



Juvenile hormone levels are increased in winners of cockroach fights

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ABSTRACT

In lobster cockroach (*Nauphoeta cinerea*) adult males, concomitant expression of attack behavior and an increase in juvenile hormone (JH) III titer can be induced by contact with an isolated antenna [Chou et al., 2007. Antenna contact and agonism in the male lobster cockroach, *Nauphoeta cinerea*. *Horm. Behav.* 52, 252–260]. In the present study, socially naïve *N. cinerea* males that were either aggressive posture-adopting (i.e., “ready-for-fight”) or not (i.e., “non-ready-for-fight”) were paired to ask if status was determined by JH III levels before the encounter and if JH III levels were altered in dominants and subordinates after the encounter. The results showed that, although in the non-aggressive posture-adopting male pairs, the one with higher JH titers before the encounter was more likely to become the dominant, this was not the case in pairs formed between aggressive posture-adopting males or between non-aggressive posture-adopting and aggressive posture-adopting males. In all types of male pairs combined, JH III levels in the dominant were significantly increased after the encounter compared with before the encounter and were significantly higher than those in the subordinates, suggesting that the JH III increase in the dominants may serve to sustain aggression. JH III application before rank formation had a significant effect on establishment of dominant status in non-aggressive posture-adopting, but not aggressive posture-adopting, males. After rank formation, JH III application to subordinates had no effect on rank switch. These results indicate that the relationship between JH and aggression in this cockroach species is broadly consistent with the vertebrate challenge hypothesis, which predicts that testosterone levels increase in response to social stimuli to modulate aggression.

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Introduction

On the subject of insect aggression, juvenile hormone (JH), secreted from the endocrine gland corpora allata (CA), has a great impact on the regulation of behavior. In the primitive social wasps *Polistes annularis*, after rank establishment, repeated topical application of JH III or JH analog to workers resulted in a disruption of colony social structure as indicated by a sharp increase in the frequency of dominance interactions (Barth et al., 1975). In the overwintered paper wasp *Polistes gallicus* foundresses, injection of JH I and 20-hydroxyecdysone, separately and simultaneously, significantly increased the probability that the treated female would be the dominant female of a test pair; also dominant females had either larger CA or more developed ovaries than subordinates (Röseler et al., 1984). In the highly eusocial honeybees *Apis mellifera*, JH III appears to be an endocrine correlate of aggression (Sasagawa et al., 1989; Huang et al., 1994; Pearce et al., 2001). In the bumble bee *Bombus terrestris*, dominant workers in queenright colonies had a higher rate of JH III biosynthesis, JH III titer

and ovarian development than low ranked workers of similar size (Bloch et al., 2000). Methoprene, a JH analog, significantly increased the proportion and the development of guarding behaviors in the neotropical social wasp *Polistes canadensis* (Giray et al., 2005). In the burying beetle *Nicrophorus orbicollis*, JH levels in both males and females increased in response to challenge by an intruder (Scott, 2006a); with the application of JH analog methoprene, a causal relationship between JH and aggression was suggested (Scott, 2006b). In the termite *Reticulitermes flavipes*, ectopic JH III is required for presoldier induction, and the ectopic JH is suggested to have a primer pheromone-like effect that subsequently stimulate endogenous JH production (Scharf et al., 2007).

In terms of hormone–behavior interactions, the challenge hypothesis (Wingfield et al., 1987, 1990), which is based on the mating systems and breeding strategies of birds, has been an important foundation for modern behavioral endocrinology in vertebrates. As described by Moore (2007), this hypothesis predicts how testosterone (T) levels will vary seasonally and how male birds will hormonally respond to social challenges from other males. The elevated T levels have an important function in “persistence of aggression” (Wingfield, 2005). In fact, the physiological function of JH in insects is not only similar to that of T in vertebrates, but is

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even broader (Trumbo, 2007). Part of the challenge hypothesis has recently been suggested to extend to an invertebrate species, as high JH levels and aggression associated with periods of high social instability have been observed in burying beetles (Scott, 2006a). However, based on the different reproductive strategies between vertebrates and invertebrates, the application of the challenge hypothesis in its entirety to JH and insects may require further investigation (Trumbo, 2007).

