

RESEARCH ARTICLE

Aggressive behavior in the antennectomized male cricket *Gryllus bimaculatus*

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SUMMARY

Male crickets (*Gryllus bimaculatus*) exhibit intensively defensive aggressive behavior towards attacking males most often culminating in fighting. After the fight, the loser no longer exhibits aggressiveness in a second, separate encounter with another male; rather, the defeated male exhibits avoidance behavior. Here, we investigated the role of sensory input from the antennae in male defensive aggressive behavior. When we removed antennae from males (antennectomized males), we found that they showed little aggressiveness towards each other whereas they continued to exhibit typical fighting behavior towards an intact male. In addition, in a second encounter, antennectomized losers showed significantly higher aggressiveness towards another male than did intact losers. We further found that antennectomized crickets do not utilize visual or palpal sensory input to elicit aggressive behavior. In contrast, intact males showed aspects of aggressive behavior to male cuticular substances before and after winning a fight, and if they lost a fight they showed avoidance behavior. It thus appears that antennal sensory information is crucial in the mediation of aggressive and avoidance behaviors. However, sensory inputs from the antennae are not necessary to elicit defensive aggressive behavior but are necessary to discriminate conspecific males and initiate attacks against them.

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INTRODUCTION

Aggression is one of the most common types of behavior in animals. Male crickets (*Gryllus bimaculatus*), as well as most other male insects, exhibit intensive aggressive behavior when they encounter another male, whereas they show courtship behavior to a conspecific female (Alexander, 1961). The aggressive behavior of male crickets in many species, including *G. bimaculatus*, towards other conspecific males is impressive and has been studied extensively (Alexander, 1961; Phillips and Konishi, 1973; Dixon and Cade, 1986; Adamo and Hoy, 1995; Hack, 1997; Hofmann and Stevenson, 2000; Stevenson et al., 2005). Males battle with each other over the acquisition of living spaces (i.e. burrows) and females (Simmons, 1986). If neither of the males retreats, they engage in a fight consisting of a sequentially escalating series of behaviors beginning with antennal fencing, which leads to spreading and finally engaging of the mandibles. The fight continues until one of the males surrenders. At this point, a behavioral hierarchy is established between the pair, with the winner initiating the singing of an aggressive song and chasing away the loser. Interestingly, in subsequent encounters with the winner, the loser exhibits avoidance behavior (e.g. running away) and thus avoids additional fights (Adamo and Hoy, 1995; Iwasaki et al., 2006). This depressive period in loser crickets continues for several hours (Hofmann and Stevenson, 2000). Furthermore, recent experience, such as copulation, flight, the opponent's size and behavior, and population density can also alter the aggressive behavior of males (Alexander, 1961; Hofmann and Stevenson, 2000; Rillich et al., 2007; Ashikaga et al., 2009). Thus, whether males decide to engage in aggressive

behaviors with each other may depend on their ability to compare their own motivation to fight with their perception of a potential opponent's fighting performance (Hack, 1997; Rillich et al., 2007).

While many of the ethological aspects of aggressive behavior and fighting are known, the causal neural mechanisms underlying aggressive behavior, including the identity of the sensory systems involved, remain largely unknown. Recently, progress has been made in delineating which sensory information is involved in the mediation of fighting behavior. For example, in *G. bimaculatus*, differences in the frequency of antennal fencing between the eventual winners and losers of a fight suggest that male crickets may 'decide' on the opponent's willingness to fight based on tactile information delivered to the central nervous system (CNS) via the antennae (Hofmann and Schildberger, 2001). Visual input also plays a role as blinded males exhibit more intense fighting than sighted males. This suggests that visual information concerning body size, mass and mandible display behavior of the opponent can suppress aggressiveness (Rillich et al., 2007). Thus, it appears that males make decisions about whether to fight or flee from a potential opponent based on different sensory modalities.

