

# Pheromone, Juvenile Hormone, and Social Status in the Male Lobster Cockroach *Nauphoeta cinerea*

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In this study, the major pheromone component, 3-hydroxy-2-butanone (3H-2B), released by dominants was measured during early scotophase. Both the JH III titer in the hemolymph and the 3H-2B content of the sternal glands of the dominants and subordinates were then measured during late scotophase and late photophase. These investigations were performed on encounter days 1, 2, 3, 5, 7, 9, 12, and 20. The results showed that, for non-aggressive posture (AP)-adopting socially naïve males (SNMs), both the 3H-2B release and the hemolymph JH III titer were maintained at a low level. Once a fight occurred, 3H-2B release was raised significantly in the AP-adopting dominants, but not in non-AP-adopting subordinates, and remained raised throughout the entire experimental period. At 30 min after the first encounter, the hemolymph JH III titer was significantly increased in dominants, but not in subordinates. A significantly higher hemolymph JH III titer was observed in dominants during late scotophase on days 3, 5, 12, and 20 and during late photophase on days 3, 5, and 20. After fighting, the sternal gland 3H-2B content of the dominants or subordinates was significantly lower than in SNMs. In dominants, the sternal gland 3H-2B content during late scotophase was significantly lower than that during late photophase in the first 9 domination days, while, in the subordinates, the 3H-2B content during late scotophase was either similar to, or significantly higher than, that in late photophase. In the dominants, 3H-2B release and JH III titer were positively correlated. In rank switchers, the switched social status was positively correlated with both 3H-2B release and JH III titer. Comparison of 3H-2B release and JH III titer in 1-time, 3-time, or 5-time dominants showed that, although winning significantly increased both 3H-2B release and JH III titer, there is no significant difference in 3H-2B release between 3- and 5-time winners, while the JH III titer was most significantly increased in the 3-time winners. The possible relationship between pheromone release, JH III titer, and social status is discussed. Arch. Insect Biochem. Physiol. 68:144–155, 2008. © 2008 Wiley-Liss, Inc.

KEYWORDS: 3-hydroxy-2-butanone; juvenile hormone III; *Nauphoeta cinerea*; RIA; social status

## INTRODUCTION

The lobster cockroach, *Nauphoeta cinerea* (Dictyoptera: Blaberidae), is well known for its male conspecific agonistic behavior (Kramer, 1964; Ewing, 1967). During the actual fight, the dominant male assumes the aggressive posture (AP), characterized by an elongated, upturned abdomen, and a stilted gait. He then usually pushes himself underneath the subordinate male and flips the subordinate up-

side down. Subsequently, the subordinate stays away from the dominant, tucking its legs and antennae close to its body (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).

The relationship between pheromone components and social status was investigated after the

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Abbreviations used: AP, aggressive posture; CA, corpora allata; 3H-2B, 3-hydroxy-2-butanone; JH, juvenile hormone; RIA, radioimmunoassay; SNMs, socially naïve males; SP, submissive posture.

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identification of sex pheromones (Sr ng, 1990; Moore et al., 1995; Everaerts et al., 1997). Sex pheromone in this species 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) (Sr ng, 1990). Previous studies on pheromone differences after rank formation resulted in no consensus (Moore et al., 1995; Everaerts et al., 1997). Recently, it was found that, during the first encounter fight, microgram levels of 3H-2B are released by the AP-adopting dominants (Kou et al., 2006); also the strategic 3H-2B release adopted by the dominants was used as a signal to determine dominance (Chen et al., 2007).

Although there are ample data on *N. cinerea* agonistic behavior and the underlying pheromonal system (Ewing, 1967; Moore et al., 1995; Everaerts et al., 1997; Moore, 1997), relatively little is known about the endocrinological factors involved in hierarchy formation or pheromone production (Hartman and Suda, 1973; Schal and Bell, 1983; Sr ng et al., 1999; Chen et al., 2005). Hartman and Suda (1973) suggested that pheromone production does not seem to be controlled by the corpora allata (CA). Schal and Bell (1983) also reported that removal of the CA does not affect the formation of hierarchies. Sr ng et al. (1999) showed that allatectomy, performed 2–3 days after emergence, led to decreased sex pheromone levels, and that administration of juvenile hormone (JH) III to allatectomized males restores both cellular differentiation of the gland and pheromone production. Chen et al. (2005) demonstrated that the CA from dominants has a significantly higher in vitro JH III release rate than that from subordinates. Recently, we further found that for SNMs, the agonism is induced by antenna contact, which results in the concomitant expression of attack behavior and an significant increase in both 3H-2B release and JH III titer (Chou et al., 2007).

In considering the dominant–subordinate hierarchy formation in *N. cinerea*, the AP is an important behavioral index of agonism. The AP is not solely a posture, as its appearance is absolutely and simultaneously associated with a large amount of

pheromone release (usually microgram levels of 3H-2B) by the dominants (Kou et al., 2006; Chen et al., 2007). Thus, both pheromone production (and/or release) and behavior development have to be considered in elucidating the endocrinological factors involved in rank formation. JH regulates pheromone production in some cockroach species, such as that of sex-specific contact pheromone in the female German cockroach *Blattella germanica* (Schal et al., 2003). In terms of insect agonistic behavior, JH also acts in behavioral development (Hartfelder, 2000), especially in adult agonistic behavior in social wasps, such as *Polistes annularis* (Barth et al., 1975) and *P. gallicus* (R seler et al., 1984), and in the eusocial honey bee, *Apis mellifera* (Huang et al., 1994).

