzed (using an autoinjector) within 24–48 h.
  (b) Analysis and quantification of sampled extracts: Analysis and quantification were performed as described previously (Ho and Millar, 2001; Kou et al., 2006). Briefly, gas chromatography-mass spectrometry (GC-MS) was used for the quantification of the collected compound(s). A DB-23 column (30 m×0.25 mm, J and W Scientific, Folsom, California) was used. The temperature of the GC was maintained at 75 °C for 1 min, then increased sequentially to 110 °C at 5 °C/min, to 113 °C at 1 °C/min, and to 240 °C at 25 °C/min, then maintained at 240 °C for 5 min, with injector and transfer line temperatures of 200 °C and 250 °C, respectively. The instrument responses to chemical standards and the internal standard, 2-phenylethanol, were determined by co-injecting 10 ng of the compounds into the GC-MS. The ratios of the peak areas in the reconstructed ion chromatogram were used to calibrate the response of the instrument to each compound. For the method of internal standard quantification, 5 doses (0.1, 0.5, 1.0, 3.0, and 5.0 μg) of the 3H-2B standard (Sigma-Aldrich Co., St. Louis, MO) were used to construct the standard curve. The 3H-2B recovery rate (90.9%) of our sampling system was measured by sampling for 10 min in an identical fashion from a glass aquarium containing 5 μl of a 400 ng/μl solution of 3H-2B placed on a piece of aluminum foil on the bottom of the aquarium, with a total of 7 replicates. All data were adjusted for the recovery rate.

(IV) Hemolymph sampling and JH III titer measurement: The hemolymph was obtained by placing the insect on its back and making a cut (of about 1 mm) with a fine pair of scissors along the connection between the tergum and the thorax tissue, and quickly collecting the hemolymph in a capillary tube. The hemolymph (4–9 μl/male) was immediately mixed with 500 μl of acetonitrile to denature any enzymes that could affect the JH titer, and the samples were placed on ice, then stored at −20 °C for subsequent JH analysis. Capillary tubes and all other glassware that were to come into contact with the JH were baked at 500 °C for 3.5 h prior to use to minimize JH adsorption (Strambi et al., 1981).
JH III, the only form of JH found in *N. cinerea* (Baker et al., 1984), was measured in individual males using a chiral-specific radioimmunoassay (RIA) (Hunnicutt et al., 1989). This assay has been specifically validated for adult worker honey bees, and yields comparative JH titers (Huang et al., 1994) to two other RIAs that have been verified by GC-MS (De Kort et al., 1985; Goodman et al., 1990). This RIA procedure has been described previously in detail (Huang and Robinson, 1995). Briefly, JH III in the hemolymph sample was extracted with 2×0.5 ml of hexane, then the pooled hexane extracts were evaporated using a vacuum centrifuge (Speedvac) linked to a condenser that trapped the solvent at −98 °C (Savant SS21). The dried JH in the sample tube was dissolved in 200 μl of premixed buffer containing anti-JH antiserum (1:14000 dilution) and 8000 DPM of [10-^{3}H(N)]-JH (647.5 Gbq/mmol; NEN), and the sample incubated at room temperature for 2 h. Dextran-coated charcoal solution (0.5 ml) was added for 2.5 min to each sample tube, which was then centrifuged (2000×*g* for 3 min), and the supernatant decanted into scintillation vials. Liquid scintillation counting was performed using a Beckman LS 6500. KaleidaGraph was used to generate the non-linear regression standard curve to estimate the amount of JH in each sample from the DPM.