It is unclear which sensory modality or combination of modalities elicits the expression of aggressive behavior in male *G. bimaculatus*. The antennae appear to be very important for male crickets to express proper aggressive behavior. For example, male crickets whose antennae have been removed (i.e. 'antennectomized' males) do not fight each other (Hofmann and Schildberger, 2001; Murakami and Itoh, 2003). Thus, sensory inputs to the CNS via antennal pathways are assumed to play a crucial role in eliciting aggressive behavior.

Interestingly, antennectomized males tend to express courtship behavior towards other antennectomized males instead of aggressiveness (Murakami and Itoh, 2003), demonstrating that males require antennal information to elicit the appropriate behaviors when they encounter conspecifics. Antennae convey both tactile and chemical sensory input to the CNS, and components of male aggressive behavior can be elicited by chemical signals released from body parts of another male, such as the forewing and antennae (Nagamoto et al., 2005; Iwasaki and Katagiri, 2008). However, Adamo and Hoy (Adamo and Hoy, 1995) reported that male crickets exhibit only weak aggressive behavior towards anesthetized males, suggesting that chemical signals alone are not sufficient for males to express proper aggressive behavior. Thus, it appears that multiple sensory cues (i.e. visual, olfactory and tactile) from a conspecific are required to elicit intensive aggressive behavior. The present study was performed to gain an understanding of the initiation mechanisms underlying aggression by observing the behavioral interactions between male *G. bimaculatus* crickets with selective sensory deprivations (e.g. antennectomy, palpectomy and darkness). Specifically, to clarify how sensory inputs from the antennae function in recognition of the opponent and/or in exhibiting aggressive behavior, we observed fighting behavior of antennectomized crickets with other intact or antennectomized crickets. Here, we show that (1) male crickets recognize each other appropriately primarily by antennal sensory input, (2) expression of aggressive behavior towards the attacking opponent does not rely on sensory information from the antennae, and (3) experience-dependent behavioral change of losers is modulated by the chemical sensory input from the antennae.

MATERIALS AND METHODS

Animals

Gryllus bimaculatus De Geer 1773 were raised in our laboratory colony. They were reared at 25–30°C under a 14h:10h light:dark cycle (lights on at 06:00h) and fed a diet of insect pellets (Oriental Koubo, Tokyo, Japan), carrots and water *ad libitum*. We used sexually mature male crickets (2–3 weeks after their imaginal molt) for all behavioral experiments. To reduce the influence of prior fighting experience and to motivate fighting (Dixon and Cade, 1986; Adamo and Hoy, 1995; Iwasaki et al., 2006), crickets were separated into individual 100 ml glass beakers lined with filter paper for 5–7 days before the experiments began. When in the glass beakers, the crickets were fed chopped carrot until 2 days before the experiments began and then starved for 2 days to motivate them for fighting.

In some cases, entire parts of both antennae and/or maxillary palpi of the crickets were cut off using fine scissors about 1 h before the experiments started to test the role of sensory input from the antennae and/or maxillary palpi for aggressive behavior. After the antennae and/or palpi had been removed, the cricket was kept isolated in a beaker for 1 h to recover and was then used for the experiment.

Behavioral experiments

All experiments were performed between 12:00h and 18:00h at 24–29°C. In most experiments, the experimental setup was illuminated by a fluorescent lamp, and the crickets' behavior was monitored using a digital video camera (NV-GS500, Panasonic, Osaka, Japan) for detailed analyses. For the experiments performed under dark conditions, an infrared LED lamp was used for illumination, and behavior of the crickets was recorded with an infrared camera (WAT-902H, Watec, Yamagata, Japan).

