Winner and loser effects in lobster cockroach contests for social dominance

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ABSTRACT

In the context of animal aggression, the winner/loser effect is a cross-taxa phenomenon. In the present study, the effect of social contest experience on winning and losing subsequent encounters was investigated in the furious male lobster cockroach, *Nauphoeta cinerea*. Dominant and subordinate individuals were generated as the result of an encounter between two socially naïve males (SNMs); the winner and loser were designated as 1st encounter dominants and 1st encounter subordinates, respectively. With these dominants and subordinates, three experiments were conducted: (I) the original pair met in a re-encounter, (II) the 1st encounter dominants and subordinates were paired with an inexperienced SNM, (III) the 1st encounter dominants and subordinates were paired with an experienced individual of the same rank. Each experiment was conducted at 1 week, 2 weeks, 3 weeks, 4 weeks and 5 weeks after the 1st encounter fight. Juvenile hormone (JH) III titer was monitored in all individuals before and after each subsequent encounter. Our results showed that, in the original pairing and in the pairing with SNMs, the probability that a 1st encounter dominant (or subordinate) would win (or lose) the subsequent encounter fit well with the 95% confidence interval of the theoretical criteria proposed by Begin et al. (1969), indicating the existence of the winning/losing effect. However, this effect was inconsistent along the five-week observation period. For all 1st encounter dominants, at each week after the 1st encounter, the before subsequent encounter JH III titers distribution was significantly different from that on the 1st encounter day; the distributions of before subsequent encounter JH III titers could be further clustered into two groups, the higher JH III group and the lower JH III group, which were significantly correlated with subsequent winning and losing, respectively. For the 1st encounter subordinates, the distributions of before subsequent encounter JH III titers were not significantly different from that of SNMs, but the titer distributions were significantly shifted to a higher level compared to the 1st encounter day. Compared with before subsequent encounter, the after subsequent encounter hemolymph JH III level was significantly increased in winners and significantly decreased in losers. From these data, we propose that instability of the winner and loser effects may occur due to physiological costs and recovery; this instability may partly explain why the social hierarchy is unstable in this cockroach species.

1. Introduction

Intra-specific aggression, which is closely related to competition for valued resources, such as mates and territories, is a well-known behavioral strategy that can serve an important adaptive function in the life of an animal. In a wide variety of animals, the experience of a previous conflict can affect the performance of an individual during a later encounter (Chase et al., 1994; Hsu et al., 2006, 2009). After winning an aggressive encounter, aggression may be enhanced in order to increase the likelihood of winning subsequent encounters – the winner effect. On the other hand, aggression may be reduced after losing a previous encounter – the loser effect. When these effects have been quantified, the loser effect tends to be of a greater magnitude and/or exhibit a longer retention time than the winner effect (Chase et al., 1994; Kasumovic et al., 2010).

In insects, the winner and loser effects have been well studied in several species, revealing that each species exhibits the effects to different magnitudes. For example, in the parasitoid wasp, *Eupelmus vuilleti*, a winner effect has been observed in the absence of any evident loser effect (Goubault and Decuignie're, 2012), while in crickets, *Gryllus bimaculatus*, both winner and loser effects exist; the winner effect is mediated by the action of octopamine (Stevenson and Schildberger, 2013), and aversive agonistic fighting experiences will invoke social defeat via the action of the NO/cGMP pathway (Rillich and Stevenson,

2017). In the olive fruit fly, *Bactrocera oleae*, aggressive behavior could be enhanced by both previous victories and defeats to improve fighting success in subsequent contests against inexperienced males (Benelli et al., 2015). In *Drosophila*, short-term loser and winner effects could result from a single fight, and repeated defeats induced a long-lasting loser effect, whereas repeated victories had no long-term behavioral consequences (Trannoy and Kravitz, 2017); moreover, it was shown that the loser effect was generated by cAMP signaling-mediated plasticity of behavioral patterns (Chouhan et al., 2017).

As a model system to study the winner/loser effect of insect aggression, the lobster cockroach, *Nauphoeta cinerea*, is exceptional. *N. cinerea* exhibits fascinating male conspecific aggressive behaviors, and the repertoire of agonistic acts between any two males encountering each other for the first time is the largest and most complex known for any cockroach species (Kramer, 1964). The outcome of these furious interactions is the formation of unstable dominant-subordinate relationships, of which changes in rank order are common after a male has been dominant for several weeks (Ewing, 1972; Bell and Gorton, 1978; Schal and Bell, 1983). During the first moments of an encounter, both fighting males assume an aggressive posture (AP), characterized by an elongated upturned abdomen. From the abdomen, the suppression pheromone 3-hydroxy-2-butanone (3H−2B) is released (identified by Sreng, 1990) in an effort to keep the rival in a submissive state. After the dominant/subordinate relationship is determined, only the dominant individual adopts an AP, and the release of both juvenile hormone III (JH III) and 3H−2B are significantly higher than in subordinates. The subordinate adopts a submissive posture, with its head and tail turned down. The significant increase in hemolymph JH III was suggested to sustain aggression (Kou et al., 2008a; Kou et al., 2008b; Kou et al., 2009b), and the release of suppression pheromone 3H−2B may serve to maintain rank and provide a signal of dominance (Chen et al., 2007; Kou et al., 2009a). The antenna contact pheromone played a central role in the aggression, since attack behavior and concomitant increases in both hemolymph JH III titer and 3H−2B release were shown to be induced within seconds solely by exposure to contact pheromone on an isolated male antenna, without the presence of any resources (Chou et al., 2007). Additionally, fighting ability is known to be strengthened by mating; there is a positive correlation between mating experience and dominant status, and JH III level and 3H−2B release rate were found to be significantly increased in mated dominants compared to subordinates (Kou and Hsu, 2013). Additionally, pre-mating wing-raising behavior with significantly increased 3H−2B release and hemolymph JH III level could be induced by contact with an isolated female antenna, in the absence of an actual female (Kou and Hsu, 2013).

Considering that this species is group-living, there are ample opportunities for males to repeatedly meet one another. Some major possibilities for secondary encounters include the same male pair meeting again with the same rival, each individual from the 1st encounter meeting a non-experienced male, or individuals meeting males with the same winning or losing experience. This social context then begs several questions: will the experience of the first encounter affect the results of further contests? If so, will this effect be long lasting? What is the role, if any, for winner and loser effects in the formation of the known unstable dominant/subordinate hierarchy? In this study, we aimed to determine whether there are winner and/or loser effects in *N. cinerea* and to describe the possible involvement of hemolymph JH III in such effects. To this end, we monitored fighting results in contests involving pre-established dominants and subordinates, in addition to measuring associated JH III titers. Observations were made during the course of several weeks following the 1st encounter fight under three experimental designs: 1) the 1st encounter male pair met again, 2) 1st encounter dominants or subordinates met an inexperienced socially naïve male (SNM), and 3) 1st encounter dominants and/or subordinates met other experienced individuals with the same social rank. Our predictions were that once an individual was established as dominant or

subordinate, a winner or loser effect would be observed; this effect would then last for some period of time, and it would be reflected by the JH III level.

2. Materials and methods

2.1. Cockroaches

Mass rearing was performed according to Kou et al. (2006). Each male was kept isolated in an individual cage (3.5 × 8.5 × 13 cm) for the 24 h period following the imaginal moult to control for the effects of learning (Manning and Johnstone, 1970); these animals are hereafter referred to as socially naïve males (SNMs). The day of emergence was considered day 1. All animals were maintained under a 16/8-h light/dark cycle (lights were on from 16:30–08:30 h) at 25–26 °C with free access to food and water. Because of the readiness with which they initiate aggressive behavior, 60–65-day-old AP-adopting SNMs were used. The AP involves standing in place and rhythmically pumping the elongated abdomen up and down. On any given day, about 40–60% of the males spontaneously adopted an AP (and the associated behavior of patrolling territory). AP adoption is an indicator of aggression (Kou et al., 2008a, 2009a). All hemolymph samples were taken at the same time of day (1–3 h into scotophase) to minimize any variation caused by circadian rhythm. The males used in all experiments were matched for size. In order to identify individual contest participants, the dominant was marked on its pronotum with white Tipp-Ex fluid. In our previous experiments, the white Tipp-Ex marking did not affect the winning or losing; as such, 24/43 (55.8%, n = 43 males) with no marking became as winners, indicating that the white Tipp-Ex fluid does not affect winning or losing frequency. In this study, the 1st encounter dominant/subordinate was determined by the result of the 1st encounter fight between any two SNMs. All subsequent fights with the 1st encounter dominants and/or subordinates resulted in winners and losers, but the dominant/subordinate experimental designation was not changed.

2.2. Experiment I (the original pairing): 1st encounter dominant/subordinate determination, re-encounter at 1, 2, 3, 4 or 5 weeks after the 1st encounter, and associated JH III titer measurements

(I) 1st encounter dominant/subordinate determination and subsequent winner/loser relationships between the same pair upon re-encounter.

(a) SNMs of 60–65 days old were isolated in a glass terrarium (12 × 12 × 12 cm) on the day before the test. On the test day, SNMs were then paired in a different aquarium for the 1st encounter fight. The 1st encounter dominant/subordinate relationship was usually determined in 1–3 s, with the dominant chasing and biting the subordinate. A total of five groups were formed (n = 10–15 pairs in each group), and each group was used in one of the five subsequent weeks for re-encounter experiments. Ten minutes after the dominant/subordinate relationship was determined, individuals were isolated in a new terrarium.