Experiment 3: role of the antenna cuticular chemical signature in increasing the hemolymph JH-III titer and 3H-2B release

To determine whether the behavioral and physiological effects were induced by contact with the cuticular chemical signature on the antenna or simply by a tactile effect, the following experiment was performed:

(i) Antenna preparation: antennae from 80- to 85-day-old SNMs were immersed in pentane for 48 h at 25–27 °C to dissolve cuticular hydrocarbons.
(ii) Hemolymph was first sampled from each SAP-adopting SNM, then, 20 min later, each individual was stimulated with a pentane-washed antenna for 1 min as described above and hemolymph samples were collected immediately after stimulation.
(iii) To check the effect of 3H-2B on inducing attack behavior, three concentrations were used (1000, 100, and 10 ng/μl CH_2Cl_2). For the test, each pentane-washed antenna was immersed in one of these solutions for 5 s, then was used to test for induction of attack behavior in SAP-adopting SNMs by contact for 1 min, each 3H-2B-coated antenna being used only once. CH_2Cl_2-coated antennae were tested as controls.

Statistical analysis

One-way ANOVA and the chi-squared test (SAS Institute, 1990) were used, respectively, to analyze the lag time and percentage of positive responses using the four different body parts. The *t*-test and chi-squared test were used, respectively, to compare lag time and percentage of positive responses of SNMs toward their own antenna or an antenna from another male. Depending on whether or not the data showed a normal distribution, the paired *t*-test or a nonparametric test (Wilcoxon rank-sum test) was used to compare hemolymph JH III titers and/or 3H-2B release in SNMs before and after antenna contact. The *t*-test was used to compare 3H-2B release before antenna contact between SAP and non-SAP SNMs. The values for the response variables are presented as the arithmetic mean and standard deviation of the mean.

Results

Effect of different body parts on induction of attack behavior

In previous unpublished observations, we found that spontaneous adoption of the AP (no lunging or biting, simply standing still, with the abdomen elongated and upturned, sometimes pumping up and down) was seen in about 35% of individuals on any given day [spontaneous AP (SAP) individuals]. Since the majority of individuals did not spontaneously adopt the AP, non-SAP SNMs were used in this experiment. Different body parts (antenna, wing, thorax tergum, and the last 2–3 abdominal tergites) from other 80- to 85-day-old SAP SNMs or an antenna from the same non-SAP individual was tested for their ability to induce attack behavior. The definition of attack behavior was lunging and biting, with an AP toward the tested body part. As shown in Fig. 2A, different body parts from another individual elicited different degrees of attack behavior, the antenna being the most effective. The average lag time from contact to appearance of attack behavior was shorter for the antenna than for other body parts ($p=0.0001$). In addition, the percentage of positive responses was significantly higher for the antenna than for other body parts ($p=0.0029$ between antenna and forewing; $p<0.0001$ between antenna and thorax tergum; $p<0.0001$ between antenna and abdominal tergum). A wooden rod with tape alone (no antennae) showed no mechanosensory effect on inducing the attack behavior ($n=20$). Presenting the wooden rod the taped antennae without actually touching the antennae of the test animal did not induce the attack behavior, demonstrating there is no olfaction, vision, or sound/vibration effect ($n=20$). Fig. 2B shows that a non-SAP SNM responded equally well to its own antenna and to an antenna from another individual, both in terms of lag time and percentage of positive responses.

Effect of antenna contact on hemolymph JH-III titer and 3H-2B release

The aim of this study was to see how different durations of contact with a foreign SAP antenna (1 or 5 min) affected the JH III titer and 3H-2B release in SAP and non-SAP SNMs. SNMs were classified as responders or non-responders after contact with the antenna (responder meaning attacking the antenna). For comparison, the effect of contact with the individual's own antenna on the JH III titer and 3H-2B release was tested on non-SAP SNMs.