To observe fighting behavior of the crickets, two randomly chosen crickets of similar body mass were introduced into a round glass

arena (12 cm diameter, 6 cm height) lined with filter paper. The body mass of the crickets used in the present study was between 0.45 and 1.01 g, and the mass difference within the pair did not exceed 0.05 g. To distinguish individuals from one another, one of each pair was marked with colored lacquer on the pronotum. The crickets were initially separated from each other by an acrylic partition in the arena to prevent any contact between them. At the start of the experiment, the partition was slowly removed, and the behavior of each cricket was observed until a dominance hierarchy within the pair was established. Dominance was indicated when one of the crickets (dominant) sang an aggressive song and chased the subordinate, while the subordinate ran away from the dominant. Experiments were terminated 5 min after the partition was removed if dominance was not established, and the data for that pair were not used for further analysis. Under the experimental conditions listed above, a pair of intact crickets usually started to fight within seconds of the partition being removed, and the dominance hierarchy was formed within 1 min (27.46 ± 28.19 s, $N=46$). Even in cases in which the dominance hierarchy was not established within the 5 min experimental period, the crickets encountered their opponent several times because the arena was sufficiently small, which means that we can exclude the possibility that they did not notice the opponent. In some experiments, to test the aggressiveness of the losers, we also observed the behavior of the same pairs in a second encounter. After the first encounter, the crickets were separated again for 15 min by inserting the acrylic partition into the arena. After this 15 min separation, at which point most intact losers show avoidance behavior towards the winners (see Iwasaki et al., 2006), the partition was slowly removed and the behavior of the pair was observed.

To test the behavioral responses elicited by male cuticular substances, a cricket was introduced into the experimental arena, and a chloroform extract of the male forewings was presented to the antennae. Chloroform is one of the solvents frequently used to analyze cuticular lipids of insects (Bagnères and Morgan, 1990). To produce the extract, the forewings of 10 crickets were isolated and washed with 5 ml of chloroform for 1 min; a strip of filter paper was then soaked in the fluid (8×2 mm). A similar strip of filter paper soaked with pure chloroform was used as a control. The strip was held with forceps and gently touched to the antennae of the cricket, and the cricket's behavior was observed over the next 2 min. Each cricket received the cuticular substance stimulus and the control stimulus, and the sequence was changed randomly during the experiments. To clarify the effects of previous fighting experience on the behavioral response elicited by the male cuticular substances, 27 losers and 28 winners were tested with the same extract after a fight with another cricket. In these experiments, a pair of crickets was placed into the arena and again each cricket was separated into a 100 ml glass beaker immediately after determination of the dominance hierarchy within the pair. After 5 min separation in the beaker, each cricket was introduced into the arena and tested with the cuticular substances and the control stimulus as described above.

The behavioral responses of each cricket towards an opponent or stimulus were categorized into the following four classes based on a previous report (Iwasaki and Katagiri, 2008): 'aggressive behavior', 'avoidance behavior', 'mating behavior' and 'no response'. Briefly, aggressive behavior included threatening posture, antennal fencing, aggressive song and/or mandible flare against the object (the opponent or the filter paper); avoidance behavior was defined by quick retreat from the object and/or kicking against the object; and mating behavior was defined as approaching the object, while singing a calling song and/or courtship song. In the experiments using the cuticular substances, crickets sometimes

Table 1. Levels of aggression of intact and antennectomized male crickets

Level	Intact	No antennae
7		Tactile combat
6		Mandible engagement (multiple)
5		Mandible engagement (single)
4		Mandible flare
3	Antennal fencing with threat posture	Threat posture
2		Avoidance after face-to-face contact with the opponent
1		Avoidance before face-to-face contact with the opponent

Fighting behavior was classified into seven levels according to the most intensive aggressiveness of the subordinate. Behavioral variation was defined based on a previous study (Stevenson et al., 2000).

showed both aggressive and avoidance behavior repeatedly. We categorized such cases into another class of ‘aggressive/avoidance’, which was treated separately from both aggressive behavior and avoidance behavior. If the dominance hierarchy was established within 5 min of the partition being removed, the level of aggression in the fight was also scored. The score depended on the maximum aggression level of the loser, indicating to what extent the fight escalated (1–7, Table 1) (modified from Stevenson et al., 2000). Using this definition, a pre-established dominance hierarchy within the pair, in which only one of the pair exhibited aggressive behavior towards the opponent, was categorized into the lowest level (Table 1, level 1). In some cases of fights between crickets with different treatments, we also evaluated the win probability as an index of aggressiveness.