The lobster cockroach, *Nauphoeta cinerea*, is well known for its male conspecific aggressive behavior (Kramer, 1964; Ewing, 1967). The outcome of these interactions is the formation of an unstable dominant/subordinate hierarchy, and changes in rank order are common after a male has been dominant for several weeks (Ewing 1972; Bell and Gorton, 1978). Our previous study showed that the *in vitro* JH III release rate is significantly higher in dominants than in subordinates (Chen et al., 2005). Concomitant expression of attack behavior and a significant increase in hemolymph JH III titer can be induced within seconds by contact with an isolated antenna (Chou et al., 2007). For any new rank formation, either for the first encounter fight or for rank switch, significantly higher JH III levels are always associated with the dominant status (Kou et al., 2008). These findings suggest that this cockroach species might be used to test the basic challenge hypothesis i.e., that the endocrine system can be quickly activated by social stimuli. In *N. cinerea*, socially naïve males aged 80- to 85-day-old show a high probability of initiating aggressive behavior and can be divided into non-aggressive posture-adopting and aggressive posture-adopting. On any given day, about 40–60% of males spontaneously adopt an aggressive posture without encountering another male. Aggressive posture adoption is an indicator of being ready to fight or aggressiveness. In our previous unpublished work, aggressive posture-adopting males were found to be more aggressive than non-aggressive posture-adopting males, the average lag time from antenna contact to initiation of attack behavior being 3.4 ± 1.6 s (mean \pm SD, $n=15$) and 9.0 ± 3.0 s (mean \pm SD, $n=20$) for aggressive posture-adopting and non-aggressive posture-adopting males, respectively. Despite the fact that any lab study may be hindered by an over-reliance on laboratory assays of behavior, there is an advantage in using this cockroach species, which allows repeated sampling of hemolymph. In the present study, we made the following two predictions. First, since the aggressive posture-adopting males were more aggressive than non-aggressive posture-adopting males, we predicted the existence of a ceiling effect of JH, i.e., application of JH III would increase aggression in the later but not in the former. Second, after the encounter, JH III titers would increase in the dominants, as with T in vertebrates. Male pairs were formed from only non-aggressive posture-adopting males, only aggressive posture-adopting males, or both non-aggressive posture-adopting and aggressive posture-adopting males.

Methods

Cockroaches

Mass rearing was carried out as described by Kou et al. (2006). Each male was isolated within the 24 h period following the imaginal molt to control for social contact (Manning and Johnstone, 1970), so all animals used in this study were socially naïve males. The day of emergence was adopted as day 1. Because of the readiness with which they initiate aggressive behavior, 80- to 85-day-old males were used, as described previously (Kou et al., 2006). All hemolymph samples were taken at the same time of day (1–3 h into scotophase) to minimize any variation caused by circadian rhythms. The males used in each experiment were all matched for size. For individual identification, the dominant was marked on its pronotum with white Tipp-Ex fluid.

Experiment 1: Hemolymph JH III titer before and after the encounter

(I) *Male pair formation and hemolymph sample collection.* The males were divided into two groups, aggressive posture-adopting and non-aggressive posture-adopting. Each male was placed in a separate glass aquarium (12 \times 12 \times 12 cm) on the day before the test and were paired in a different aquarium on the test day. Three kinds of male pairs were formed consisting of: (i) two non-aggressive posture-adopting males ($n=30$ pairs), (ii) two aggressive posture-adopting males ($n=35$ pairs), and (iii) a non-aggressive posture-adopting and an aggressive posture-adopting male ($n=30$ pairs). For each kind of male pair, the following procedures were carried out:

- (1) Before the encounter, a hemolymph sample was collected from each male during early scotophase (1–3 h into scotophase under a 16 h light:8 h dark photoperiod),
- (2) Thirty minutes after hemolymph collection, two males were paired for a 10 min fight period, in which the dominant/subordinate status was usually determined within a few seconds of pairing, with the dominant chasing and biting the subordinate. Hemolymph samples were collected from both the dominant and subordinate at the end of the fight period.

(II) *Hemolymph sampling and JH III titer measurement.* The hemolymph was obtained by placing the insect on its back and making a cut (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 (3–8 μ l/male) was immediately mixed with 500 μ l of acetonitrile to denature any enzymes that could affect JH and the samples placed on ice, then stored at -20 °C for subsequent JH analysis. Capillary tubes and all other glassware that came into contact with 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 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 \times 0.5 ml of hexane, then the pooled hexane extracts were evaporated using a vacuum centrifuge (Speedvac) linked to a condenser (Savant SS21), which trapped the solvent at -98 °C. The dried JH in the sample tube was dissolved in 200 μ l of premixed buffer containing anti-JH antiserum (1:14000 dilution) and 8000 DPM of [10^{-3} H(N)]-JH (NEN, 647.5 GBq/mmol) and the mixture was incubated at room temperature for 2 h, then 0.5 ml of dextran-coated charcoal suspension (Sigma) was added for 2.5 min to each sample tube, which was then centrifuged (2000 \times g for 3 min), and the supernatant decanted into scintillation vials. Liquid scintillation counting was performed using a Beckman LS 6500. KaleidaGraph (Synergy Software, PA, USA) was used to generate a standard curve. The standard curve was obtained by using DPM bound as the dependent variable, JH amount (after log transformation) as the independent variable, and 5 unknown parameters, using nonlinear regression. The five parameter formula was described by Huang and Robinson (1996). Excel (Microsoft, USA) was used to calculate the amount of JH in each sample, by reversing the five parameter formula (solving for JH with known DPM and the five fitted parameters). The recovery of the radiolabeled JH III was 98.2%. The intra- and inter-assay variabilities were 6.2% and 8.4%, respectively.