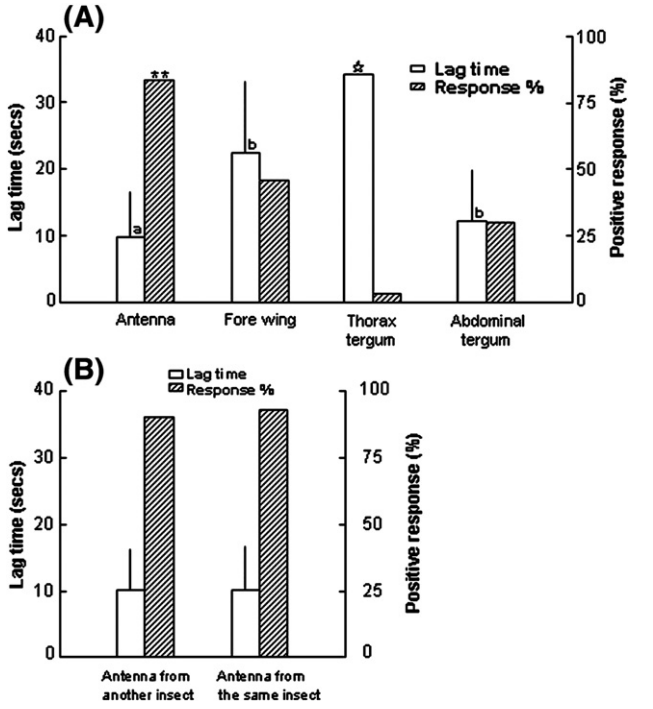

Fig. 2. Lag time and percentage of positive responses of non-SAP SNMs to different body parts from other SAP SNMs or to an antenna from the same or a different non-SAP SNM. (A). Lag time and percentage of positive responses of non-SAP SNMs toward different body parts from other SAP SNMs. For the lag time, means with different letters (a, b) indicate a significant difference ($p=0.0001$). ☆: only 1 out of 30 males showed attack behavior and was not included in the statistical analysis. ✳: For the positive response (%), $p=0.0029$ between antenna and forewing, $p<0.0001$ between antenna and thorax tergum, and $p<0.0001$ between antenna and abdominal tergum. (B) Lag time and percentage of positive responses of non-SAP SNMs to an antenna from the same or a different non-SAP SNM. Lag time: time from antenna contact to expression of attack behavior. Positive response: chasing and biting by adopting the AP. $N=30$ for each group.

Using a foreign antenna for contact

Using a foreign SAP antenna and comparing the responders in both groups (SAP and non-SAP), there was no difference in hemolymph JH III titer before antenna contact (Figs. 3A and B), although 3H-2B release was significantly higher in the SAP SNMs in the 5 min group (Fig. 3B; $p=0.0002$). Before antenna contact, the much higher 3H-2B release in the 5 min group when compared to the 1 min group, might be mainly attributed to the time duration for air collection, one was 1 min (in the 1 min antenna contact), and the other was 5 min (in the 5 min antenna contact).

After 1 min of antenna contact with a foreign SAP antenna, attack behavior was induced in 76.2% of non-SAP individuals, and the hemolymph JH III titer showed significant increase in both the responders ($p=0.02$) and the non-responders ($p=0.03$). In non-SAP individuals, the small increase in 3H-2B release in the responders was not significant, and there was no increase in non-responders. In the non-SAP group, the JH III titer before antenna contact was significantly lower in non-responders than in responders ($p<0.0001$). Although the increase in JH III titer after antenna contact was similar in these responders and non-responders ($p=0.54$), the final JH III titer in non-responders was