Statistical analysis

The χ^2 test was used for independent pairs of the proportion of the behavioral responses. For the scored aggression levels, the median with the lower and upper quartiles was calculated, and the Mann–Whitney *U*-test and Wilcoxon’s test were used for independent and dependent pairs of data, respectively. The Kruskal–Wallis test was used for multigroup data for the aggression level, and Scheffe’s test was used for *post hoc* comparisons.

RESULTS

Fights between pairs of antennectomized crickets

Aggressive behavior was observed in the following four groups: intact crickets, intact crickets in the dark, crickets with both palpi removed (palpectomy) and crickets with both antennae removed (antennectomy).

In all groups, crickets were always paired with another cricket with the same treatment (e.g. antennectomized crickets with antennectomized crickets). When both antennae were removed, these crickets exhibited a significantly lower percentage of aggressive behavior than intact crickets under either light or dark conditions. In addition, the antennectomized crickets also exhibited less aggression than palpectomized crickets (Fig. 1A; $P < 0.0001$, χ^2 test). Among intact crickets, 92% and 93% exhibited aggressive behavior towards the opponent under light and dark conditions, respectively (Fig. 1A, intact/light, intact/dark). The remaining crickets exhibited avoidance behavior towards their opponent, suggesting that there may have been a pre-established dominance hierarchy between the two crickets. Palpectomized crickets were as aggressive as the intact crickets towards their opponents (Fig. 1A, NP/light). Thus, it is clear that the palpectomy operation did not affect the aggressive behavior of the crickets. In contrast, only 10 of 42 (23.8%) antennectomized crickets showed aggressive behavior towards their opponent (Fig. 1A, NA/light). Consequently, the typical fighting behavior

observed in the intact crickets, in which both members of the pair exhibited aggressive behavior, was not seen in most antennectomized cases (18 of 21 pairs). Rather than exhibiting aggressive behavior, these crickets either totally ignored the other cricket or exhibited courtship behavior. The data presented in Fig. 1B show the aggression levels of the fights between intact or palpectomized crickets in cases when both crickets showed aggressive behavior. We did not find any significant differences in aggressive behavior between intact in the light *versus* intact in the dark *versus* palpectomized crickets ($P = 0.1743$, Kruskal–Wallis test). Thus, deprivation of visual or palpal sensory input does not affect aggressive behavior in crickets.

Fights between antennectomized and intact crickets

In all of the above experiments, we studied aggressive behavior only between crickets that had been treated in the same manner (e.g. both had their antennae removed). In the next series of experiments, we studied aggressive behavior of pairs in which one cricket had been antennectomized while the other was intact. This enabled us to better determine whether antennectomy seriously impaired the elicitation of aggressive behavior. In addition, we also observed aggressive behavior in antennectomized *versus* intact crickets in the dark and in antennectomized–palpectomized (i.e. crickets that had both the antennae and the palpi removed) *versus* intact crickets in the light to examine the effects of blocking visual or palpal sensory input for antennectomized crickets.