In *N. cinerea*, the only product released by the CA is JH III (Baker et al., 1984) and the main pheromone component released by AP-adopting dominants is 3H-2B (2-MT and 4E-2M being detected in only a few cases and in small quantities within the 1-h sampling period) (Kou et al., 2006). To provide basic information about the neuroendocrine factors underlying hierarchy formation, the daily patterns of both 3H-2B release in dominants and hemolymph JH III titer changes in both dominants and subordinates were investigated over a period of 20 days after encounter. In addition, the 3H-2B content of the sternal glands was measured in both dominants and subordinates.

## MATERIALS AND METHODS

### Chemicals

All chemicals used for pheromone analysis and quantification were from Sigma (Natick, MA). For hemolymph sample preparation and radioimmunoassay (RIA), all solvents and chemicals were HPLC grade from Fisons or Merck Chemical Co.

### Cockroaches

Mass rearing was basically according to Kou et al. (2006) on a reversed 16-h light/8-h dark photoperiodic cycle. Each male was isolated within the

24 h following the imaginal molt in order to control for the effects of learning (Manning and Johnstone 1970), and these are referred to hereafter as socially naïve males (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). In *N. cinerea*, about 93% ( $n = 320$ ) of adults emerge during photophase, so, in this experiment, each experimental day started with photophase and ended with scotophase. All pheromone or hemolymph samples were taken at the same time of day (1–4 h into scotophase) to minimize any variation caused by circadian rhythms.

**Experiment 1: Measurement of 3H-2B release by dominants and hemolymph JH III titer and sternal gland 3H-2B content in both dominants and subordinates.**

**Male group formation.** In this experiment, each group was formed by randomly placing two non-AP-adopting SNMs of identical age (80–85 days) and similar size in a glass aquarium ( $12 \times 12 \times 12$  cm) to generate one dominant and one subordinate male on the encounter day, (300 male groups). For individual identification, each male was marked on its pronotum with white Tipp-Ex fluid.

**Pheromone sampling and analysis.** Pheromone samples were taken during early scotophase on days 1, 2, 3, 5, 7, 9, 12, and 20, with day 1 being the day of the first encounter fight. For pheromone sampling, the air 1.5–2 cm above an individual dominant was sampled for 10 min. Air samples were taken using adsorbent sample tubes, each packed with activated charcoal granules (# 226-01, 20–40 mesh, 150 mg, SKC Co., Eighty-Four, PA, USA), connected to an air sampling pump (model 210-1000, SKC Co.) operating at 200 ml/min. During the first encounter fight, if initial grappling occurred (lasting about 3–10 s), sampling was only started once the final dominant–subordinate relationship had been established, then samples were collected using the sample tube, following the dominant if it moved. To ensure sampling only collected pheromones from the dominant, the subordinate was temporarily moved to another glass aquarium; sometimes, the non-AP-adopting sub-

ordinate was also sampled in the new aquarium to provide background data. After sampling, the subordinates were moved back to the original glass aquarium. On the day of rank switch, samples were taken from the new dominant (originally the subordinate) and the step-down dominant (now the subordinate) in separate glass aquaria, as described above. SNMs of the same age were also sampled individually. At the end of each sampling session, the collecting tube was eluted with 4 ml of dichloromethane and 2,000 ng of 2-phenylethanol added to the eluate as the internal standard. The eluates were stored in glass vials with Teflon-lined screw caps at  $-20^{\circ}\text{C}$  and were analyzed within 24–48 h.

For analysis and quantification of air samples, the eluates were concentrated by evaporation under a gentle stream of nitrogen minutes before analysis, then analyzed by splitless coupled gas chromatography-mass spectrometry (GC-MS) using a Thermo Quest Trace GC interfaced with a Finnigan Trace Mass (electron impact ionization, 70 eV) (Ho and Millar, 2001; Ho et al., 2003). The temperature of the GC was held at  $75^{\circ}\text{C}$  for 1 min, then increased sequentially to  $110^{\circ}\text{C}$  at  $5^{\circ}\text{C}/\text{min}$ , to  $113^{\circ}\text{C}$  at  $1^{\circ}\text{C}/\text{min}$ , and to  $240^{\circ}\text{C}$  at  $25^{\circ}\text{C}/\text{min}$ , then maintained at  $240^{\circ}\text{C}$  for 5 min, with injector and transfer line temperatures of  $200^{\circ}\text{C}$  and  $250^{\circ}\text{C}$ , respectively. A DB-23 column ( $30 \text{ m} \times 0.25 \text{ mm}$ , J & W Scientific, Folsom, California) was used, with helium as carrier gas. Compounds were tentatively identified from the interpretation of the mass spectra and from matches with the NBS-NIH mass spectral data base. Identification was confirmed by comparison of the retention times and spectra with those of authentic standards. GC-MS was used for quantification of the collected compound. The instrument responses to chemical standards and the internal standard, 2-phenylethanol, were determined by coinjecting 10 ng of the compounds onto 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 internal standard quantification, a total of 6 doses of the standard (100, 1,000, 3,000, 5,000, 7,000, and 10,000 ng of 3H-2B) were used to build the standard curve. The recovery rate

(0.909) of our sampling system was measured by sampling for 10 min in an identical fashion from a glass aquarium containing 5  $\mu$ l of a 400 ng/ $\mu$ l 3H-2B solution placed on a piece of aluminum foil on the aquarium bottom, with a total of 7 replicates. All data were adjusted for the recovery rate (0.909).