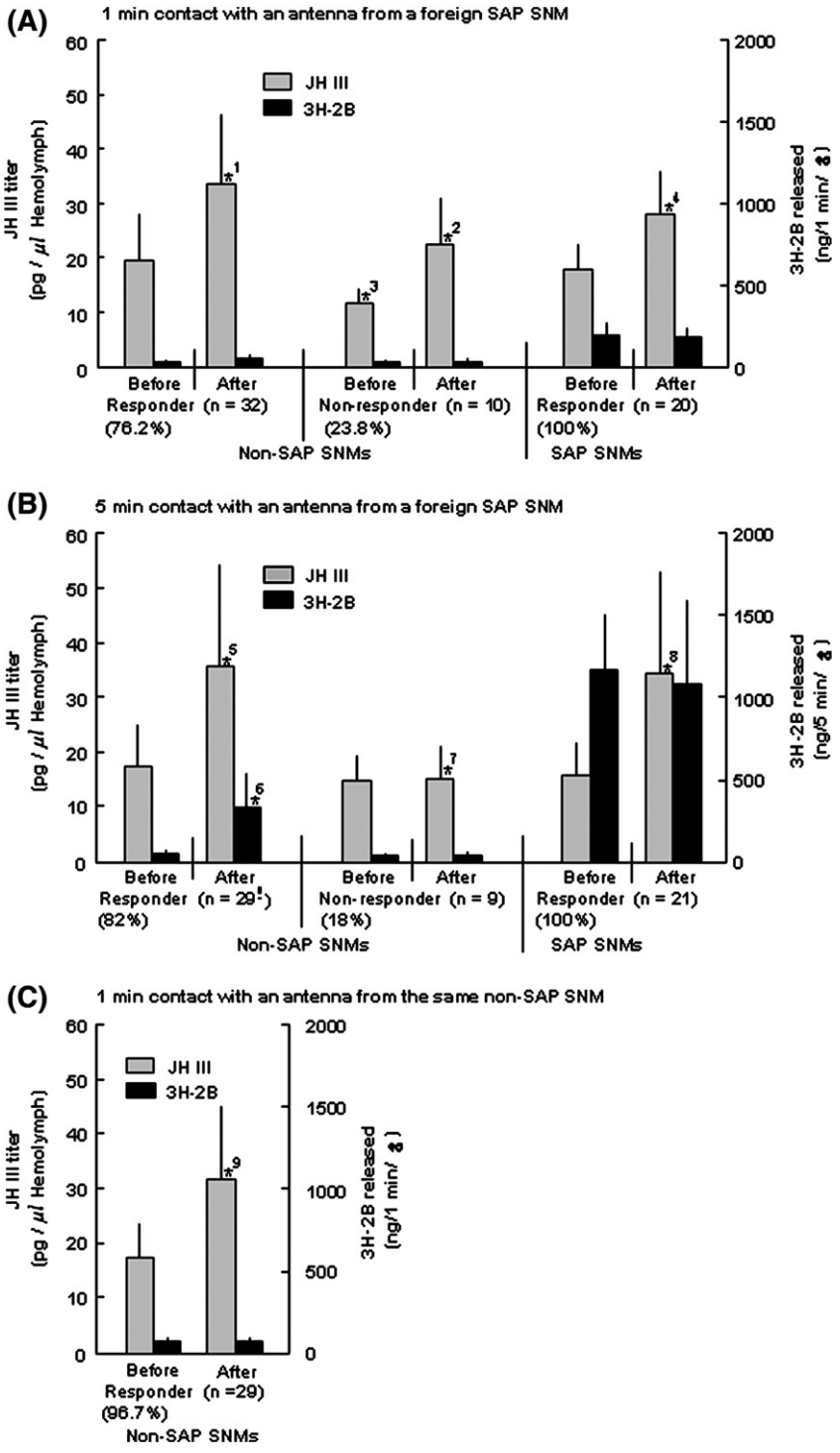

Fig. 3. Effect of contact with a foreign antenna or the individual's own antenna on hemolymph JH III titer and 3H-2B release in SAP or non-SAP SNMs. (A and B) 1 min (A) or 5 min (B) contact with a foreign SAP antenna; (C) 1 min contact with the non-SAP individual's own antenna. Before: before antenna contact. After: after antenna contact. SNMs: socially naïve males. SAP: spontaneous aggressive posture. ▒: JH III titer. ■: 3H-2B released. For the comparison of the JH III titer increase after antenna contact, $*^1$: $p=0.02$, $*^2$: $p=0.03$, $*^4$: $p=0.0001$, $*^5$: $p=0.0004$, $*^8$: $p=0.0008$, $*^9$: $p<0.0001$. For the comparison of the JH III titer between responders and non-responders before antenna contact in the 1 min non-SAP group, $*^3$: $p<0.0001$. For the comparison of the JH III titer between responders and non-responders after antenna contact in the 5 min non-SAP group, $*^7$: $p=0.0065$. For the comparison of the increase in 3H-2B release after 5 min antenna contact, $*^6$: $p<0.0001$. !: Twenty-nine individuals were randomly chosen from the 41 (82%) responders.

significantly lower ($p=0.04$) than that in responders. In the SAP SNMs, all individuals showed attack behavior and the hemolymph JH III titer was significantly increased ($p=0.0001$), but 3H-2B release was not significantly altered (Fig. 3A).