(b) At 1 week (n = 15 pairs), 2 weeks (n = 15 pairs), 3 weeks (n = 10 pairs), 4 weeks (n = 15 pairs) or 5 weeks (n = 15 pairs) after the 1st encounter fight, hemolymph was collected from each 1st encounter dominant and subordinate at 1–3 h into scotophase. Thirty minutes after hemolymph collection, the 1st encounter dominant and the corresponding subordinate were moved into a new glass terrarium for a 10-min re-encounter fight period. Again, the winner/loser status was usually determined within a few seconds of pairing. The win/loss percentage of 1st encounter dominants and subordinates was calculated

(c) Hemolymph samples were then collected from both the winners and losers at the end of the 10-min re-encounter fight period

(II) Hemolymph sampling and JH III titer measurement

As described previously (Kou et al., 2008a; Kou et al., 2008b; Kou and Hsu, 2013), 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. Hemolymph was then quickly collected in a capillary tube. The hemolymph (7 μl/ male) was immediately mixed with 500 μl acetonitrile to denature any enzymes that could affect JH. The samples were placed on ice and 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, in order 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 JH titers (Huang et al., 1994) that are comparable to two other RIAs, which have been verified by GC–MS (De Kort et al., 1985; Goodman et al., 1990). The RIA procedure has been described previously in detail (Huang and Robinson, 1995). Briefly, JH III in the hemolymph sample was extracted twice with 0.5 ml of hexane, and 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 III 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 (NEN, 647.5 Gbq/mmol). The mixture was incubated at room temperature for 2 h; then 0.5 ml of dextran-coated charcoal suspension (Sigma) was added to each sample tube, followed by incubation for 2.5 min. Samples were centrifuged ($2000 \times g$ for 3 min), and the supernatant was 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 nonlinear regression with five unknown parameters, using 'DPM bound' as the dependent variable and 'JH amount' (after log transformation) as the independent variable. The five-parameter formula was described in Huang and Robinson (1996). Excel (Microsoft, USA) was used to calculate the amount of JH in each sample, by solving for JH with known DPM and the five fitted parameters. The recovery of the radiolabeled JH III was 98.2%. Intra- and inter-assay variability were 6.2% and 8.4%, respectively.

2.3. Experiment II (paired with SNM): 1st encounter dominants or subordinates encounter a new SNM opponent at 1, 2, 3, 4 and 5 weeks after the 1st encounter, and the associated JH III titer measurement

(I) 1st encounter dominant/subordinate group formation and later fight with a new SNM opponent

(a) 1st encounter dominant/subordinate groups formation: in this experiment, the initial dominant/subordinate designations were made as described in Experiment I. A total of 5 groups were formed (n = 10–15 pairs for each group), each group was used in one of the five subsequent encounter experiments. After the 10-min fighting period, each of the 1st encounter dominants and subordinates were immediately separated to a new terrarium. At the same time, 60–65-day-old SNMs were kept individually in separate glass terraria for 1 week (n = 15), 2 weeks (n = 15), 3 weeks (n = 10), 4 weeks (n = 15) or 5 weeks (n = 15).

(b) At 1 week (n = 15 pairs), 2 weeks (n = 15 pairs), 3 weeks (n = 10 pairs), 4 weeks (n = 15 pairs) or 5 weeks (n = 15 pairs) after the 1st encounter fight, hemolyph was collected from each 1st encounter dominant (and/or subordinate) and a matched SNM. Thirty minutes after the hemolymph collection, both males were moved into a new glass terrarium for the 10-min fight period. The win/loss percentages for 1st encounter dominants and subordinates with a new inexperienced SNM opponent were recorded.

(c) Hemolymph samples were collected at the end of the 10-min fight period from both the winner and loser.

(II) Hemolymph sampling and JH III titer measurement were performed as described above in Experiment I.

2.4. Experiment III (same rank pairing): 1st encounter dominants and subordinates encounter an opponent with the same rank at 1, 2, 3, 4 and 5 weeks after the 1st encounter, and the associated JH III titer measurement

(I) 1st encounter dominant/subordinate group formation and subsequent fight with an opponent of the same rank.

(a) 1st encounter dominant/subordinate group formation: in this experiment, the initial generation of dominant and subordinate pairs was the same as described in Experiment I. A total of two sets (set A and set B) of male pairs were created. Each set was comprised of 5 groups of male pairs (n = 10 pairs for each group). Each group in A set and B set was used in one of the subsequent encounter experiments. After the 10-min fighting period, each 1st encounter dominant and subordinate was immediately removed to a new terrarium as described above in Experiment I.

(b) At 1 week (n = 10 pairs), 2 weeks (n = 10 pairs), 3 weeks (n = 10 pairs), 4 weeks (n = 10 pairs) or 5 weeks (n = 10 pairs) after the 1st encounter fight, hemolymph was collected from matched 1st encounter dominants (and/or subordinate) from set A and corresponding individuals from set B. Thirty minutes after the hemolymph collection, both males (set A 1st encounter dominant vs. set B 1st encounter dominant, or set A 1st encounter subordinate vs. set B 1st encounter subordinate) were moved into a new glass terrarium for the 10-min fight period.

(c) Hemolymph samples were collected again from each individual winner and loser at the end of the 10-min fight period. The win/loss percentage of 1st encounter dominants and 1st encounter subordinates (in A set) with a same rank male (in B set) was recorded.

(II) To provide the 1st encounter fight background data for all above three Experiments, a male group (n = 30 pairs) composed of 60–65-day-old SNMs was formed for the 1st encounter fight, and the hemolymph samples were collected from each male before and after the 1st encounter.

(III) Hemolymph sampling and JH III titer measurement were the same as described above in Experiment I.

2.5. Statistical analysis

For the analysis of winning and losing probabilities in each week of the three Experiments, the experimental data were compared to the 95% confidence interval of theoretical criteria (i.e., 83%, 67% and 50% for the original pairing, paired with SNM and same-rank pairing, respectively) proposed by Bégin et al. (1996). A proportion test was used to (1) compare winning or losing percentage among the three experimental groups (the original pairing, paired with SNM and same rank pairing) at each week after the 1st encounter and (2) test whether the relatively higher before 1st encounter JH III level was associated with the > 50% winning probability in the eventual dominants. Since all JH data sets were paired, the Wilcoxon signed rank test was used to compare JH III titers (1) before or after the 1st encounter, comparing between 1st encounter dominants and 1st encounter subordinates, (2) in 1st encounter dominants and/or 1st encounter subordinates, comparing between before and after the 1st encounter, (3) before and after the subsequent encounter, comparing between subsequent winners and losers, and (4) in winners and/or losers, comparing between before and after the subsequent encounter. Since some data sets did not follow a normal distribution, non-parametric statistical analyses were applied. The *p*-values were subjected to FDR adjustment in comparisons that
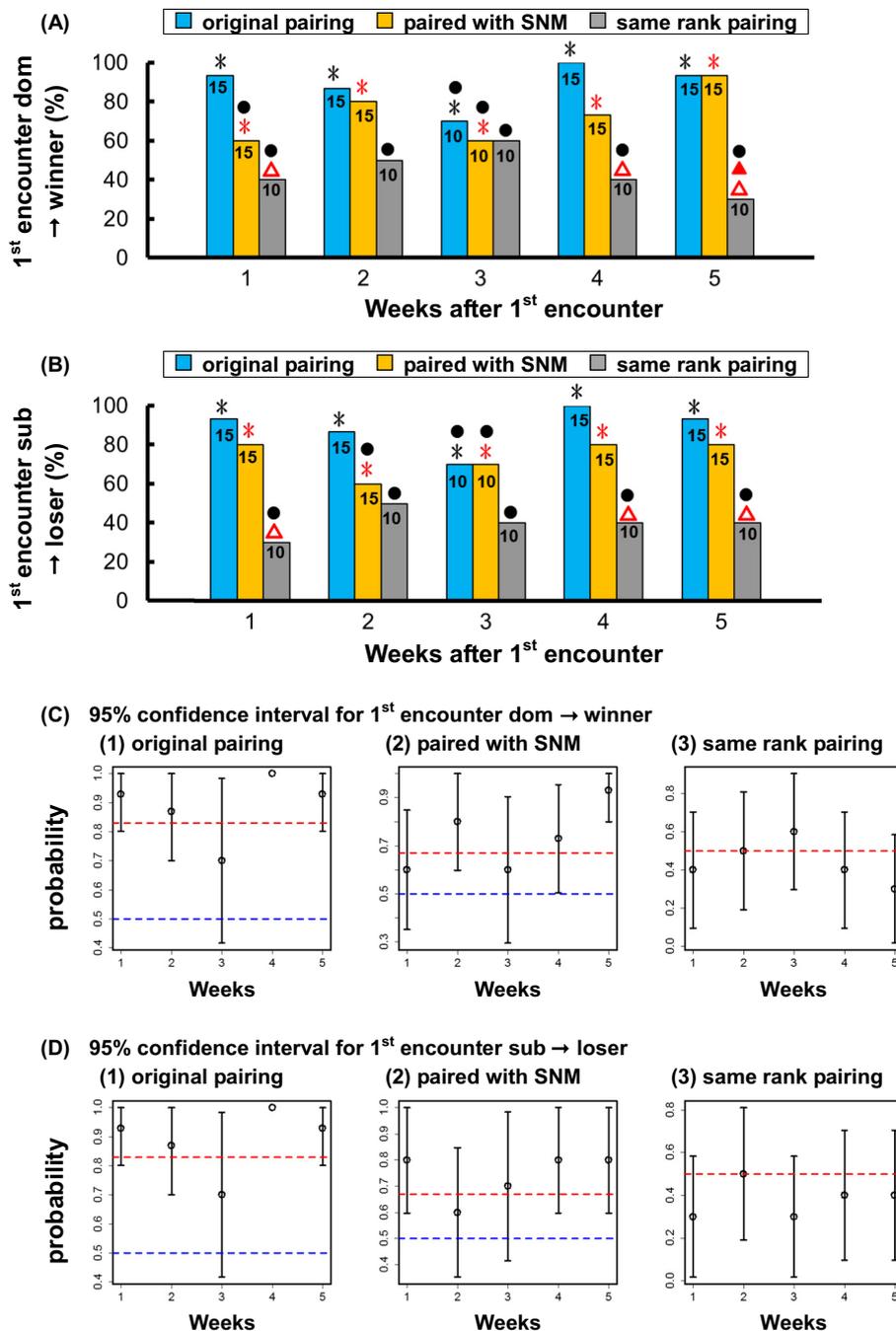
Fig. 1. (A) The winning percentage (%) of the 1st encounter dominants; (B) the losing percentage (%) of the 1st encounter subordinates, at 1 week, 2 weeks, 3 weeks, 4 weeks and 5 weeks after the 1st encounter fight; (C) 95% confidence interval for the 1st encounter dominant to be winner; and (D) 95% confidence interval for the 1st encounter subordinate to be loser.