**Measurement of the hemolymph JH III titer in dominants and subordinates.** For hemolymph JH III titer measurement, hemolymph samples were prepared from each dominant and/or subordinate during both late (13–15 h into) photophase and late (5–7 h into) scotophase on days 1, 2, 3, 5, 7, 9, 12, and 20. Hemolymph was also sampled from dominants and subordinates 30 min after the first fight (early scotophase of day 1). The hemolymph was obtained by placing the insect on its back, making a cut with a fine pair of scissors along the connection between the tergum and thorax tissue, and quickly collecting the hemolymph with a capillary tube. Hemolymph from individual SNMs was also sampled immediately (within 1 min), and 30 min, after the cut to provide background data about whether the JH III titer change was caused by the cut. The hemolymph (6–8  $\mu$ l/male) was immediately mixed with 500  $\mu$ l of acetonitrile to denature any enzymes that could affect JH and the samples immediately placed on ice, then stored at  $-20^{\circ}\text{C}$  for subsequent JH analysis. Capillary tubes and all other glassware that would come into contact with the JH were baked at  $500^{\circ}\text{C}$  for 3.5 h before 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 RIA (Hunnicuttt 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 hemolymph samples was extracted twice, using 0.5 ml of hexane for each extraction, then the hexane extract was evaporated using a vacuum centrifuge (Speedvac) linked to a condenser that trapped the solvent at

$-98^{\circ}\text{C}$  (Savant SS21). The dried JH in the sample tube was dissolved in a 200- $\mu$ l premixed buffer containing anti-JH antiserum (1:14,000 dilution) and 8,000 DPM of  $[10^{-3}\text{H}(\text{N})]$ -JH (NEN, 647.5 Gbq/mmol), and the sample incubated at room temperature for 2 h. Dextran-coated charcoal (DDC) solution (0.5 ml) was added to each sample tube for 2.5 min, which was then centrifuged (2,000g 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.

**Analysis and quantification of 3H-2B in whole gland extracts from dominants and subordinates.** After hemolymph sampling, whole gland extracts were prepared during late scotophase and late photophase on days 1, 3, 5, 7, 9, 12, and 20. Whole gland extraction was performed after the hemolymph samples were collected and after pheromone sampling from the dominants. The sternum of each dominant and/or subordinate was rapidly removed and completely submerged in 1 ml of dichloromethane for 24 h at room temperature, then the sternum was removed and 2,000 ng of 2-phenylethanol added to the extract as the internal standard. The extracts were stored at  $-20^{\circ}\text{C}$  in glass vials with Teflon-lined screw caps and analyzed within 24–48 h, as described above.

**Experiment 2: Pheromone release and hemolymph JH III titer in dominants with different numbers of wins.** To investigate whether the number of wins affected pheromone release and hemolymph JH III titer, the following experiment (shown in Diagram 1) was performed. Two SNMs were paired to fight for 5 min to obtain a 1-time dominant (1-time winner); then the subordinate was removed and the dominant allowed to rest for 5 min. Two 1-time dominants were then paired for another 5 min fight to get a 2-time dominant (2-time winner), and this process was repeated to obtain up to 5-time winners. One-time, 3-time, and 5-time dominants ( $n = 15$ –18 insects in each group) were then compared for pheromone release and hemolymph JH III titer, as described above.

**Statistical Analysis**

The *t*-test (SAS Institute, 1990) (for a normal distribution of data) or nonparametric test (if the data were not a normal distribution) was used to compare 3H-2B release between SNMs and dominants on day 1, JH III titers between SNMs and dominants (or subordinates) at 30 min after the first encounter fight, 3H-2B content between SNMs and dominants (or subordinates) on day 1, and sternal gland 3H-2B content between photophase and scotophase.

The paired *t*-test (normal distribution) or nonparametric test (if the data were not a normal distribution) was used to compare daily JH III titers between dominants and subordinates, sternal gland 3H-2B content between dominants and subordinates, and 3H-2B release and/or JH III titer between

new dominants and step-down dominants when rank switched.

The chi-squared test was used to analyze the association between the JH III titer in SNMs and subsequent social status. For rank switchers, the correlation between JH titer and 3H-2B release in dominants was analyzed by Pearson correlation analysis, and that between social status and 3H-2B release (or between social status and JH III titer) by Spearman correlation analysis. One-way analysis of variance (ANOVA) was used to compare the JH III titer between dominants with different numbers of wins.

In Diagram 1, two 80- to 85-day-old SNMs fought for 5 min to create a 1-time dominant; then the subordinate was removed and the dominant allowed to rest for 5 min. Two randomly chosen 1-time dominants were then paired to fight for an-