After 5 min of antenna contact, attack behavior was induced in 82% of non-SAP individuals, and the hemolymph JH III titer ($p=0.0004$) and 3H-2B release ($p<0.0001$) were significantly increased in these responders, but not in the non-responders. In

the non-SAP group, before antenna contact, the JH III titer in the non-responders was not significantly different than that in responders ($p=0.32$). After antenna contact, the JH III titer in these non-responders was not increased, and was significantly lower than that in the responders ($p=0.0065$). In the SAP SNMs, the results were similar to those for 1 min of antenna contact, i.e., attack behavior was induced in 100% of individuals, the hemolymph JH III titer was significantly increased ($p=0.0008$), and there was no significant difference in 3H-2B release (Fig. 3B). The increase in JH III titer was not due to the cut made to sample the hemolymph before contact, the titer being 16.3±1.2 pg/μl ($n=25$) and 14.8±1.5 pg/μl ($n=25$) ($p=0.67$), respectively, immediately after, and 20 min after, making the cut in insects with no antenna contact.

Using the individual's own antenna for contact

When non-SAP SNMs were tested for attack behavior induced by contact with their own antenna, 96.7% of individuals responded. In these responders, the hemolymph JH III titer was significantly increased ($p<0.0001$), but the increase in 3H-2B release was not significant (Fig. 3C). For the one that did not respond, the JH III titer before and after antenna contact was 15.0 and 12.3 pg/μl, respectively. There were no significant differences in the increase in hemolymph JH III titer and 3H-2B release when comparing these results with those for non-SAP SNMs using a foreign SAP antenna (Fig. 3A).

Role of the antenna cuticular chemical signature in the increase in hemolymph JH-III titer and 3H-2B release

Using SAP SNMs, a pentane-washed antenna induced attack behavior in only 10% (3 out of 30) of instances and caused no significant increase in hemolymph JH III titer (15.5±1.0 pg/μl and 13.3±1.0 pg/μl before and after antenna contact, respectively) ($p=0.56$). In the 3 responsive individuals, the hemolymph JH III titer before and after antenna contact was 15.3±2.2 pg/μl and 17.8±2.1 pg/μl, respectively (difference not significant) ($p=0.48$). A pentane-washed antenna coated with different concentrations of 3H-2B (1000, 100, and 10 ng/μl CH_2Cl_2) failed to induce attack behavior.

Discussion

The lobster cockroach, *N. cinerea*, is fascinating because of its well-known male conspecific agonistic behaviors, which are characterized by a complex repertoire of agonistic acts (Kramer, 1964; Ewing, 1967). Although the evolutionary relevance of this agonistic behavior and the underlying pheromonal system are well established (Moore et al., 1995; Moore, 1997), how agonistic behavior is initiated between two males meeting for the first time is not known. We found recently that following the first agonistic encounter, the hemolymph JH III titer was significantly increased, regardless of whether the insect was a dominant or subordinate. This phenomenon fits well with the vertebrate challenge hypothesis, which explains species-level and individual-level temporal patterns of variation in plasma testosterone (T) and predicts that T levels will respond to prevailing social conditions (Wingfield et al., 1987, 1990). JH and testosterone have parallel functions in insects and vertebrates respectively, as they are responsible for the development of the male reproductive system, as well as spermatogenesis. The challenge hypothesis has recently been extended to invertebrates, as high JH levels and aggression are associated with periods of high social instability in burying beetles (Scott, 2006).