-----: 83%, 67% and 50% probability in (C) (1,2,3) and (D) (1,2,3), respectively.

-----: 50% probability in (C) (1,2) and (D) (1,2), respectively.

*: In the original pairing (■), the winning probability for the 1st encounter dominants (or the losing probability for the 1st encounter subordinates) fit into the 95% confidence interval of the theoretical criteria (83%) proposed by Bégin et al. (1996) on weeks 1, 2, 3, 4 and 5.

*: In the pairing with SNM (■), the winning probability for the 1st encounter dominants (or the losing probability for the 1st encounter subordinates) fit into the 95% confidence interval of the theoretical criteria (67%) proposed by Bégin et al. (1996) on weeks 1, 2, 3, 4 and 5.

●: the winning or losing probability fit into the 95% confidence interval of the 50% criteria.

△: 1. For the 1st encounter dominants, the winning percentage in the original pairing group (■) was significantly higher than that in the same rank pairing group (■) at weeks 1($p = 0.04$), 4 ($p = 0.009$) and 5 ($p = 0.01$). 2. for the 1st encounter subordinates, the losing percentage in the original pairing group (■) was significantly higher than that in the same rank pairing group (■) on weeks 1 ($p = 0.01$), 4 ($p = 0.009$) and 5 ($p = 0.04$).

▲: the winning percentage in the pairing with SNM group (■) was significantly higher than that in the same rank pairing group (■) on week 5 ($p = 0.01$).

used the same data sets. A Kolmogorov-Smirnov test was used to compare the JH III titer distribution between 1st encounter dominants (and/or 1st encounter subordinates) and SNMs before the new encounter. K-means clustering was performed on before subsequent encounter JH III titers in 1st encounter dominants. Pearson's Chi-squared test was used for the correlation test between before subsequent encounter JH III level and winning/losing. For all data sets, values are presented as the mean ± SE.
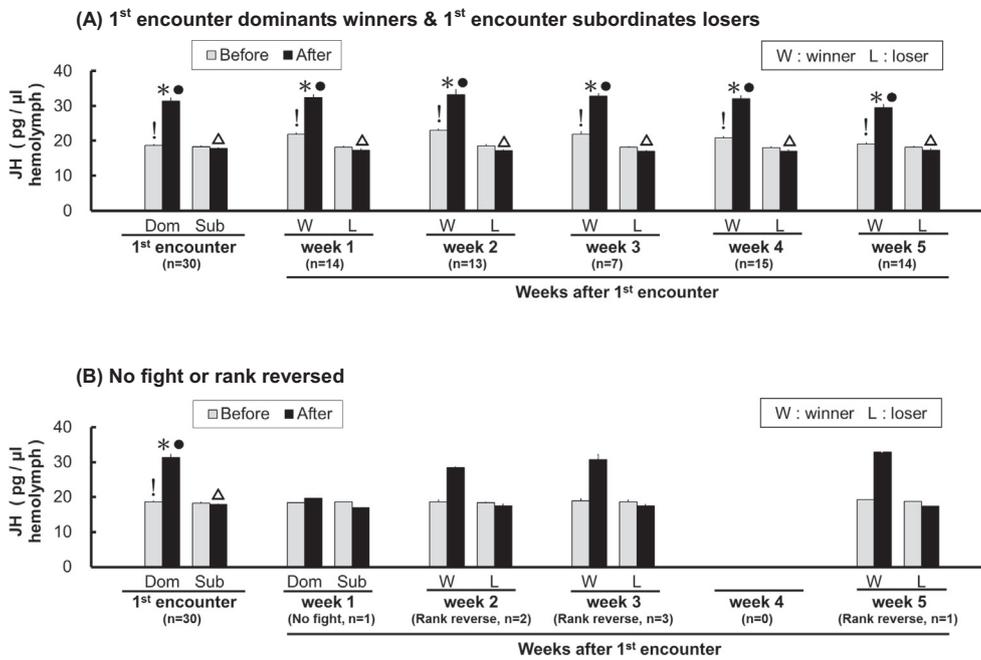
3. Results

3.1. Experiment I (the original pairing): Re-encounter of dominant and subordinate after 1st encounter

When original pairs meet again during the five-week period after the 1st encounter, the winning probability of all dominants fit into the

95% confidence interval of the theoretical criteria (83%) proposed by Bégin et al. (1996) and showed significantly higher than 50% probability, except for week 3 (Fig. 1A, C1); although week 3 fit into the 95% confidence interval of the theoretical criteria, it showed no significant difference with 50% probability (Fig. 1C1). Since the same pairings were used in the re-encounter, the losing probability of 1st encounter subordinates was the same as the winning probability of the 1st encounter dominants, and comparison to the theoretical criteria (the 95% confidence interval of the theoretical criteria, 83%, proposed by Bégin et al., 1996, Fig. 1B, D) also yielded the same results, with all groups fitting the model except week 3. Upon re-encounter, one pair (7%) stopped fighting at week 1; meanwhile, two pairs (13%), three pairs (30%) and one pair (7%) reversed the original dominant/subordinate status at weeks 2, 3 and 5, respectively.

Before two SNMs experienced a 1st encounter, the before fight JH III titer was significantly higher in the eventual dominants than in the

(A) 1st encounter dominants winners & 1st encounter subordinates losers

(B) No fight or rank reversed

Fig. 2. JH III titers before and after the 1st encounter or the re-encounters in the original pairing group (Experiment I). (A) 1st encounter dominants and subordinates that maintained rank. (B) No fight or rank reversed. The values are the mean ± SE.

!: In 1st encounter dominants, the before 1st encounter JH III titer was significantly higher ($p = 0.049$) than that in the 1st encounter subordinate opponents on the 1st encounter day; in 1st encounter dominants that won the re-encounter, the before re-encounter JH III titer was significantly higher than that in the 1st encounter subordinate opponents at all five weeks ($p = 0.001$, 0.001, 0.02, 0.0007 and 0.004 on weeks 1, 2, 3, 4 and 5, respectively).

*: JH III titer was significantly increased in the 1st encounter dominants on the 1st encounter day (n = 30, $p < 0.0001$), and after the subsequent re-encounter ($p = 0.001$, 0.001, 0.02, 0.0007 and 0.001 on weeks 1, 2, 3, 4 and 5, respectively).

●: After 1st encounter, JH III titer in the 1st encounter dominant was significantly higher than that in the 1st encounter subordinate on the 1st encounter day

($p < 0.0001$); and after the subsequent re-encounter, JH III titer in the 1st encounter dominant winners was significantly higher than that in the 1st encounter subordinate losers ($p = 0.001$, 0.001, 0.02, 0.0007 and 0.001 on weeks 1, 2, 3, 4 and 5, respectively).