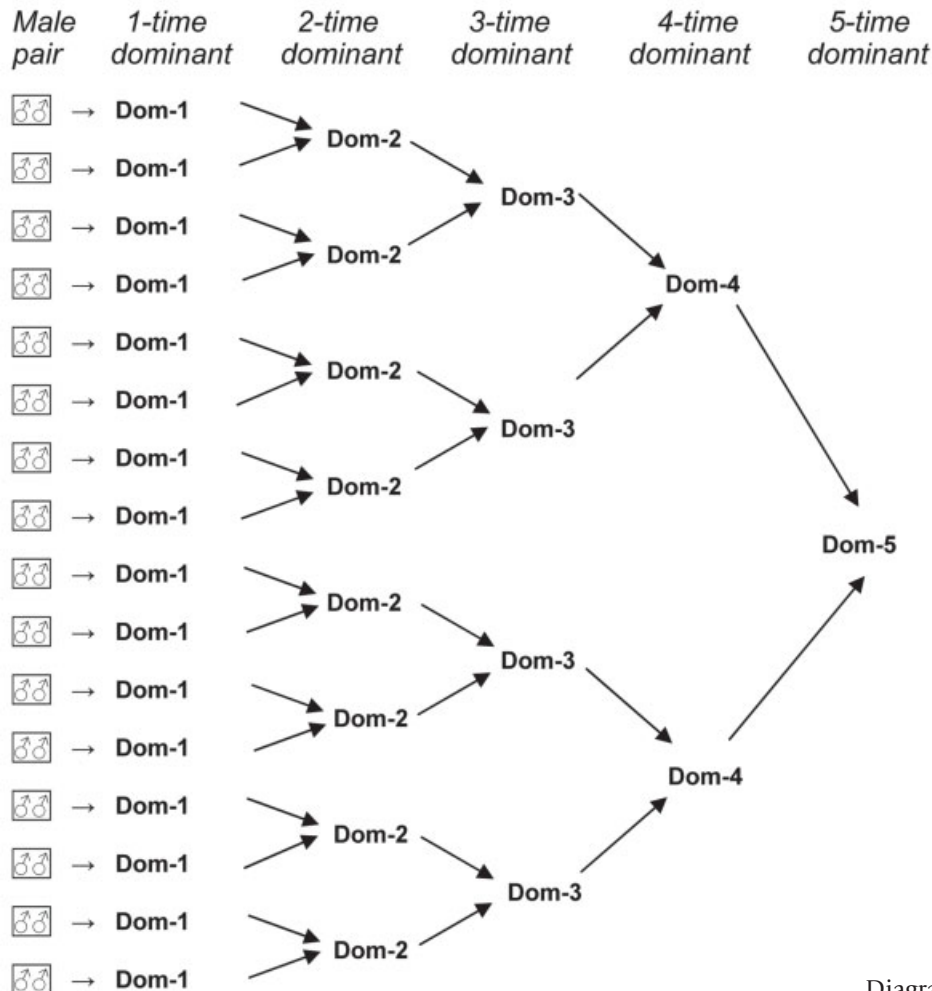


Diagram 1.



other 5 min to create a 2-time dominant, and so on. Thirty-two SNMs were used to create one 5-time dominant (see Diagram 1).

## RESULTS

### Pheromone Release by Dominants

As reported previously (Kou et al., 2006), the mass spectra data showed that the major pheromone component collected was 3H-2B. Compared to non-AP-adopting SNMs, 3H-2B release in AP-adopting dominants was significantly increased ( $t = 17.0$ , d.f. = 11,  $P < 0.0001$ ) during the first encounter fight (Fig. 1A). 3H-2B release in the AP-adopting dominants fluctuated within the range of 2800–5000 ng on the test days during the 20 days investigation period except for a decrease on day 12. 3H-2B release in subordinates adopting the submissive posture (SP), 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, fluctuated within a lower range (87.3–169.6 ng/male/10 min). On the day of rank switch (average rank switch day:  $17.2 \pm 1.1$  days,  $n = 14$ ), the new dominants exhibited significantly ( $\chi^2 = 17.3$ , d.f. = 1,  $P < 0.0001$ ) higher 3H-2B release than the new subordinates (originally dominant, now stepped down). For rank switchers, there was a significant correlation ( $r = 0.9$ ,  $P < 0.0001$ ) between social status (switched dominant or subordinate status) and 3H-2B release.

### Hemolymph JH III Titer in Dominants and Subordinates

Figure 1A shows that the JH III titer in SNMs was low. At 30 min after the first encounter fight, the JH III titer was significantly increased in the dominants ( $t = 7.0$ , d.f. = 18,  $P < 0.0001$ , compared to SNMs;  $t = 9.2$ , d.f. = 9,  $P < 0.0001$ , compared to subordinates), but not in the subordinates. In hemolymph samples obtained during late scotophase, the JH III titer of dominants fluctuated within the range of 11.7–23.5 pg/ $\mu$ l during the first 9 days, fell sharply on day 12, and peaked again

on day 20. In the subordinates, the JH III pattern was similar, although the titers were usually lower. During late scotophase, a significant difference in JH III titer between the dominants and subordinates was seen on days 3 ( $t = 3.1$ ,  $P = 0.007$ ), 5 ( $t = 2.6$ ,  $P = 0.02$ ), 12 ( $t = 2.6$ ,  $P = 0.02$ ), and 20 ( $t = 2.7$ ,  $P = 0.015$ ). On the day of rank switch, the new dominants exhibited a significantly ( $t = -4.3$ ,  $P < 0.001$ ) higher JH III titer than the new subordinates (originally dominant, now stepped down). For rank switchers, there was a significant correlation ( $r = 0.78$ ,  $P < 0.0001$ ) between social status (switched dominant or subordinate status) and the hemolymph JH III titer. For hemolymph samples obtained during late photophase (Fig. 1B), the JH III pattern change was similar to that during late scotophase; a significant difference in JH III titer between dominants and subordinates was seen on days 3 ( $t = 2.8$ ,  $P = 0.014$ ), 5 ( $t = 4.2$ ,  $P = 0.0006$ ), and 20 ( $t = 3.9$ ,  $P = 0.002$ ). Pearson correlation analysis showed that, in the dominants, 3H-2B release was positively correlated with hemolymph JH III titer ( $r = 0.8$ ,  $P = 0.01$ ). The hemolymph of SNMs sampled immediately after (within 1 min) and 25–30 min after the cut was made showed similar JH III titers (unpublished data).