The results of the present study showed that, in *N. cinerea*, male–male agonistic interactions are based on antenna contact, which results in attack behavior and increased JH levels, and in some instances, increases in 3H-2B release (for the 5 min stimulation in non-SAP SNMs). In SNMs, although SAP individuals released significantly more 3H-2B after 5 min contact with a foreign antennae than non-SAP individuals, there was no difference in hemolymph JH III titer between the two groups, showing that spontaneous adoption of the AP was not directly related to the hemolymph JH III titer. The key factor in inducing the initial attack was antenna contact and pheromone, not the mechanosensory, olfactory, vision or sound/vibration effects. Also the initial attack was not induced by the 3H-2B released from the abdominal sternal glands upon adopting the AP (Kou et al., 2006), contrasting with the results of Moore et al. (1997). The function of the released 3H-2B by the dominants is still under investigation. The effect of antenna contact on induction of attack behavior and the increase in both hemolymph JH III titer and 3H-2B release (for the 5 min stimulation in non-SAP SNMs) was similar to that seen after fighting. For the non-SAP SNMs, since the AP still could be induced, the non-significant increase of 3H-2B release in the 1 min stimulation might be attributed to the short time period (only 1 min) of air collection. For the SAP SNMs, it is possible that since the insects were physiologically already in a state of readiness for fighting (AP-adopting) which was accompanied with high levels of 3H-2B release could not be stimulated further by 1 min and 5 min antenna contact.

The importance of the antenna was demonstrated in a previous study (Schal and Bell, 1983) in which agonism failed to occur when the antennae of both males were removed. In the context of agonism, the importance of the antennae has also been demonstrated in the cricket, *Gryllus bimaculatus*. In *G. bimaculatus*, the duration of antennal fencing, which is necessary to initiate a fight, is independent of the experience and weight asymmetry of the contestants (Hofmann and Schildberger, 2001). Also in *G. bimaculatus*, expression of male–male aggression is significantly reduced in antennectomized males, and brain serotonin levels are significantly reduced at 7 days after removal of the antennae (Murakami and Itoh, 2003). In a previous study (Adamo et al., 1994), allatectomized (removal of CA by microsurgery) crickets were shown to exhibit normal aggressive behavior, but we think this might partly be due to the existence of the intact antenna.

The fact that similar attack responses were obtained regardless of whether the antenna came from the tested animal himself or from another individual indicates that the key factor inducing the attack response must be a component that is common to the different antennae. Why is antenna contact so important and so effective in eliciting the behavioral and physiological responses? According to Wyatt (2003), recognition of kin or fellow group

members is central to social behavior in both a colony of millions or a small family group. How animals distinguish members of their group from non-members is a key behavior allowing them to favor the offspring and other relatives (kin) or fellow group members (Sherman et al., 1997). In our *N. cinerea*, the SNMs had no opportunity to meet (or learn from) other individuals, so there was no learning experience of the contact pheromones of other individuals. The fact that attack behavior could be induced in a SNM by contact with an isolated antenna suggests an inherent predisposition toward agonism, as reported in some crayfish species (Issa et al., 1999; Goessmann et al., 2000). This suddenly induced behavioral response (the attack behavior) might be due to the recognition allele mechanism, i.e., an individual with allele Z recognizes an animal carrying allele Z, whether or not kin, without learning (Hamilton, 1964, Dawkins, 1976; Haig, 1997). Such genes have been proposed on theoretical grounds to mediate both altruism and intragenomic conflicts (Hamilton, 1964, Dawkins, 1976), and were first demonstrated in the imported red fire ant, *Solenopsis invicta* (Keller and Ross, 1998). In *S. invicta*, all egg-laying queens are Bb heterozygotes at the Gp-9 locus, and BB queens initiating reproduction are killed by Bb workers on the basis of a transferable odor cue. In fact, cuticular hydrocarbons used for kin recognition via contact have been reported in many insect species, such as the honey bee, *Apis mellifera* (Getz, 1991), the paper wasp, *Polistes fuscatus* (Arathi et al., 1997), the sweat bee, *Lasioglossum zephyrum* (Greenberg, 1979), and some ants, such as *Pachycondyla apicalis* (Soroker et al., 1998; Lenoir et al., 2001). The ability to recognize these cuticular hydrocarbons is thought to be under genetic control (Wyatt, 2003).