Δ: JH III titer was significantly decreased in the 1st encounter subordinates on the 1st encounter day ($p = 0.01$), and after the subsequent re-encounter ($p = 0.001$, 0.001, 0.02, 0.0007 and 0.001 on weeks 1, 2, 3, 4 and 5, respectively).

subordinates, although the statistical confidence was marginal ($p = 0.049$, V = 309, n = 30 pairs). Moreover, a higher before 1st encounter JH III level was significantly associated with higher winning probability ($> 50\%$, Z = 1.86, $p = 0.03$, Proportion test). After the 1st encounter, the JH III titers were significantly increased in 1st encounter dominants ($p < 0.0001$, V = 0) and were significantly higher in dominants than in subordinates ($p < 0.0001$, V = 465). Furthermore, JH III titers were significantly decreased in the subordinates ($p < 0.0001$, V = 0) (Fig. 2A). For the 1st encounter dominants that won the subsequent re-encounter, the before re-encounter JH III titer was significantly higher than that in the 1st encounter subordinate opponents at all five time-points (week 1: n = 14, $p = 0.001$, V = 105; week 2: n = 13, $p = 0.001$, V = 91; week 3: n = 7, $p = 0.02$, V = 28; week 4: n = 15, $p = 0.0007$, V = 120 and week 5: n = 14, $p = 0.004$, V = 99). Upon re-encounter, the vast majority (90%, 63/70) of 1st encounter dominants were ranked as winners. The JH III titers in the these 1st encounter dominant winners were significantly increased compared to before re-encounter (week 1: n = 14, $p = 0.001$, V = 0; week 2: n = 13, $p = 0.001$, V = 0; week 3: n = 7, $p = 0.02$, V = 0; week 4: n = 15, V = 0, $p = 0.0007$; week 5: n = 14, $p = 0.001$, V = 0), and was significantly higher than that in the 1st encounter subordinate losers (week 1: n = 14, $p = 0.001$, V = 105; week 2: n = 13, $p = 0.001$, V = 91; week 3: n = 7, $p = 0.02$, V = 28; week 4: n = 15, $p = 0.0007$, V = 120 and week 5: n = 14, $p = 0.001$, V = 105). The JH III titers in the 1st encounter subordinate losers was significantly decreased compared to before re-encounter (week 1: n = 14, $p = 0.001$, V = 105; week 2: n = 13, $p = 0.001$, V = 91; week 3: n = 7, $p = 0.02$, V = 28; week 4: n = 15, $p = 0.0007$, V = 120 and week 5: n = 14, $p = 0.001$, V = 105) (Fig. 2A).

In the week 1 pair that stopped fighting during the re-encounter, the JH III titer after re-encounter was slightly increased (19.6 pg/μl) compared to before re-encounter (18.4 pg/μl) in the 1st encounter dominant, and the titer was slightly decreased compared to before re-encounter in the 1st encounter subordinate (from 18.6 to 17.0 pg/μl) (Fig. 2B). Two pairs, three pairs and one pair reversed the dominant/subordinate status at weeks 2, 3 and 5, respectively. In these rank-reversed male pairs, the JH III titer in all 1st encounter dominants was decreased after loss of the re-encounter (week 2: from 18.4 ± 0.0 to 17.5 ± 0.2, n = 2; week 3: from 18.6 ± 0.3 to 17.5 ± 0.1, n = 3; week 5: from 18.8 to 17.4 pg/μl hemolymph, n = 1). Conversely, the titer was increased in 1st encounter subordinate after winning the re-encounter (week 2: from 18.7 ± 0.2 to 28.5 ± 0.1, n = 2; week 3: from 18.9 ± 0.4 to 30.8 ± 1.1, n = 3; week 5: from 19.2 to 32.9 pg/μl hemolymph, n = 1; data were not statistically analyzed, because n < 4) (Fig. 2B).

3.2. Experiment II (paired with SNM): 1st encounter dominants and subordinates encounter new SNM opponent

When the 1st encounter dominants encountered an inexperienced SNM, the winning probability on weeks 2, 4 and 5 fit well with the 95% confidence interval of the theoretical criteria (67%) proposed by Bégin et al. (1996), and was significantly higher than 50% (Fig. 1A, C2), while 1st encounter subordinates exhibited an enhanced losing probability on weeks 1, 4 and 5 (Fig. 1B, D2).

For most 1st encounter dominant winners, the before subsequent encounter JH III titer was significantly higher than that in SNM opponents (week 1: n = 9/15, 60% of total, $p = 0.01$, V = 45; week 2: n = 12/15, 80% of total, $p = 0.0025$, V = 78; week 3: n = 6/10, 60% of total, $p = 0.036$, V = 21; week 4: n = 11/15, 73% of total, $p = 0.004$, V = 66; week 5: n = 14/15, 93% of total, $p = 0.001$, V = 105) (Fig. 3A). The JH III titers in the these 1st encounter dominant winners were significantly increased compared to before subsequent encounter (week 1: n = 9, $p = 0.01$, V = 0; week 2: n = 12, $p = 0.02$, V = 0; week 3: n = 6, $p = 0.036$, V = 0; week 4: n = 11, $p = 0.004$, V = 0; week 5: n = 14, $p = 0.0005$, V = 0), and was significantly higher than that in the 1st encounter SNM losers ($p = 0.01$, V = 45; $p = 0.0025$, V = 78; $p = 0.036$, V = 21; $p = 0.004$, V = 66 and $p = 0.001$, V = 105 at weeks 1, 2, 3, 4 and 5, respectively) (Fig. 3A). In the SNM losers, the after encounter JH III titer was significantly decreased compared with before the encounter ($p = 0.01$, V = 44; $p = 0.0025$, V = 78; $p = 0.036$, V = 21; $p = 0.004$, V = 66;
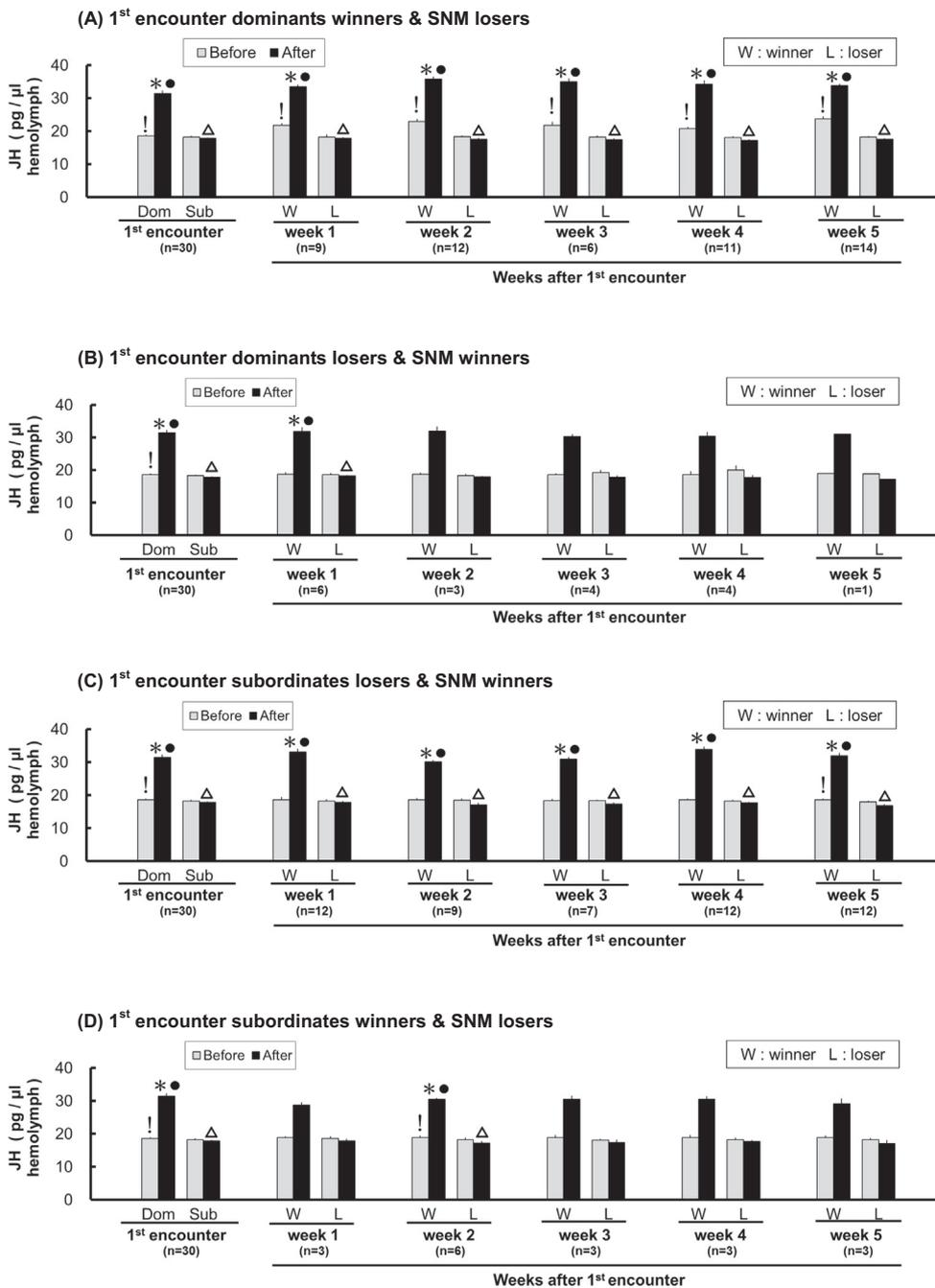
Fig. 3. JH III titers before and after subsequent encounters between 1st encounter dominants and subordinates with opponent SNMs (Experiment II). (A) 1st encounter dominant winners and the opponent SNM losers; (B) 1st encounter dominant losers and the opponent SNM winners; (C) 1st encounter subordinate losers and the opponent SNM winners; (D) 1st encounter subordinate winners, and the opponent SNM losers. The values are the mean ± SE.