### Sternal Gland 3H-2B Content in Dominants and Subordinates

As shown in Figure 2, in non-AP-adopting SNMs, the 3H-2B content of the sternal glands during late photophase was similar to that during late scotophase. Once fighting occurred (day 1), the 3H-2B content in both dominants and subordinates in late scotophase was significantly reduced ( $\chi^2 = 21.0$ , d.f. = 1,  $P < 0.0001$  and  $\chi^2 = 19.3$ , d.f. = 1,  $P < 0.0001$  for the dominants and subordinates, respectively, compared to SNMs in late photophase). In the dominants, the sternal gland 3H-2B content during late photophase was significantly higher than that during late scotophase in the first 9 days ( $t = 3.2$ , d.f. = 13.8,  $P = 0.006$ ;  $t = 8.6$ , d.f. = 23,  $P < 0.0001$ ;  $t = 2.4$ , d.f. = 28,  $P = 0.022$ ; or  $t = 2.8$ , d.f. = 17,  $P = 0.012$  for domination days 3, 5, 7, or 9). In contrast, in the subordinates, the 3H-

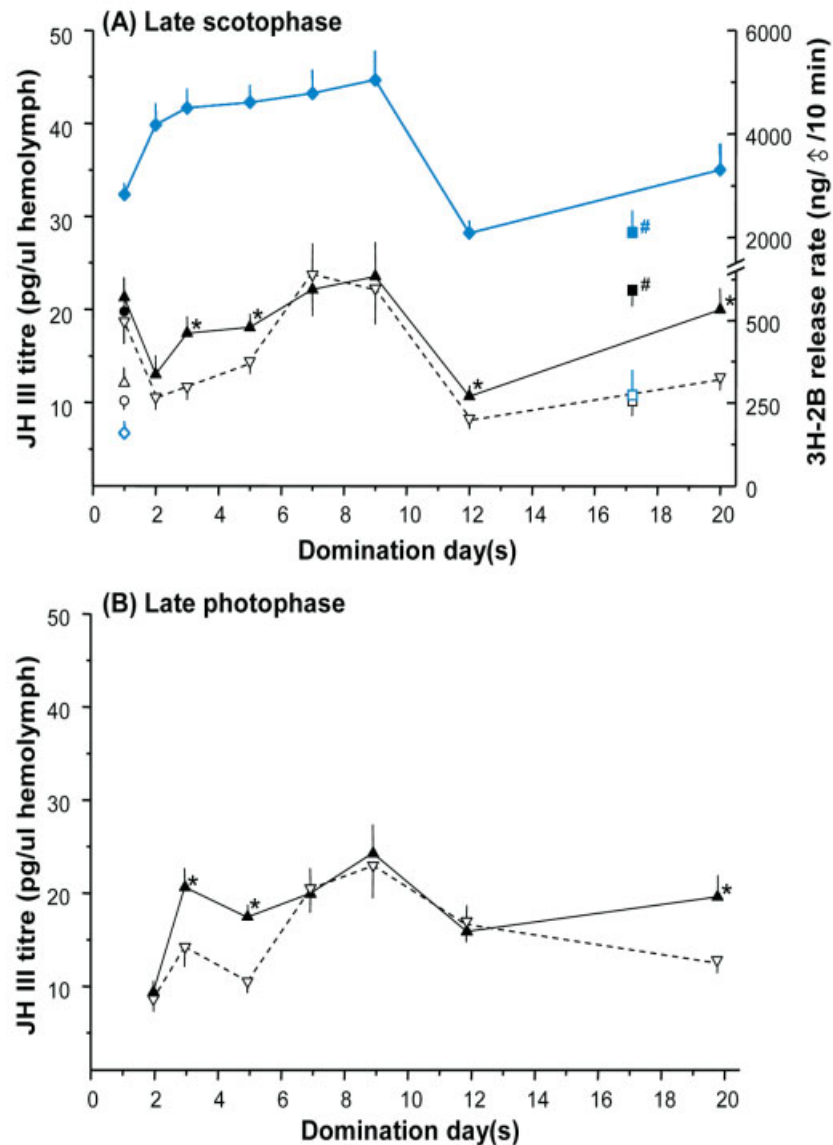


Fig. 1. A: 3H-2B release by dominant *N. cinerea* males during early scotophase, and hemolymph JH III titers in dominants and subordinates during late scotophase on the indicated day. B: Hemolymph JH III titer in dominants and subordinates during late photophase on the indicated days. The average duration before rank switch was  $17.2 \pm 1.1$  days. 3H-2B release: ◇, indicates 3H-2B released by non-AP-adopting SNMs ( $n = 15$ ); ◆, 3H-2B release in dominant males ( $n = 12-15$  for each data point); ■, 3H-2B release after rank switch to a dominant ( $n = 14$ ); □, 3H-2B release after rank switch to a subordinate ( $n = 14$ ). For the hemolymph JH III titer: △, indicates non-AP-adopting SNMs ( $n = 40$ ); ●, the dominant at 30 min after the first encounter fight ( $n = 18$ ); ○, the subordinate at 30 min after the first encounter fight ( $n = 18$ ); ▲, the JH III titer in dominants on