Experiment 2: Effect of JH III application on initial rank establishment

Non-aggressive posture-adopting male pairs and aggressive posture-adopting male pairs were tested separately.

JH III (1 or 2.5 μ g in 1 μ l of mineral oil) was injected through the intersegmental membrane between the second and third sternites of

the test male, while the control group for each dose was injected with 1 μ l of mineral oil. Seven days after this injection, each JH III-injected male was paired with a mineral oil-injected male and the status observed. The sample size for each JH treatment was 50 pairs.

Experiment 3: Effect of JH III application to subordinates on rank switch

In this experiment, the effect of JH III on rank maintenance was investigated separately in aggressive posture-adopting male pairs and non-aggressive posture-adopting male pairs. Each male pair was placed in a glass aquarium (12 \times 12 \times 12 cm) on the pairing day. Starting on the second pairing day, each subordinate was injected with 1 μ l of JH III (1 or 2.5 μ g in 1 μ l of mineral oil) or 1 μ l of mineral oil alone (separate control groups for each dose) as described above, but the

injection was repeated another two times at 10 day intervals. Rank switch (the original subordinate became dominant) was examined daily from day 2 of treatment. The sample sizes for the 1 μ g and 2.5 μ g JH-treated groups were 42 and 30 pairs, respectively.

Statistical analysis

To compare hemolymph JH III titers before and after the encounter in the same individuals, between the dominants and subordinates after the encounter, or between higher JH and lower JH dominants, the data were log transformed for statistical analysis, then back transformed for graphical depiction. The Z-test was used to determine whether the proportion of male pairs in which the male with the higher JH levels became the dominant was significantly greater than that in which the male with the lower JH levels became dominant. The Chi-squared test was used to determine whether, for any given male pair, the one with higher JH levels before the encounter had a significantly higher probability of becoming the dominant. The Z-test was also used to compare the percentage of winners in the JH-treated and mineral oil-treated insects. The t-test was also used to compare the percentage of pairs showing a rank switch or the day of rank switch between the JH-treated (1 μ g or 2.5 μ g) and control groups in either the non-aggressive posture-adopting or aggressive posture-adopting male pairs. The values for the response variables are presented as the mean \pm SD.

Results

Experiment 1: Hemolymph JH III titer before and after the encounter

Three kinds of male pairs were formed:

(I) Pairs formed only from non-aggressive posture-adopting males.

Among the 30 male pairs, the male with the higher JH titers before the encounter became the dominant in 53.3% (16/30), while the male with the lower JH titers before the encounter became the dominant in only 20% (6/30) [Fig. 1(I)], the difference being significant ($Z=2.7$, $P=0.004$). In one pair (3.3%), the males had the same JH titers before the encounter and, after the encounter, the JH levels were increased in the dominant, but not the subordinate. The other 23.3% of pairings (7/30) resulted in no agonistic interactions. In the 22 pairs in which fighting occurred, the male with the higher JH titers before the encounter was more likely to become the dominant ($\chi^2=4.6$, $P=0.03$). Males with the higher JH titers before the encounter that became dominant, their JH levels were significantly ($t=-5.5$, $P<0.0001$) increased after the encounter. In the males with the lower JH titers before the encounter that became dominant, although their JH titers before the encounter was significantly ($t=3.9$, $df=20$, $P=0.001$) lower than that in the high titers group, it was significantly ($t=-4.7$, $P=0.005$) increased after the encounter to a similar level as that in high titer group. In all subordinates, JH levels after the encounter were not significantly increased.

(II) Pairs formed only from aggressive posture-adopting males.

In this experiment, fighting occurred in all 35 pairs formed. The percentages of encounters in which the male with the higher JH titers became dominant (45.7%, 16/35 pairs) and in which the male with the lower JH titers became dominant (48.6%, 17/35 pairs) were almost equal [Fig. 1(II)]. Before the encounter, JH levels in the males with the higher JH titers that became dominant were significantly higher than those in the males with lower JH titers that became dominant ($t=2.6$, $df=31$, $P=0.01$). After the encounter, JH levels in both groups were significantly increased to a similar level ($t=-8.3$, $P<0.0001$ and $t=-8.6$, $P<0.0001$, respectively). In 2 male pairs (5.7%), both males had the same JH titers before the encounter, and JH levels in the dominant, but not the subordinate, were increased after the encounter. In the groups in which the male with the higher JH titer became dominant, although JH levels in the subordinates were significantly increased by