!: In 1st encounter dominants, the before 1st encounter JH III titer was significantly higher ($p = 0.049$) than that in the 1st encounter subordinate opponents on the 1st encounter day; in (A), among the 1st encounter dominants that won the subsequent encounter, the before subsequent encounter JH III titer was significantly higher than that in the SNM opponents at all 5 weeks ($p = 0.01, 0.0025, 0.036, 0.004$ and 0.001 on weeks 1, 2, 3, 4 and 5, respectively). In (C), on week 5, the before encounter JH III titer in the SNMs was significantly higher than that in 1st encounter subordinates, $p = 0.025$. In (D), among the 1st encounter subordinates that won the subsequent encounter, the before encounter JH III titer was significantly higher than that in the SNM opponents on week 2 ($p = 0.036$).

*: JH III titer was significantly increased in 1st encounter dominants on the 1st encounter day ($n = 30, p < 0.0001$), and in (A) 1st encounter dominant winners after the subsequent encounter ($p = 0.01, 0.02, 0.036, 0.004$ and 0.0005 on weeks 1, 2, 3, 4 and 5, respectively); (B) SNM winners on week 1 ($p = 0.048$); (C) SNM winners ($p = 0.003, 0.01, 0.03, 0.05$ and 0.003 on weeks 1, 2, 3, 4 and 5, respectively); (D) 1st encounter subordinate winners on week 2 ($p = 0.036$).

●: After the 1st encounter, the JH III titer in the 1st encounter dominant was significantly higher than that in the 1st encounter subordinate on the 1st encounter day; ($p < 0.0001$); after the subsequent encounter, JH III titer was significantly higher in winners than in losers: (A) ($p = 0.01, 0.0025, 0.036, 0.004$ and 0.001 on weeks 1, 2, 3, 4 and 5, respectively); (B) $p = 0.048$, on week 1; (C) ($p = 0.003, 0.01, 0.03, 0.05$ and 0.003 on weeks 1, 2, 3, 4 and 5, respec-

tively); (D) $p = 0.036$, on week 2.

Δ: JH III titer was significantly decreased in the 1st encounter subordinates on the 1st encounter day ($n = 30, p = 0.01$), and after the subsequent encounter: (A), in SNM losers ($p = 0.01, 0.0025, 0.036, 0.004$ and 0.001 on week 1, 2, 3, 4 and 5, respectively); (B), in 1st encounter dominant losers ($p = 0.036$, on week 1); (C), in 1st encounter subordinate losers ($p = 0.003, 0.01, 0.03, 0.05$ and 0.003 on weeks 1, 2, 3, 4 and 5, respectively); (D), in SNM losers ($p = 0.036$, on week 2).

and $p = 0.001$, $V = 105$ at weeks 1, 2, 3, 4 and 5, respectively) (Fig. 3A). Some of the 1st encounter dominants lost the encounter with SNMs (Fig. 3B); in these 1st encounter dominant losers, the before subsequent encounter JH III titer was not significantly different from the SNM for any of the time-points measured; the after fight JH III titer in the SNM winners was consistently increased (week 1: from 18.7 ± 0.2 to 31.9 ± 3.7, n = 6, $p = 0.048$, V = 0; week 2: from 18.7 ± 0.2 to 32.0 ± 7.3, n = 3; week 3: from 18.5 ± 0.1 to 30.3 ± 5.3, n = 4; week 4: from 18.6 ± 0.3 to 30.5 ± 5.3, n = 4; week 5: from 19.0 to 31.1 pg/µl hemolymph, n = 1; data were not statistically analyzed when n ≤ 4). In the 1st encounter dominant losers, the after fight JH III titer was decreased to a lower level (week 1:

from 18.6 ± 0.2 to 18.2 ± 0.1, n = 6, $p = 0.048$, V = 21; week 2: from 18.4 ± 0.3 to 17.9 ± 0.1, n = 3; week 3: from 19.2 ± 0.3 to 17.8 ± 0.2, n = 4; week 4: from 20.1 ± 0.5 to 17.7 ± 0.2, n = 4; week 5: from 18.9 to 17.1 pg/µl hemolymph, n = 1).

Upon meeting a SNM, 1st encounter subordinates mostly lost. The before subsequent encounter JH III titer was not significantly different between SNMs and 1st encounter subordinates, except at week 5 (JH III titer in the SNM was significantly higher than that in 1st encounter subordinate, $p = 0.025$, V = 68) (Fig. 3C). The after encounter JH III titer was significantly higher in the SNM winners than that in the 1st encounter subordinate losers ($p = 0.003$, V = 78; $p = 0.01$, V = 45; $p = 0.03$, V = 28; $p = 0.05$, V = 78 and $p = 0.003$, V = 78 on weeks 1,

2, 3, 4 and 5, respectively). In the 1st encounter subordinate losers, the after encounter JH III titer was significantly decreased when compared to before encounter ($p = 0.003$, V = 78; $p = 0.01$, V = 45; $p = 0.03$, V = 28; $p = 0.05$, V = 66 and $p = 0.003$, V = 78 at weeks 1, 2, 3, 4 and 5, respectively). Some of the 1st encounter subordinates won the encounter with an SNM (Fig. 3D). Although the before encounter JH III titer was similar between the 1st encounter subordinate winners and SNM losers, statistical analysis was not conducted due to the low number of individuals in the groups. The exception was week 2, where 1st encounter subordinate winners had significantly higher before encounter JH III titer than that SNM losers ($p = 0.036$, V = 21, n = 6). The after subsequent encounter JH III titer was generally increased in 1st encounter subordinate winners (week 1: from 18.9 ± 0.1 to 28.7 ± 0.4, n = 3; week 2: from 18.9 ± 0.2 to 30.6 ± 0.1, n = 6, $p = 0.036$, V = 0; week 3 from 18.9 ± 0.3 to 30.5 ± 0.4, n = 3; week 4: from 18.9 ± 0.2 to 30.6 ± 0.3, n = 3; week 5: from 18.9 ± 0.3 to 29.2 ± 1.6 pg/μl, n = 3), however, the data were mostly not statistically analyzed due to the low numbers in each group. Likewise, the after encounter JH III titer was generally decreased in SNM losers (week 1: from 18.6 ± 0.3 to 17.8 ± 0.2, n = 3; week 2: from 18.2 ± 0.2 to 17.2 ± 0.2, n = 6, $p = 0.036$, V = 21; week 3: from 18.1 ± 0.1 to 17.3 ± 0.4, n = 3; week 4: from 18.3 ± 0.2 to 17.7 ± 0.1, n = 3; week 5: from 18.2 ± 0.2 to 17.0 ± 0.4 pg/μl hemolymph, n = 3).

3.3. Experiment III (same rank pairing): 1st encounter dominants and subordinates encounter an opponent with the same rank

In this experiment, two groups each of 1st encounter dominants and 1st encounter subordinates were generated (set A and set B). At 1, 2, 3, 4 and 5 weeks after the 1st encounter, each 1st encounter dominant from set A was paired with another 1st encounter dominant from set B, and 1st encounter subordinates were similarly paired. The results showed that there was no statistical preference (1) for 1st encounter dominants from either set to win the encounter (Fig. 1A, C3); or (2) for 1st encounter subordinates from either set to lose the encounter (Fig. 1B, D3). All data fit well with the 95% confidence interval of 50% probability.

For meetings between 1st encounter dominants, each pair had a winner and a loser. Thus, for statistical analysis of the JH III data, at each week, all 1st encounter dominant winners were pooled as one group (n = 10), and 1st encounter dominant losers were also grouped together (n = 10). Similarly, 1st encounter subordinate losers were pooled as a group (n = 10), and the 1st encounter subordinate winners were pooled as another group (n = 10).

For 1st encounter dominant winners, the before encounter JH III titer was significantly higher than 1st encounter dominant loser opponents ($p < 0.008$, V = 55, at each of the five time-points) (Fig. 4A). After the same-rank encounter, JH III titer was also significantly higher in the winners than in the losers ($p < 0.008$, V = 55, at each of the five time points). Compared with before encounter, the after encounter JH III titer was significantly increased in the 1st encounter dominant winners ($p < 0.008$, V = 0, at each of the five time points); for the 1st encounter dominant losers, the after encounter JH III titer was either similar to the before encounter levels ($p = 0.22$, V = 40, at week 1) or significantly decreased (week 2: $p = 0.014$, V = 52; weeks 3, 4 and 5: $p < 0.006$, V = 55).