the indicated day ( $n = 12-15$  for each data point); ▽, the JH III titer in subordinates on the indicated day ( $n = 12-15$  for each data point); ■, the JH III titer on the day rank switched to a dominant ( $n = 14$ ); □, the JH III titer on the day rank switched to a subordinate ( $n = 14$ ). \*Significant difference in JH III titer between dominants and subordinates [during late scotophase,  $t = 3.1$ ,  $P = 0.007$ ;  $t = 2.6$ ,  $P = 0.02$ ;  $t = 2.6$ ,  $P = 0.02$ ; or  $t = 2.7$ ,  $P = 0.015$  for day 3, 5, 12, or 20, respectively; during late photophase,  $t = 2.8$ ,  $P = 0.014$ ;  $t = 4.2$ ,  $P = 0.0006$ ; or  $t = 3.9$ ,  $P = 0.002$  for day 3, 5, or 20, respectively]. # and #: Significant difference for 3H-2B release ( $\chi^2 = 17.3$ ,  $d.f. = 1$ ,  $P < 0.0001$ ) and for JH III titer ( $t = -5.7$ ,  $P < 0.0001$ ) respectively between the new dominant and new subordinate on the day of rank switch. SNMs: socially naïve males. All values are means  $\pm$  SE.

2B content during late scotophase was similar to, or significantly higher than ( $t = -2.4$ , d.f. = 23,  $P = 0.024$  and  $t = -2.7$ , d.f. = 17,  $P = 0.015$  for day 3 or 20, respectively), during late photophase. After a fight, the 3H-2B content in the glands during late photophase on each day in both dominants and subordinates was similar, except on day 20 when the 3H-2B content in the subordinates was higher than in the dominants ( $t = -1.9$ ,  $P = 0.07$ ). However, in late scotophase, the 3H-2B content was significantly lower in dominants than in subordinates in the first 7 days and on day 20 ( $\chi^2 = 13.8$ ,  $P = 0.0002$ ;  $t = -6.9$ ,  $P < 0.0001$ ;  $t = -6.6$ ,  $P < 0.0001$ ;  $t = -2.4$ ,  $P = 0.03$ ; or  $t = -2.4$ ,  $P = 0.03$  for day 1, 3, 5, 7, or 20, respectively).

### 3H-2B Release and Hemolymph JH III Titer in Dominants with Different Numbers of Wins

Table 1 shows that, in non-AP-adopting SNMs, both the 3H-2B release and hemolymph JH III titer were low. Once fighting occurred (for only 5 min), both 3H-2B release and JH III titer were significantly increased in the 1-time winners and were further significantly increased in the 3-time winners. In the 5-time winners, 3H-2B release was similar to that in the 3-time winners, but the JH III titer was close to that of the 1-time winners (ANOVA,  $F_{3,60} = 52.8$ ,  $P < 0.0001$  and  $F_{3,60} = 48.4$ ,  $P < 0.0001$  for comparison of 3H-2B release and JH III titer, respectively, between SNMs and 1-time, 3-time, or 5-time winning dominants).

## 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, 1972). The outcome of the agonistic interactions is the formation of an unstable dominant-subordinate relationship. Although the evolutionary relevance of *N. cinerea* agonistic behavior is well established (Schal and Bell, 1983; Moore et al., 1997), physiological factors involved in agonism have seldom been investigated, except for some

studies on the role of the CA. Hartman and Suda (1973) suggested that pheromone production does not seem to be controlled by the CA. Schal and Bell (1983) reported that removal of the CA does not affect the formation of hierarchies. Sréng et al. (1999) indicated that JH III is involved in the differentiation of sternal glands. Chen et al. (2005) reported that the CA from dominants shows a significantly higher JH III in vitro release rate than the CA from subordinates. Recently, we further found that for SNMs, the agonism is induced by contacting with the antenna contact pheromone(s), which results in the concomitant expression of attack behavior and an significant increase in both 3H-2B release and JH III titer (Chou et al., 2007). Since 3H-2B release is absolutely associated with the dominant during the first encounter fight (Kou et al., 2006; Chen et al., 2007), both pheromone (3H-2B) release and JH III titer need to be reinvestigated to firmly establish the physiological factors involved in hierarchy formation.

Our present results showed that, although only a small amount of 3H-2B was released by non-AP-adopting SNMs, the AP was induced in 100% of dominants during the first encounter fight (100% of the corresponding subordinates adopted the SP) and the accompanying 3H-2B release was significantly increased, an effect which lasted for the entire experimental period. The reason why we focused on one pheromone component, 3H-2B, is that, in our previous study (Kou et al., 2006), 2-MT and 4E-2M were detected in only a few cases and in small quantities (85–160 ng for 2-MT and 3–6 ng for 4E-2M in the 1-h sampling period); this may be due to an age effect and selective traps. The fact that the hemolymph JH III titer was significantly increased in the dominants and not the subordinates at 30 minutes after the first encounter indicates that social conflict may have a rapid and significant activating effect on the winner's endocrine system. Based on the positive correlation between the 3H-2B release and hemolymph JH titer in the dominants and the fact that the dominants have higher JH III titers than the subordinates (especially after rank switching), we hypothesized that mutual contact (such as via the action of at-