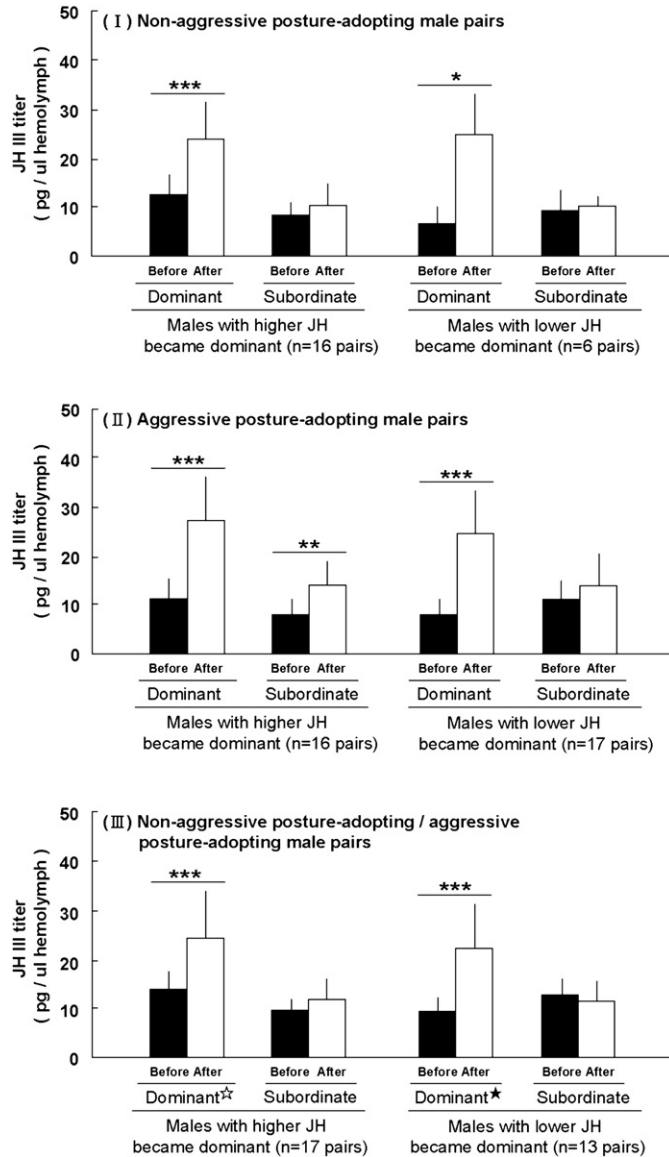


Fig. 1. Hemolymph JH III titer before and after the encounter in male pairs formed between (I) non-aggressive posture-adopting males, (II) aggressive posture-adopting males, or (III) non-aggressive posture-adopting and aggressive posture-adopting males. All males used were 80- to 85-day-old. Before: before the encounter (■). After: after the encounter (□). ☆: consisting of 11 that were aggressive posture-adopting and 6 that were non-aggressive posture-adopting males before the encounter. ★: consisting of 11 that were aggressive posture-adopting and 2 that were non-aggressive posture-adopting before the encounter. The data were log transformed for statistical analysis and back transformed for graphical depiction. The values are the mean \pm SD. The bars with the asterisks indicate statistical differences between 2 means, *, **, and *** = $P=0.005$, $P=0.002$, and $P<0.0001$ respectively.