For 1st encounter subordinate contests, some resulted in no fighting, (n = 2, 4, 3, 2 and 3 pairs at weeks 1, 2, 3, 4 and 5, respectively). Among the pairs between which fighting occurred, the 1st encounter subordinate losers only exhibited before encounter JH III titer that was significantly lower than the opponent on week 4 (n = 8, $p = 0.04$, V = 1.5); the after encounter JH III titer was significantly lower in losers than in winners (week 1: n = 8, $p = 0.019$, V = 0; week 2: n = 6, $p = 0.048$, V = 0; week 3: n = 7, $p = 0.03$, V = 0; week 4: n = 8, $p = 0.019$, V = 0; week 5: n = 7, $p = 0.03$, V = 0) (Fig. 4B). JH III titer was significantly decreased in the losers after the encounter as

compared to before the encounter (week 1: n = 8, $p = 0.019$, V = 36; week 2: n = 6, $p = 0.048$, V = 21; week 3: n = 7, $p = 0.03$, V = 28; week 4: n = 8, $p = 0.019$, V = 36; week 5: n = 7, $p = 0.03$, V = 28), but it was significantly increased in the winners (week 1: n = 8, $p = 0.019$, V = 0; week 2: n = 6, $p = 0.048$, V = 0; week 3: n = 7, $p = 0.03$, V = 0; week 4: n = 8, $p = 0.019$, V = 0; week 5: n = 7, $p = 0.03$, V = 0). In the male pairs that did not fight, the before encounter JH III titer was similar between the contestants, and the JH III titer was also similar for all individuals when comparing between before and after the encounter (Fig. 4C).

3.4. Comparison of the win/loss percentage among the three experimental groups

When comparing the winning or losing percentages between the three experiments, the 1st encounter dominant and/or subordinates in the first two experiments generally showed more extreme winning or losing percentages than the third group. The 1st encounter dominants in the original pairing group (**EXP I**) showed significantly higher winning percentage than those in the same rank pairing group (**EXP III**) at weeks 1, 4 and 5 ($p = 0.04$, 0.1, 0.87, 0.009 and 0.01 at weeks 1, 2, 3, 4 and 5, respectively); also the 1st encounter dominants paired with SNMs (**EXP II**) showed a significantly higher winning percentage than the same rank pairing group (**EXP III**) at week 5 ($p = 0.01$). There was no significant difference in the winning percentage between 1st encounter dominants in the original pairing (**ExP I**) and the pairing with SNM (**EXP II**) experiments (Fig. 1A).

For the 1st encounter subordinates, the original pairing group (**EXP I**) also showed significantly higher losing percentage than those in the same rank pairing group (**EXP III**) on weeks 1, 4 and 5 ($p = 0.01$, 0.1, 0.1, 0.009 and 0.04 at weeks 1, 2, 3, 4 and 5, respectively) (Fig. 1B). There was no significant difference in the losing percentage between the original pairing (**EXP I**) and the pairing with SNMs (**EXP II**) experiments (Fig. 1B).

3.5. Distribution of the before subsequent encounter JH III titers in the 1st encounter dominant/subordinate

With regard to the 1st encounter, the JH III titer distribution was significantly shifted from a lower level in before 1st encounter SNMs to a higher level in after 1st encounter dominants ($p < 10^{-12}$) (Fig. 5). Then, in 1st encounter dominants before subsequent encounter (pooled from all three experiments), the JH III titer distributions showed significant differences from before ($p < 0.0001$ for all time points, higher than before) and after ($p < 0.0001$ for all time points, lower than after) the 1st encounter (Fig. 5). At each week, the before subsequent encounter JH III titer could be further clustered into two groups, i.e., the low JH III titer group and the high JH III titer group, with the low and/ or high JH III titer significantly correlated with subordinate losing and dominant winning ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$, $p = 0.01$ and $p = 0.0008$ at weeks 1, 2, 3, 4 and 5, respectively).

For the 1st encounter subordinates, on the 1st encounter day, the after 1st encounter JH III titer distribution was shifted to lower than, but not significantly different from the before 1st encounter (SNM) distribution ($p = 0.07$) (Fig. 6). Then, at each week after the 1st encounter, when the before subsequent encounter JH III titers of 1st encounter subordinates in all three experiments were pooled, the JH III titer distributions showed no significant difference with that of SNMs before the 1st encounter ($p = 0.85$, 0.30, 0.11, 0.75 and 0.50 on weeks 1, 2, 3, 4 and 5, respectively); however the distributions were significantly shifted to a higher level than that of the 1st encounter subordinates (i.e., SNMs after losing the 1st encounter) on the 1st encounter day ($p = 0.015$, $p = 0.0008$, $p < 0.0001$, $p = 0.013$, and $p = 0.025$ at weeks 1, 2, 3, 4 and 5, respectively) (Fig. 6). At each week after the 1st encounter, the before subsequent encounter JH III distribution in the 1st encounter subordinates was different from the two
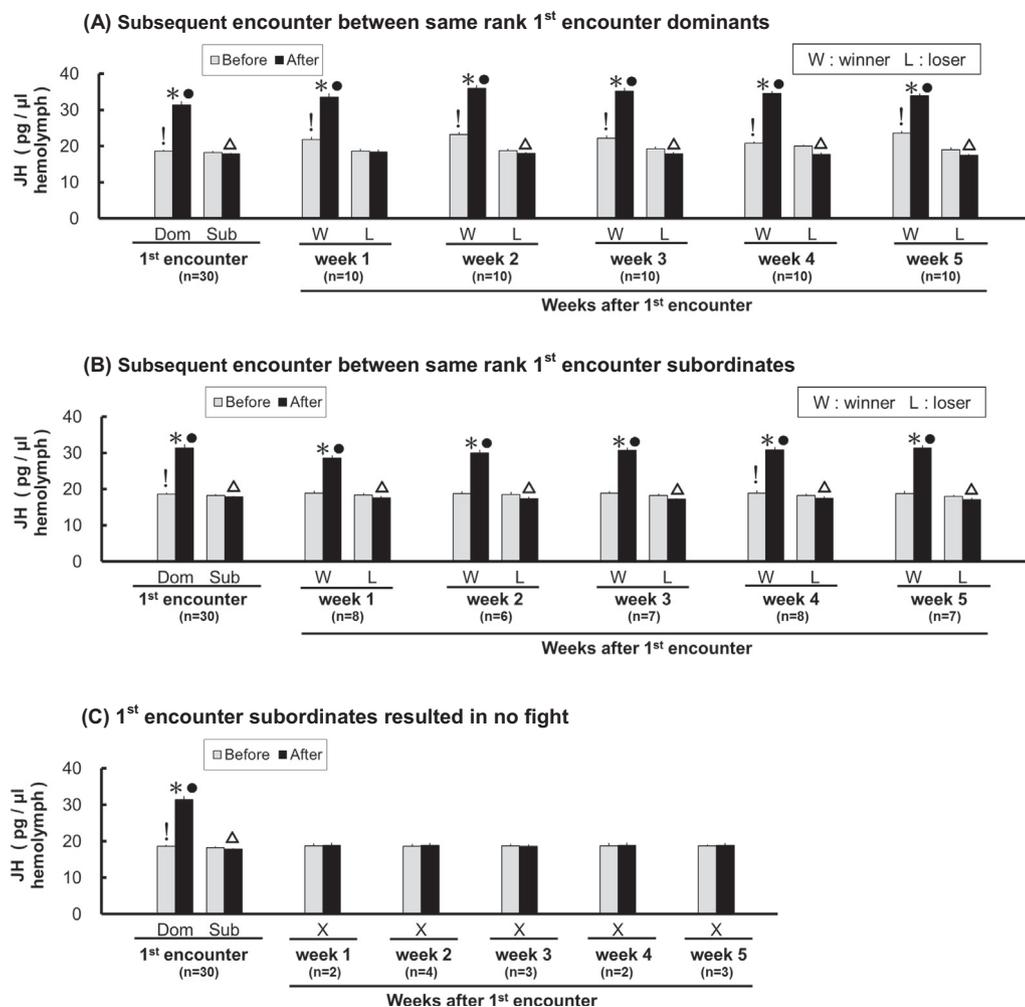
Fig. 4. JH III titers before and after the subsequent encounter between (A) same rank 1st encounter dominants and (B) same rank 1st encounter subordinates (Experiment III); (C) 1st encounter subordinates that did not fight in the subsequent encounter. The values are the mean ± SE.

!: In 1st encounter dominants, the before 1st encounter JH III titer was significantly higher ($p = 0.049$) than that in the 1st encounter subordinate opponents on the 1st encounter day; the before subsequent encounter JH III titer was significantly higher in winners than in losers in (A), for the 1st encounter dominant winners ($p < 0.008$ at each of the 5 weeks); (B) for the 1st encounter subordinate winners ($p = 0.04$, week 4).

*: JH III titer was significantly increased in the 1st encounter dominants after the 1st encounter (n = 30, $p < 0.0001$), and in (A) 1st encounter dominant winners ($p < 0.008$ at each of the 5 weeks); and (B) 1st encounter subordinate winners ($p = 0.019, 0.048, 0.03, 0.019$ and 0.03 on week 1, 2, 3, 4 and 5, respectively).