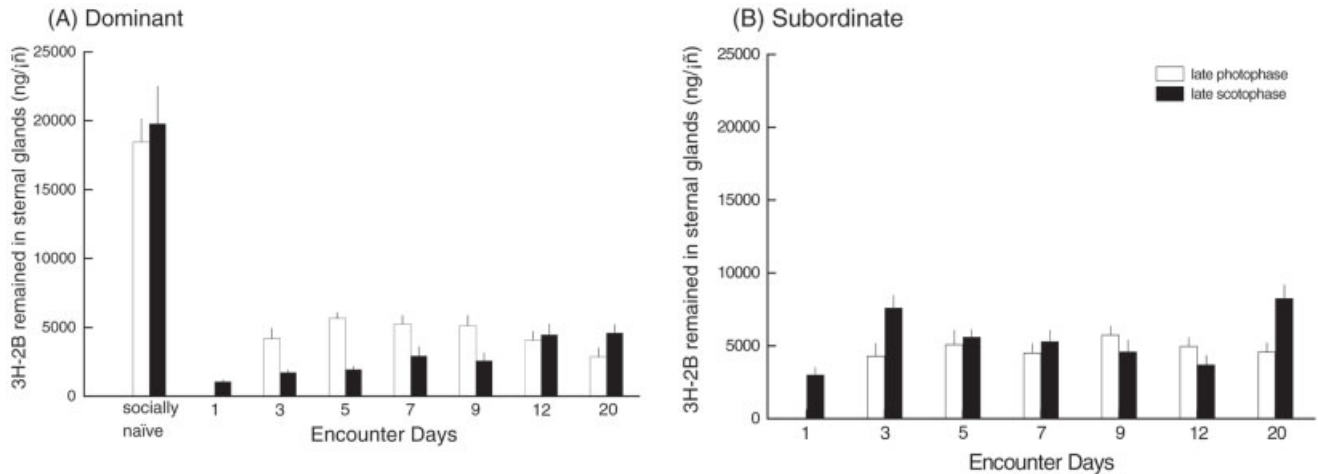


Fig. 2. 3H-2B content (ng/male) of the sternal glands of SNMs ( $n = 15$ ), dominants ( $n = 11-15$ ), and subordinates ( $n = 11-15$ ) during late scotophase and late photophase on the indicated days. ■, Late scotophase. □, Late photophase. Since about 93% ( $n = 320$ ) of adults emerged during photophase, each experimental day was started with the photophase and ended with the scotophase. The photophase data for day 1 were the same as those in SNMs, which were paired to fight during early scotophase and their sternal glands extracted during late scotophase. In the late scotophase of day 1, there was a significant difference in the 3H-2B content between dominants and SNMs ( $\chi^2 = 21$ , d.f. = 1,  $P < 0.0001$ ) and between subordinates and SNMs ( $\chi^2 = 19.3$ , d.f. = 1,  $P < 0.0001$ ). In the dominants, the 3H-2B content during late photophase was significantly higher than during late scotophase in the first

9 days ( $t = 3.2$ , d.f. = 13.8,  $P = 0.006$ ;  $t = 8.6$ , d.f. = 23,  $P < 0.0001$ ;  $t = 2.4$ , d.f. = 28,  $P = 0.022$ ; or  $t = 2.8$ , d.f. = 17,  $P = 0.012$  for day 3, 5, 7, or 9). In the subordinates, the 3H-2B content on days 3 and 20 was significantly higher in scotophase ( $t = -2.4$ , d.f. = 23,  $P = 0.024$  and  $t = -2.7$ , d.f. = 17,  $P = 0.015$  for day 3 or day 20, respectively) than in late photophase. During photophase on day 20, subordinates had a higher amount of 3H-2B content than dominants ( $t = -1.9$ ,  $P = 0.07$ ). During late scotophase, the 3H-2B content was significantly less in dominants than in the subordinates in the first 7 days and on day 20 ( $\chi^2 = 13.8$ ,  $P = 0.0002$ ;  $t = -6.9$ ,  $P < 0.0001$ ;  $t = -6.6$ ,  $P < 0.0001$ ;  $t = -2.4$ ,  $P = 0.03$ ; or  $t = -2.4$ ,  $P = 0.03$  for day 1, 3, 5, 7, or 20, respectively). SNMs: socially naïve males. All values are means  $\pm$  SE.

tack or being attacked) could activate CA activity in the dominants. In the dominants, the higher CA activity may further regulate 3H-2B biosynthesis and release. Following release of 3H-2B by the dominants, the CA activity of the loser might be

temporarily suppressed at a lower level via olfactory-mediating allatostatic factors.

For rank formation, there might be two levels, i.e., rank establishment and rank maintenance. Our further study indicated that, except to be used as a

TABLE 1. 3H-2B Release (ng/5 min/male) and Hemolymph JH III Titers (pg/ $\mu$ l Hemolymph) in Dominants With Different Numbers of Wins\*

	Status			
	Socially naïve	1-time dominant	3-times dominant	5-times dominant
3H-2B emitted (range, n)	80.1 $\pm$ 7.2 <sup>c</sup> (38.8–113.0, n=18)	834.3 $\pm$ 167.3 <sup>b</sup> (414.5–1948.6, n=16)	2296.4 $\pm$ 168.2 <sup>a</sup> (1147.8–3587.0, n=15)	2304.1 $\pm$ 298.2 <sup>a</sup> (1078.2–3255.8, n=15)
JH III titer (range, n)	12.9 $\pm$ 1.0 <sup>c</sup> (8.0–17.2, n=18)	20.1 $\pm$ 1.2 <sup>b</sup> (14.5–28.3, n=16)	43.1 $\pm$ 2.9 <sup>a</sup> (31.9–58.0, n=15)	22.0 $\pm$ 1.8 <sup>b</sup> (15.8–35.0, n=15)

\*The method to generate dominants with different numbers of wins is described in Materials and Methods, in Diagram 1.