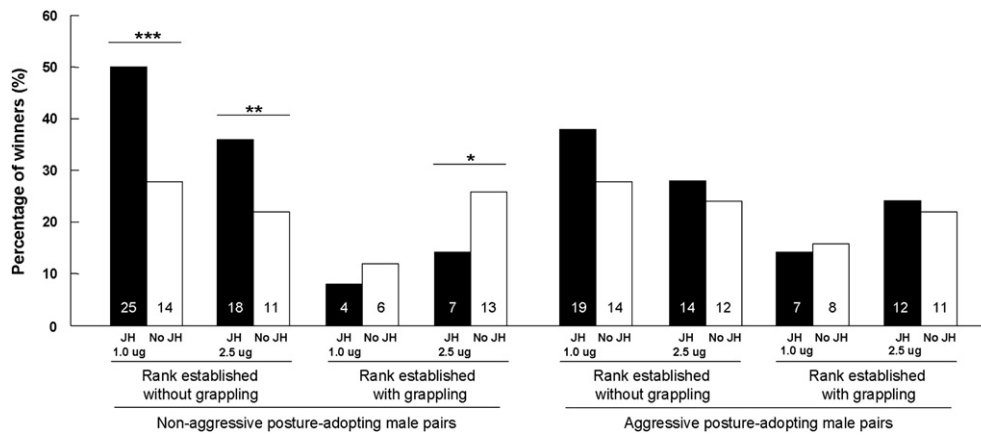


Fig. 2. Effect of JH III application on rank establishment. Two doses of JH III (1.0 µg or 2.5 µg) were tested. For each JH III dose, pairs were formed between one male injected with 1 µl of JH III and one injected with 1 µl of mineral oil. The pairs, consisting either of non-aggressive posture-adopting or aggressive posture-adopting males, were formed 7 days after injection and the status was observed. All males used were 80- to 85-day-old. The bars with the asterisks indicate statistical differences between 2 means, *, **, and ***: $P=0.042$, $P=0.037$, and $P=0.0025$ respectively. JH: JH III-treated (■). No JH: mineral oil-treated (□). The number in each column is the number of pairs in which fighting occurred. For each JH dose, the original sample size was 50 male pairs. The sum of all numbers within the columns for the same JH dose (including non-grappling and grappling male pairs) is the total number of male pairs in which fighting occurred. In the non-aggressive posture-adopting male pairs, the number of non-fighting male pairs was 1 in both the 1.0 µg JH-treated and 2.5 µg JH-treated groups. For the aggressive posture-adopting male pairs, the numbers of non-fighting male pairs in the 1.0 µg JH-treated and 2.5 µg JH-treated groups were 2 and 1, respectively.

the encounter ($t=-3.7$, $P=0.002$), they were still significantly ($t=5.9$, $df=30$, $P<0.0001$) lower than those in the dominants. In the groups in which the male with the lower JH titer became dominant, there was no significant increase in JH levels in the subordinates after the encounter.

(III) Pairs formed from non-aggressive posture-adopting and aggressive posture-adopting males.

In this experiment, in which pairs were formed by pairing non-aggressive posture-adopting with aggressive posture-adopting males, fighting occurred in all pairs. Among the 30 pairs, 56.7% (17/30 pairs) and 43.3% (13/30 pairs) resulted in the male with the higher or lower JH titer, respectively, becoming dominant, indicating that the relative JH levels had no obvious effect on status determination ($\chi^2=0.31$, $P=0.58$) [Fig. 1(III)]. Of the dominants which had a higher JH titer before the encounter, significantly more were aggressive posture-adopting before the encounter [64.7% (11/17 pairs)] ($Z=1.7$, $P=0.04$) and the same applied to the dominants which had lower JH titers before the encounter [84.6% (11/13 pairs)] ($Z=3.5$, $P=0.0002$). In significantly ($Z=3.6$, $P=0.0002$) more of the pairs (73.3%, 22/30 pairs), the dominant was aggressive posture-adopting before the encounter,

indicating that aggressive posture-adopting males had a significantly higher probability of becoming winners ($\chi^2=7.8$, $P=0.005$). Again, in all dominants, JH levels were significantly increased by the encounter ($t=-5.4$, $P<0.0001$ and $t=-7.6$, $P<0.0001$ for the males with higher or lower JH titers that became dominants, respectively). Although JH levels before the encounter were significantly higher in the males with higher JH titers that became dominants than in those with lower JH titers that became dominants ($t=4.1$, $df=27$, $P=0.0004$), JH levels after the encounter were similar in both kinds of dominants. JH levels were not significantly increased in any of the subordinates by the encounter.

Among the 87 male pairs from all three kinds of pairings combined in which fighting occurred, the male which attacked first had a significantly greater probability of being the winner [$\chi^2=19.2$, $P<0.0001$; $\chi^2=14.2$, $P=0.0002$, and $\chi^2=22.5$, $P<0.0001$ for the non-aggressive posture-adopting pairs (21/22 pairs), the aggressive posture-adopting pairs (28/35 pairs), and the non-aggressive posture-adopting/aggressive posture-adopting pairs (28/30 pairs), respectively].

Experiment 2: Effect of JH III application on initial rank establishment

In this experiment, a single injection of 1.0 or 2.5 µg of JH III (in 1 µl mineral oil) was given to one animal from a pair of either non-aggressive posture-adopting males or aggressive posture-adopting males and the other received 1 µl of mineral oil. Seven days after injection, each JH-treated male was paired with a corresponding mineral oil-treated male and the status observed. The results are shown in Fig. 2. Because of the high percentage of pairs showing grappling (two males catching and biting each other and rolling around) in the 2.5 µg JH-treated non-aggressive posture-adopting male pairs, we categorized the fighting conditions as “without grappling (dominant/subordinate determined instantly at the moment of mutual contact)” and “with grappling”.

In those non-aggressive posture-adopting male pairs in which rank was established without grappling, the percentage of JH-treated winners was significantly higher than the percentage of mineral oil-treated winners ($Z=3.2$, $P=0.0025$ and $Z=2.2$, $P=0.037$ for the 1.0 and 2.5 µg JH-treated group, respectively).