Turning to another aspect, why are some individuals non-responders? Looking at the levels of JH, our present results suggest two possible explanations. The first, observed in the 1 min antenna contact experiments, is that before antenna contact, basal or starting level of the JH III titer in the non-responders was significantly lower than that in the responders. After antenna contact, although the JH III increase was similar in the responders and non-responders, the final JH III titer in the non-responders was still significantly lower than the levels observed in the responders. The second possibility, observed in the 5 min antenna contact experiments, is that the JH III titer in the non-responders was similar to that in the responders before antenna contact, the final JH III titer in the non-responders could not be elevated above a threshold level by antenna contact. Through either mechanism, the JH III titer in the non-responders did not reach the threshold needed to generate a response to the attack-inducing stimulus (such as antenna contact pheromone). Further investigations in our laboratory have shown that, during the first encounter fight, dominant status was dependent on whether the JH III titer showed a significant increase. In non-SAP SNMs, injection into the abdomen of JH III dissolved in mineral oil significantly increased the probability of winning without grappling [the percentage of non-grappling winners was significantly higher in JH III-injected SNMs ($p=0.0025$ with 1.0 μg of JH III ($n=50$ pairs) and $p=0.037$ with 2.5 μg of JH III ($n=50$ pairs)] and of grappling winning [the percentage of grappling winners was significantly higher (40%, $n=50$ pairs; $p=0.001$) in the 2.5 μg JH III treatment than that of the control (18.0%, $n=50$ pairs)].

In the responders, the signal transduction pathway between antenna contact and the induced behavioral and physiological responses is of interest. Our hypothesis is that, during antenna contact, the cuticular chemical signature (such as cuticular hydrocarbons) might be detected by olfactory receptors on the dendrites in the antenna, and the activated receptors induce a signal transduction cascade in the central nervous system. The result is activation of the agonistic system, both behavioral and physiological (activation of the CA, release of 3H-2B, etc.). Since attack behavior was induced within seconds of antenna contact, the amount of contact pheromone-binding protein (CPBP) or the activation level of CPBP receptors in the central nervous system might be important in determining how quickly an individual shows the attack response. This may explain why, when two males of approximately equal strength (or size) meet, the winner is always (100%, $n=100$ male pairs) the one who attacks first. The fact that the first attacker becomes the winner was also reported in the lobster, *H. americanus* (Breithaupt and Atema, 2000), in which the relationship between urine release and threat display is very similar to that between 3H-2B release and attack strength in *N. cinerea* (Kou et al., 2006). The neurophysiologic response threshold to the contact pheromone might be lower in the winner than in the loser, or attack and JH III and/or 3H-2B release might be more quickly induced to suppress the rival's fighting ability. These possibilities are being investigated.

The present results demonstrating antenna contact-induced behavioral and hormonal responses show that agonism in *N. cinerea* is not only expressed at the behavioral level, but also at the physiologic level. This is reasonable, since any sudden behavioral response might not consist solely of the superficial behavior change, but also some underlying or accompanying physiological changes, either just before exhibiting the behavior or in the maintenance of the behavior. Our result that agonism was induced by antenna contact is consistent with a previous report (Schal and Bell, 1983) that CA does not directly influence the ontogeny of agonism. Contact with cuticular components may also result in the release of some types of neurohormones, which, in turn, stimulate CA biosynthetic activity and increase JH III release into the hemolymph. The increased JH III release may be involved in either dominant-status maintenance (for dominant males) or in compensating for the physiological changes caused by stress (for subordinate males). Based on the marked accelerative effect of early social contact experience on aggressiveness, Manning and Johnstone (1970) hypothesized that social contact operates by initiating the development or activation of some endocrine system and suggested that the onset of aggressiveness in *N. cinerea* is associated with activation of the CA, which is, in turn, caused by neural and neurohumoral activity in the brain initiated by social contact. The neuroendocrine cascade from antenna contact to the increase in hemolymph JH levels requires further investigation. Since altering the levels or function of amine neurons causes important changes in aggression in all invertebrate systems examined (Kravitz and Huber, 2003; Stevenson et al., 2005), changes in the amine system after antenna contact will also be investigated.

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