All values are means  $\pm$  SE.

Means with different superscript letters (a,b,c) were significantly different (ANOVA,  $F_{3,60} = 48.4$ ,  $P > 0.0001$  and  $F_{3,60} = 52.8$ ,  $P > 0.0001$  for 3H-2B release and JH III titer, respectively).

signal to determine dominance (Chen et al., 2007), the released 3H-2B was not related to rank establishment, but to rank maintenance; for rank maintenance, 3H-2B functioned as a submission-inducing pheromone, which suppressed the fighting capability of rivals and kept them in a submissive state (Kou et al., 2008). In *N. cinerea*, the initiation of a fight between two males has nothing to do with the released 3H-2B. For rank establishment, the initial fight was induced by antenna contact (contacting with antenna cuticular components), which in turn induced significant JH III titer increase (Chou et al., 2007). After rank switching, both 3H-2B release and JH III titer were significantly increased in the new dominant (originally subordinate) and both parameters correlated positively with the switched social status. The present result is consistent with our previous report (Chen et al., 2005) that the CA from dominants exhibits a significantly higher JH III in vitro release rate than the CA from subordinates. The positively correlated relationship between 3H-2B release and JH III titer in dominants also indicates regulatory relationships between these two elements. If pheromone biosynthesis and release are regulated by JH III, whether JH III exerts its pheromonotropic effects directly on the pheromone gland cells or whether it acts indirectly via other pathways requires further investigation.

In insects, the most well understood pheromone-related endocrine regulatory system is the sex pheromones. Diverse endocrine regulatory mechanisms are adopted by different insect species for sex pheromone biosynthesis and release, such as regulation of pheromone production by pheromone biosynthesis activating neuropeptide (PBAN) in some moth species (Raina et al., 1989; Rafaeli and Jurenka, 2003) and by JH III in some female cockroaches (Smith and Schal, 1990). Sréng (1998) showed that decapitation or allatectomy (removal of the CA) of *N. cinerea* males before a critical point during pheromone gland maturation completely blocks the apoptotic process and proposed that brain factors mediate these maturational changes of the glandular cells, this brain factor was shown not to be PBAN-like neuropeptides. On the other hand, JH III administered to allatectomized males


restored both cellular differentiation of the gland and pheromone production (Sréng, 1999).

In the case of non-AP-adopting SNMs, the sternal gland 3H-2B content during late photophase was similar to that during late scotophase and pheromone sampling showed that usually less than 200 ng/male/10 min was collected during scotophase, showing that only a small amount of 3H-2B was released during photophase or scotophase. In the first 9 days after the first encounter fight, the sternal gland 3H-2B content of dominants was significantly higher during late photophase than during late scotophase, indicating great release during scotophase. Sirugue et al. (1992) also found that the amount of 3H-2B in the glands falls progressively during scotophase, and suggested that this change was due to a diel pattern in pheromone biosynthesis. In accordance with the significantly decreased 3H-2B release on days 12 and 20, the sternal gland 3H-2B content in dominants during late scotophase was not significantly lower than that during late photophase. In subordinates, the first encounter fight might induce decomposition of the photophase-accumulated 3H-2B. In dominants, the significant decrease in gland 3H-2B content on the first fighting day (compared to that of SNMs) may also give a hint about the amount of 3H-2B released for a new rank formation. In considering the 3H-2B released in dominants [thousands of nanograms are released in 10 min by the dominant and the AP is adopted for almost 80% of the scotophase duration], biosynthetic activity may still be continued during scotophase. In contrast, the 3H-2B content in non-AP-adopting subordinates was usually maintained at almost the same level in scotophase and photophase or was even higher in scotophase, directly demonstrating the very minor amount of 3H-2B released during scotophase. This phenomenon also agrees well with a previous report that pheromone is released during adoption of the AP (Sréng, 1990; Moore et al., 1995; Kou et al., 2006). In *N. cinerea*, the AP is not solely a behavior, but is accompanied by immediate pheromone release. The AP and immediate 3H-2B release (or possibly the JH III titer increase) in *N. cinerea* are two (or three) facets of

one thing. In considering the control mechanism, whether the AP and 3H-2B release (or JH III increase) are controlled by the same mechanism is worthy of further investigation.

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. They also suggested the possibility 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. Our present results further indicated that, during onset of aggressiveness, activation of CA activity was initiated by social contact. Whether social contact first initiated brain or neural activity, then activated both CA activity and aggressiveness requires investigation. The result for dominants with different numbers of wins showed that both pheromone release and CA activity have a physiological limitation. The fact that the JH III titer in 5-time winners was significantly less than in 3-time winners, may indicate a negative feed-back regulation after the endocrine system (JH III) reached a maximal activation in 3-time winners. We are currently carrying out further investigations into the regulatory mechanism of both physiological responses.

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