In those non-aggressive posture-adopting male pairs in which rank was established with grappling, surprisingly, significantly fewer ($Z=2.2$, $P=0.042$) of the 2.5 µg, but not the 1 µg, JH-treated animals than mineral oil-treated animals won the fight. The percentage of fights with grappling (40%, with 14% JH-treated winners and 26%

Table 1
Effect of JH III treatment on rank switch

	Rank switch (%) ^a	Rank switch day ^{a,b}
<i>Non-aggressive posture-adopting dominant/subordinate pairs</i>		
JH-treated (1 µg) (n=42 pairs)	21.4	17.8±8.0
Control (n=42 pairs)	14.3	18.8±9.2
JH-treated (2.5 µg) (n=30 pairs)	13.3	18.3±6.8
Control (n=30 pairs)	13.3	14.4±4.4
<i>Aggressive posture-adopting dominant/subordinate pairs</i>		
JH-treated (1 µg) (n=48 pairs)	18.8	15.1±7.7
Control (n=48 pairs)	18.8	17.7±11.0
JH-treated (2.5 µg) (n=30 pairs)	20.0	18.0±3.0
Control (n=30 pairs)	14.0	10.6±6.4

Dominant/subordinate male pairs were formed from either non-aggressive posture-adopting or aggressive posture-adopting males. Starting on the 2nd day of pairing, the subordinate in the JH-treated group was injected once every 10 days for a total of 30 days with 1 or 2.5 µg of JH III in 1 µl of mineral oil, while the subordinate in the control group was injected with 1 µl of mineral oil. Rank switch was examined daily.

^a There was no significant difference (t -test, $P>0.05$) in the percentage of pairs showing a rank switch or the day of rank switch between the JH-treated (1 µg or 2.5 µg) and control groups in either the non-aggressive posture-adopting or aggressive posture-adopting male pairs.

^b Rank switch day was the day of the switch counted from the first pairing day (Mean ± SD). The first pairing day was day 1.

mineral oil-treated winners) was significantly ($Z=3.4$, $P=0.001$) higher than that in untreated pairs (18.0%, 9/50 non-aggressive posture-adopting male pairs; these 50 male pairs were observed, paralleled to Experiment 2, to provide background data).

In the aggressive posture-adopting males, regardless of whether rank was established with or without grappling, the percentage of winners was not influenced by JH treatment. The percentage of fights with grappling was not different in the 2.5 μg JH-treated group (46%, with 24% JH-treated winners and 22% mineral oil-treated winners) ($P=0.39$) from that in untreated pairs (40.0%, 20/50 aggressive posture-adopting male pairs). In untreated pairs, the percentage of grappling fights in the aggressive posture-adopting pairs (40%) was almost double that in the non-aggressive posture-adopting pairs (18%).

Experiment 3: Effect of JH III application to subordinates on rank switch

In this experiment, the effect of JH III on rank maintenance was examined separately in aggressive posture-adopting or non-aggressive posture-adopting pairs. For each kind of male pair, each subordinate was injected at 10 day intervals for 30 days, starting on the 2nd pairing day, with 1 μl of JH III (1 or 2.5 μg in 1 μl of mineral oil) or 1 μl of mineral oil alone (separate control groups for each dose). Table 1 shows that there was no significant difference in either the percentage of rank switching pairs or the day of rank switch (time from the 1st pairing day to rank switch) between the JH-treated and mineral oil-treated control groups for either the non-aggressive posture-adopting or aggressive posture-adopting pairs.

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). The outcome of these interactions is the formation of an unstable dominant/subordinate hierarchy, and changes in rank order are common after a male has been dominant for several weeks (Ewing 1972; Bell and Gorton, 1978). Our recent results showed that, for socially naïve *N. cinerea* males, both attack behavior and an increase in JH III levels can be induced solely by contact with an isolated antenna (Chou et al., 2007). For any new rank formation, significantly higher JH III levels are always associated with the dominant status (Kou et al., Accepted). In the present study, during a fight between two males, several additional phenomena were observed.

(A) *The relative JH III levels in the paired males before the encounter were not the absolute determinant of winning or losing*

Although, in the non-aggressive posture-adopting male pairs, the one with higher JH III levels before the encounter was more likely to be the dominant, this was not the case in pairs formed between aggressive posture-adopting males or non-aggressive posture-adopting/aggressive posture-adopting males. In the non-aggressive posture-adopting male pairs, the fact that the one with higher JH III levels than its rival was more likely to win may indicate an activation effect of JH, i.e., activation of aggression. The influence of being in an aggressive condition on winning or losing was clearly seen in the non-aggressive posture-adopting/aggressive posture-adopting pairs. In these pairs, the relative JH III levels before the fight were not related to the social status, while significantly more individuals (especially those with relatively lower JH III levels) that were aggressive posture-adopting before the encounter achieved dominant status (64.7% and 84.6% of winners in the males with higher or lower JH III titers, respectively, were aggressive posture-adopting). In the aggressive posture-adopting male pairs, no association was seen between the relative JH III levels before the encounter and dominant/subordinate

status; this may be because both sides were already in the physiological condition of “ready for fight” before the encounter and therefore JH III could not exert an activation effect. Since JH III levels were not the determining factor, we must ask what determines winning or losing in aggressive posture-adopting male pairs or even in all fighting pairs. Based on the fact that nearly 88.5% of dominants (77/87 male pairs) in the present study and 100% (100 male pairs) in our previous unpublished results were the animal which attacked first, it seems that the first attacker usually claims superiority. Thus, the sensitivity of the periphery nervous system or the speed of the signal transduction cascade from the social cue to the attack behavior, together with the attack strength, might be the most important determinant for winning or losing. All these possibilities require further investigation.