●: After 1st encounter, JH III titer in the 1st encounter dominant was significantly higher than that in the 1st encounter subordinate ($p < 0.0001$); after subsequent encounter, JH III titer was significantly higher in winners than in losers: (A) ($p < 0.008$ at each of the 5 weeks); (B) ($p = 0.019, 0.048, 0.03, 0.019$ and 0.03 on week 1, 2, 3, 4 and 5, respectively)

Δ: JH III titer was significantly decreased in the 1st encounter subordinates on the 1st encounter day ($p = 0.01$), in (A) 1st encounter dominant losers ($p = 0.014, < 0.006, p < 0.006$ and < 0.006 on week 2, 3, 4 and 5, respectively); and (B) 1st encounter subordinate losers ($p = 0.019, 0.048, 0.03, 0.019$ and 0.03 on week 1, 2, 3, 4 and 5, respectively).
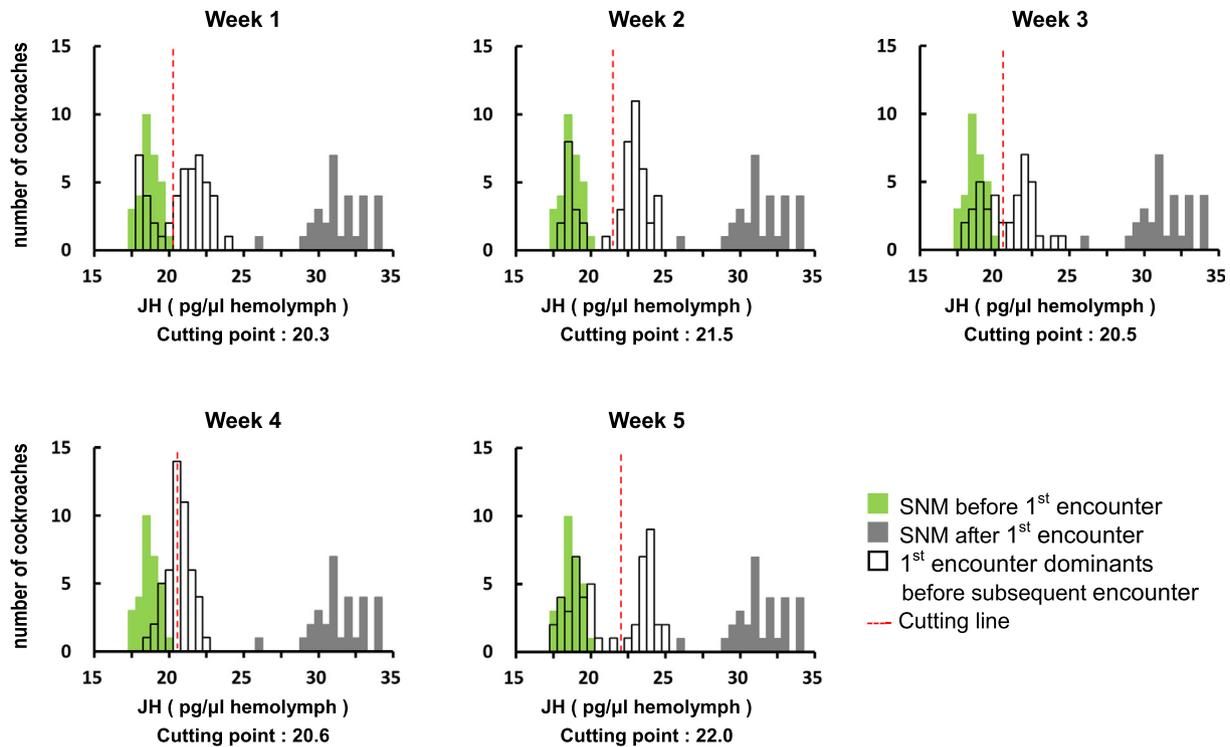
distribution clusters of 1st encounter dominants.

4. Discussion

In the context of intra-species aggression, winner and loser effects are widespread throughout the animal kingdom (Hsu et al., 2006; Rutte et al., 2006) and strongly impact hierarchy formation in socially living species (Dugatkin and Druen, 2004). The existence of winner and loser effects indicates that contest resolution is not only based on the fighting abilities of contestants, but it is also influenced by previous contest experiences. In insects, the magnitude of the winner or loser effect seems to depend on the species. For example, only a winner effect has been observed in the parasitoid wasp, *E. vuilleti* (Goubault and Decuignie're, 2012), but only the loser effect was long-lasting in *Drosophila* (Trannoy and Kravitz, 2017). Moreover, both winner and loser effects have been shown in the cricket species, *G. bimaculatus* (Rillich and Stevenson, 2017).

In our present results, we observed the existence of winner and loser effects within five weeks following a 1st encounter fight. These effects were supported by multiple lines of evidence. 1) In the repeated original pairing experiment, there was a significant tendency for the 1st encounter dominant/subordinate to keep their initial rank. 2) The results of the original pairing experiment might not be due to the recognition of each other, since pairing experienced dominants/subordinates with inexperienced SNMs still generated similar results. 3) When 1st encounter dominants/subordinates were paired with an

experienced male of the same rank, the winner/loser effect disappeared. Our results also indicated that in this species, the winner/loser effect may not be consistent over time, being observed during some weeks and not in other weeks. This instability may result from the physiological costs for maintaining an aggressive state of dominance and by physiological recovery from existing as a subordinate. The conclusion that the effect is unstable can be strengthened by our previous result, which showed that release of the dominance-signaling suppression pheromone 3H−2B fluctuated daily, and the rank rotated frequently, corresponding to the pheromone release (Chen et al., 2007).

When looking at the hormone level, we found that the JH III level change may further explain the processes of physiological cost and recovery. First, there was a significant difference ($p = 0.049$, marginal statistical significance) between the JH III titers of two inexperienced SNMs in before 1st encounter, corresponding to eventual dominant/subordinate status ($p = 0.03$, > 50% probability). This observation indicated that the SNMs with higher before fight JH III are likely to be more aggressive than their opponents. Since the before 1st encounter JH III titer was already higher in the dominants, why is JH III titer still significantly raised (almost two-fold) in dominants after the 1st encounter? According to the vertebrate challenge hypothesis (Wingfield et al., 1987, 1990) and our previous results (Kou et al., 2008a; Kou et al., 2008b; Kou et al., 2009b; Kou and Hsu, 2013), the raised hormone level stimulates persistence of aggression. The challenge hypothesis (Wingfield et al., 1987, 1990; Wingfield, 2017), which is based on the mating systems and breeding strategies of birds, has been an

Fig. 5. JH III titer distribution of the SNMs that became 1st encounter dominants on the 1st encounter day and later weeks before subsequent encounter. The before subsequent encounter JH III titers of the 1st encounter dominants were pooled from all three experiments. █: JH III titer distribution of the SNMs on the 1st encounter day, before the 1st encounter. █: JH III titer distribution of SNMs that became 1st encounter dominants after the 1st encounter. □: JH III titer distributions of the 1st encounter dominants at each of the 5 weeks after the 1st encounter before subsequent encounters. ---------: line indicating the two clusters.

important foundation for modern behavioral endocrinology in both vertebrates and invertebrates (Tibbetts and Crocker, 2014). This hypothesis predicts how testosterone (T) levels will vary seasonally and how male birds will hormonally respond to social interactions with other males and receptive females (Wingfield et al., 1990; Moore, 2007). In fact, competitive interactions which rapidly potentiate T
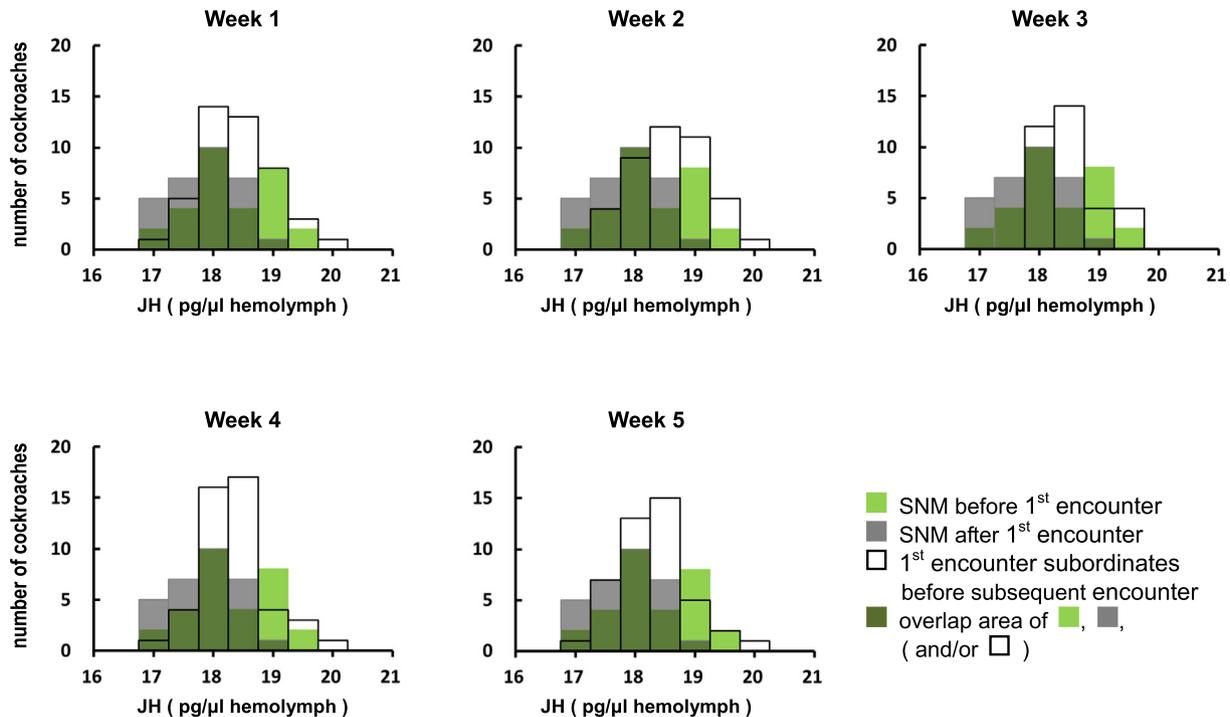
Fig. 6. JH III titer distribution of the SNMs that became 1st encounter subordinates on the 1st encounter day and on later weeks before subsequent encounter. Before subsequent encounter JH III data of 1st encounter subordinates were pooled together from all three experiments to create the distribution. █: JH III of the SNMs on the 1st encounter day, before the 1st encounter. █: JH III of the 1st encounter subordinates after the 1st encounter. □: JH III titer distribution of the 1st encounter subordinates at each of the 5 weeks after the 1st encounter before subsequent encounters. █: overlap area of the █, █ and/or □.