Whether an individual male was physiologically aggressive (i.e., aggressive posture-adopting) before the encounter was not JH III-related, since (i) about 50% of individuals in the aggressive posture-adopting pairs had higher or lower JH III levels before the encounter and (ii) there was no difference in JH III levels between aggressive posture-adopting and non-aggressive posture-adopting males before the encounter. The situation seems a little more complex in the non-aggressive posture-adopting males, as, before the encounter, whether a male was aggressive posture-adopting or not was not JH III-related, whereas, after the encounter, whether he was a dominant was determined by whether he attacked first. We hypothesize that antenna contact between two males (perception of social stimuli) activates a signaling cascade from the periphery nervous system to the central nervous system to activate the aggression system and that JH III is involved in this pathway. Briefly, after perception of social stimuli, the expression of attack behavior in the “not ready for fight” individuals may involve a series of JH III-related signaling events, leading to the activation of JH receptors in the central processing system and of aggressive ability. We speculate that all of these events are dependent on JH III, but not on JH III levels; even low JH levels exceed a threshold for activation in most males. The reason why, in non-aggressive posture-adopting male pairs, the winner was more likely to be the one with the higher JH III levels before the encounter could be because more JH III molecules were available, increasing the possibility that they would bind to the JH receptor and activate aggression, but not guaranteeing a more rapid response in the whole signal transduction cascade and leading to the animal attacking first, since any problem in the signal transduction cascade before, during, or after JH receptor binding would stop further signaling. On the other hand, lower JH III levels do not necessarily mean less JH receptor activation, as, for instance, the presence of more JH binding proteins could still lead to activation of the signaling pathway. This could explain why some individuals in the non-aggressive posture-adopting pairs with relatively lower JH III levels before the encounter were still able to be winners. External stimulus perception by the periphery nervous system (such as neurophysiological sensitivity) and central processing mechanisms might be similar in higher and lower JH dominants. In *N. cinerea*, a physiologically aggressive condition does not necessarily equate with attack, but makes it much more likely for attack to occur if stimulated with some external cue to which the insect is inherently predisposed. This is similar to a person in an angry mood, who does not necessarily attack, but is in a dangerous state and is much more likely to attack if given an appropriate stimulus. Our present result in *N. cinerea* showing that lower or higher JH III levels before the encounter did not determine winning or losing may be a quite general phenomenon, since in burying beetle females, natural JH titers also do not predict the outcome of aggressive interactions (Scott, 2006b). Our result is also very similar to previous results in the cichlid fish (*Oreochromis massambicus*) (Oliveira et al., 1996), in which steroid concentrations prior to male group formation are not a good predictor of the social status attained by each fish and social status itself can modulate sex steroid concentrations after male group formation. In fact, androgens in *O. massambicus* are viewed not only as sex steroids, but also

as competition hormones that respond to the social environment and prepare the individual for competitive situations (Oliveira et al., 2001).

(B) In all first encounter fights between any of the three types of male pairs, JH III levels after the encounter were always significantly higher in the dominant than in the subordinate

(a) In the subordinates

In most males, the attack response and increase in JH III levels can be rapidly induced within seconds solely by contact with an isolated antenna (Chou et al., 2007). In the subordinate, a lack of response (no attack behavior or JH III increase) after exposure to male–male challenge might indicate suppression of responsive capability by the dominant. This suppression might be mainly due to the fierce attack, which is accompanied by the release of large amounts of the submission-inducing pheromone 3H-2B (Kou et al., 2006; Chen et al., 2007; and unpublished data) or to other attack-related factors. Alternatively, the subordinate may realize that he will lose during the first seconds of the encounter, resulting in no increase in hormone levels. This assessment of “information on the likely outcome of a contest” was first proposed in *O. massambicus* (Oliveira et al., 2005), in which information on the likely outcome of a contest is required before the androgen response is triggered in a combatant.

(b) In the dominants

The rapid increase in JH III levels after winning suggests that it may play an important role in sustaining aggressive behavior and the physiological activities that accompany rank formation. It is therefore highly possible that, after the encounter, the rapid increase in JH III levels is associated with either fighting ability or rank maintenance. A similar phenomenon of the JH titer being significantly increased in aggressive or dominant individuals is seen in both the honey bee, *A. mellifera*, (Pearce et al., 2001) and the burying beetle, *N. orbicollis* (Scott, 2006a). In the song sparrow, *Melospiza melodia*, T appears to increase the persistence of aggression following an intrusion, rather than activating aggression per se (Wingfield et al., 1987; Wingfield, 2005). Similarly, in *O. massambicus*, it has been suggested that there is an adaptive mechanism that allows individuals to mount an androgen response for the purpose of maintaining their social status after they have assessed the relative fighting ability of their challenger (Oliveira et al., 2005). In vertebrates, there are potential adaptive benefits by increasing androgen levels during social challenge, such as enhancing cognitive tasks, social attention, learning, and memory (Andrew and Rogers, 1972; Cynx and Nottebohm, 1992).

(C) JH III application had no absolute effect on initial rank establishment

The result of JH III treatment before the encounter showed that, in non-aggressive posture-adopting pairs, significantly more JH-treated than control individuals won a non-grappling fight and also the probability of grappling was significantly increased in control winners, indicating that fighting ability might be increased in JH-treated individuals, so the oil-treated control rival must grapple with the JH-treated animal if he wants to win. This effect of JH on rank establishment in these “not-ready-for-fight” individuals is consistent with the results of Experiment 1, which showed that significantly more non-aggressive posture-adopting males with a higher JH titer than their rival before the encounter achieved dominant status. The result of JH III treatment strengthens our speculation about the activation effect of JH, i.e., raising the physiological condition to a status of “ready-for-fight” or more aggressive behavior. The ability of JH treatment to increase the probability of winning has been reported in the social wasp *P. annularis* (Barth et al., 1975) and paper wasp *P. gallicus* (Röseler et al., 1984). However, even so, a significantly increased probability does not mean it is the absolute determinant. This raises the question of why the aggressive posture-adopting males

were not affected by JH treatment (although the JH-treated group seemed to win a little more frequently). We speculate that, because the aggressive posture-adopting males were physiologically already ready for fight, additional JH supplementation before the encounter would only have a limited strengthening effect, if any, on the already activated aggression system.

(D) JH III application to subordinates had no effect on rank switch

The fact that JH application to the subordinates resulted in no change in the original dominant/subordinate relationship is similar to the observation of Röseler (1985) that treatment with JH does not alter dominant status once determined. This result further indicates that the increased endocrine activity in the dominants was a consequence, and not a cause, of dominant rank formation. After the encounter, the significantly raised JH levels are absolutely dominant rank-associated (Kou et al., 2008; also in the present study). In vertebrates, although males behavior can be strongly influenced by androgens, the release of androgens and other hormones can also be triggered by the behavior (Wingfield and Hahn, 1994; Oliveira et al., 2001; Canoine and Gwinner, 2002). In fact, social interactions have shown rapid effects on plasma androgen concentrations in many vertebrate taxa, such as fish, amphibians, reptiles, birds, and mammals (Archer, 2006; Hirschenhauser and Oliveira, 2006). Furthermore, gene activity changes in both participants can be induced from social cue-induced steroid level changes (Adkins-Regan, 2005). Our present results showed that, in *N. cinerea*, the interplay between behavior and hormones can be as complex as that in vertebrates. For males, isolation since emergence may provide conditions in which the endocrine system is free of social stimuli. Although JH levels may regulate behavioral outcomes (after the encounter, aggression might be more easily activated in non-aggressive posture-adopting males with higher JH levels), the converse is even more obvious, i.e. social interactions can have profound effects on hormone levels, since JH levels were significantly increased in all dominants, regardless of their having relatively higher or lower JH levels before the encounter. Behaviorally, the aggressive posture, which is accompanied by a large amount of pheromone release (microgram amounts of 3H-2B in 10 min) and abdomen pumping, was much strengthened after the encounter in the dominants and was temporarily suppressed in the subordinates. There is an obvious difference in aggressive posture behavior before and after challenge. Before the encounter, the aggressive posture is not adopted daily, but discontinuously, being seen for a few days, then not for the next few days (Chen et al., 2007), whereas, after the encounter, it is seen daily, even in the absence of the subordinate, for at least 2 months. Considering the energy required for elongating and pumping the abdomen, making attacks, and 3-hydroxy-2-butanone (3H-2B) biosynthesis and release, rank maintenance in *N. cinerea* is physiologically costly. The signaling pathways by which JH causes activation and promotion of the aggression system (such as aggressive posture or attack behavior, accompanied by 3H-2B release) in the dominant and by which the aggression system is temporarily silenced in all subordinates require further investigation.

In summary, the lobster cockroach is a very good model system for studying social stimuli–hormone responses in invertebrates. It provides both a non-ready-for-fight (i.e., non-aggressive posture-adopting) and a ready-for-fight (i.e., aggressive posture-adopting) behavioral phenotype for the investigation of activation (for rank establishment) and promotion (for rank maintenance) effects of JH on aggression. Although the theoretical axis of social stimuli–behavioral responses has been well established in vertebrates, there is little evidence for it in invertebrates. Our present findings on social stimuli and the hormonal response in insects are very similar to those in vertebrates, although application of the challenge hypothesis in its entirety to insects and JH still awaits further study using Trumbo's criteria (Trumbo, 2007).

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