release can be observed in both humans (Archer, 2006; Oliveira and Oliveira, 2014; Carré and Olmstead, 2015) and non-human species, such as birds (Wingfield et al., 1990) and fish (Oliveira et al., 2009). This robust hypothesis can even be applied to insects (Scott, 2006; Kou et al., 2008a, 2008b; Tibbets and Huang, 2010). In *N. cinerea*, we speculate that the 1st encounter fight strengthens the aggression system in 1st encounter dominants, maintaining the individuals in a physiologically demanding state, where they are more sensitive to initiate attack after stimulation by antenna contact pheromone, show significantly increased hormone titer, and are more capable of both winning subsequent fights and experiencing reproductive success (Breed et al., 1980; Schal and Bell, 1983).

At each week after the 1st encounter, the JH III titer distribution in 1st encounter dominants before subsequent encounter was lower than after the 1st encounter but still significantly higher than before 1st encounter SNMs. This decreased shift might be due to physiological depletion, signaling that dominants cannot maintain such a high JH III level as that of the 1st encounter day. At each week after the 1st encounter and in all three experiments, the 1st encounter dominants that became subsequent winners exhibited before subsequent encounter JH III titers that were routinely higher than the opponents. After the 1st encounter, a similar frequency distribution of the before subsequent encounter JH III titer in winners appeared at almost all time-points, indicating it is a very stable physiological response. Each week after the 1st encounter day, the before subsequent encounter JH III titer distribution for all 1st encounter dominant winners could be clearly clustered into two groups, with the relatively higher JH III group significantly correlated with subsequent winning and the lower group correlated with losing. Before subsequent fight, why do some 1st encounter dominants maintain a higher hormone level and others do not? Our explanation is that some individuals may be physiologically depleted and the aggression reduced after the 1st encounter fight, so hormone level is reduced to that similar to before the 1st encounter. For the still physiologically more aggressive dominants, the hormone level was still maintained at a relatively higher level than its opponent. After the 1st encounter, there may be a reciprocal interaction between aggression and physiologically activated factors, including hormones and other related factors. Briefly stated, aggression-induced physiological factors were raised by successful aggression in the 1st encounter, after which the elevated factors may have potentiated further aggression.

In the 1st encounter dominants, the relatively high before subsequent encounter JH III level may only be one of several physiological factors which function together to confer an improved fighting ability. The significantly higher hormone level is just one indicator that the competitor is more aggressive than its opponent. This explanation is supported by our observation that the 1st encounter dominant losers usually had similar before subsequent fight JH III level as its opponents. These similar levels may be observed because the 1st encounter dominants that lost were not physiologically more aggressive than their opponents; for such 1st encounter dominants, the winning/losing of a subsequent fight might be determined by the sensitivity and neurophysiological response speed after contact with the aggression initiator (i.e., the antenna contact pheromone), and by the attacking strength of both sides.

The involvement of hormones in winner and loser effects has long been studied in vertebrates. In male California mice (*Peromyscus californicus*), injections that produced a transient increase in T following an aggressive encounter caused males to behave more aggressively in subsequent encounters (Trainor et al., 2004). The acute T response to victory was speculated to reinforce learning processes associated with winning the competition (Gleason et al., 2009). The winner effect of *P. californicus* was suggested to be in part due to an upregulation of androgen receptors in several key brain regions involved in reward and motivation as well as social aggression (Fuxjager et al., 2010). In humans, recent evidence indicated that although competition outcome modulates T reactivity patterns, the resulting neuroendocrine responses

have similar effects on subsequent behavior in winners and losers (Carré and Olmstead, 2015). Another interesting issue is the rapidity of increase in hormone levels in both vertebrates (including human) and the invertebrate cockroach we studied. The fact that JH III level increases very rapidly and could be detected immediately at the end of the fight has reported before (Kou et al., 2008a; Kou et al., 2008b; Kou et al., 2009b; Kou and Hsu, 2013). This rapid response implies a possible involvement of cell surface voltage-dependent channel opening either in the nervous system or on the endocrine organ (the corpora allata) (Hsieh et al., 2002), but evaluation of this hypothesized mechanism will require further study. In humans (Jezova and Vigas, 1981; Flinn et al., 2012) and non-human primates (Sapolsky, 1986), a rapid T increase was suggested to be mediated by sympathetic catecholamines, not via the hypothalamic–pituitary–gonadal axis. In *N. cinerea*, the neural circuitry underlying the rapid hormone increase and winner effect may be examined in further investigations.

On the other hand, for the 1st encounter subordinates, the aggression system may be suppressed by the 1st encounter defeat, and the physiological submissive response may create more reluctance to respond to subsequent aggression initiation (i.e., the antenna contact pheromone). This submissive state may be characterized by lower physiological aggression-related features, such as lower hormone level, so most 1st encounter subordinates will lose a subsequent fight. A few weeks after the 1st encounter, no matter whether the 1st encounter subordinates won or lost the subsequent encounter, almost all the before subsequent encounter JH III levels were similar to the opponents (except for the group of week 4 subsequent encounter of same rank subordinates), indicating the before subsequent fight JH III level is not a major determinant for winning/losing. So what is the determinant for the winning or losing in these subordinates? We speculate that winning capability may be dependent on the physiological recovery of each individual. With different levels of recovery (which may partly be reflected in the hormone levels), physiological factors may recover normal function, responding to aggression initiators with an attack.

Each week after the 1st encounter day, the before subsequent encounter JH III titer distribution of 1st encounter subordinates was quite different from that of dominants, with no obvious JH III clustering. The 1st encounter defeat made the post-1st encounter JH titer distribution shift toward a lower level, although the difference before and after the encounter was not significant. Interestingly, the before subsequent encounter JH III titer distributions in the later five weeks were significantly shifted to a higher level than that of the 1st encounter day (after they being defeated). This shifting to a higher level implied physiological recovery had taken place for further fighting. Based on the fact that for any SNM, antenna contact induced genetically coded innate aggression, this natural born reaction to the aggression initiator must not function for the 1st encounter subordinate to consistently lose subsequent contests, and this lack of function may serve to promote the submissive state, allowing the individual to avoid further attack. Thus, for a subordinate that is not well prepared for further fights, the defeat syndrome may serve as a protective adaptive function.

For those 1st encounter subordinate winners of subsequent contests, the JH III level was still significantly increased after winning the subsequent fight, indicating a recovery of aggressive capability. Our present results showed that for most 1st encounter subordinates, the loser effect could last for weeks; this long lasting loser effect might be due to learning-related mechanisms that suppress recovery, or the blocking of aggression-related neurocircuitry may be involved. These mechanisms still require further elucidation. In contrast to the winner effect, some 1st encounter subordinates never recovered within the 5-week observation period, indicating the naturally born aggressive response to the aggression initiator (i.e., the antenna contact pheromone) was completely aborted. For many animals, losing a fight (social defeat) induces a period of suppressed aggressiveness and general behavior, often with symptoms common to human psychiatric disorders (Rose et al., 2017). In *Drosophila*, cAMP signaling was shown to be involved in

mediating the plasticity of behavioral patterns that generate the loser effect (Chouhan et al., 2017). In *G. bimaculatus*, the effects of social defeat are mediated by the NO/cGMP pathway (Rillich and Stevenson, 2017), and after being defeated, dopamine is necessary for the recovery of aggression (Rillich and Stevenson, 2014). In mice, glutamatergic and GABAergic neuronal metabolism and neurotransmitter cycling are decreased in concert with a depression-like phenotype (Veeraiah et al., 2014). Furthermore, treatments with anti-androgen showed that in Mozambique tilapia *Oreochromis mossambicus* androgens are critical for the winner effect but not the loser effect (Oliveira et al., 2009). In *N. cinerea*, contest experience may be mediated via the activation (the winner effect) or de-activation (the loser effect) of the aggression system. The underlying functional defects in cellular mechanism that drive the loser effect in this cockroach species are still unknown.

In summary, the winner/loser effect exists in both vertebrates and invertebrates as a cross-taxa phenomenon. In the furious cockroach species, *N. cinerea*, we suggest that instability of the winner and loser effects may be due to physiological costs and recovery. For the winner effect, the JH III level before subsequent fights may represent a single marker of enhanced aggression. Meanwhile, the loser effect may be due to the blockage of innate aggression responsive pathways. The concept of instability in winner and loser effects could provide further understanding of the unstable social hierarchies in this species.

Acknowledgments

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