In all experiments, intact crickets showed as high a percentage of aggressive behavior towards antennectomized crickets as they did towards intact crickets (Fig. 1C, intact/light, intact/dark, NP/light *versus* Fig. 1A, intact/light: $P = 0.1985$, $P = 0.1391$, $P = 0.7211$, respectively, χ^2 test). Interestingly, in the light, antennectomized crickets exhibited a significantly higher percentage of aggressive behavior when they made contact with an intact cricket compared with the situation with two antennectomized crickets (Fig. 1C, NA/light *versus* Fig. 1A, NA/light, $P = 0.0005$, χ^2 test). However, the aggressiveness shown by the antennectomized crickets against intact crickets was still significantly lower than that of intact crickets (Fig. 1A, intact/light *versus* Fig. 1C, NA/light, $P = 0.0019$, χ^2 test). These data clearly indicate that, in crickets, the antennae are not necessary to elicit aggressive behavior towards another male. As the antennectomized males could not initiate fights against other antennectomized males (see Fig. 1A, NA/light), the antennae must be necessary to recognize an aggressively attacking opponent. In the dark, the antennectomized crickets showed as high a percentage of aggressive behavior as they showed under the light conditions (Fig. 1C, NA/dark *versus* NA/light, $P = 0.0605$, χ^2 test). The antennectomized–palpectomized crickets also showed no significant difference in the percentage of aggressive behavior compared with

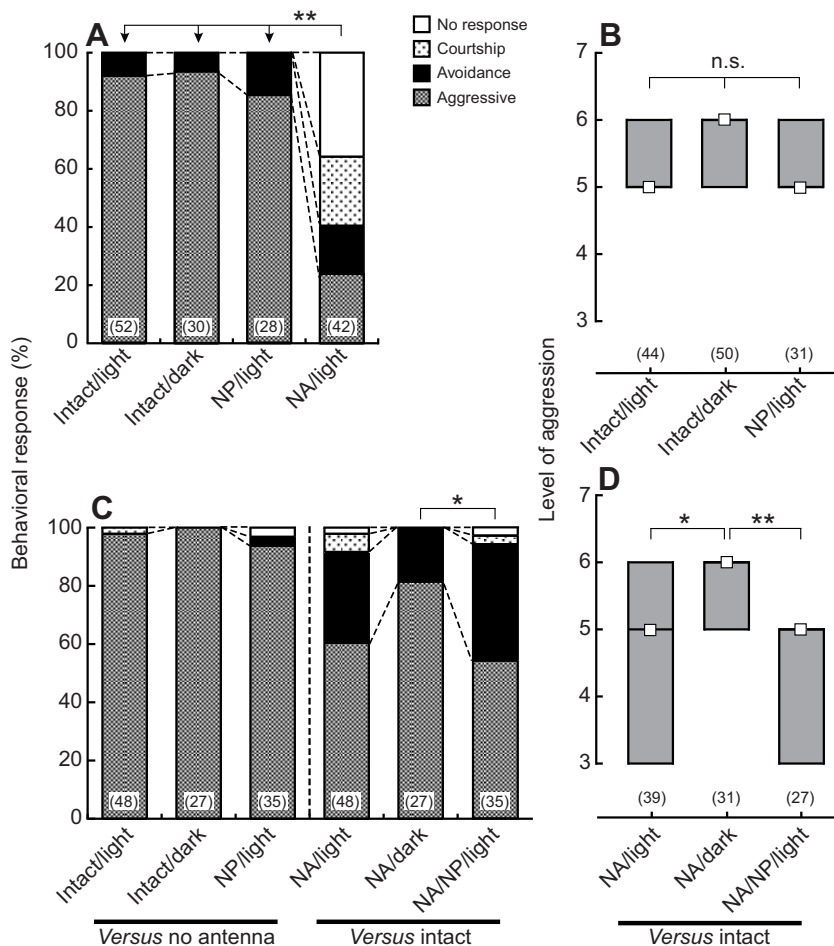


Fig. 1. Fighting behavior between pairs of male crickets treated the same (A,B) and differently (C,D). Treatments for the experimental crickets are shown under each column. NP, no palpi (palpectomized); NA, no antennae (antennectomized). Numbers in parentheses indicate the number of experimental individuals. (A,C) Behavioral responses of the male towards his opponent. Behavior of the individual crickets was categorized into four classes: aggressive, avoidance, courtship and no response. The significance of differences in the number of crickets showing aggressiveness was examined by the χ^2 test (* P <0.05, ** P <0.01). (B,D) Bar graph (median \pm interquartile range) of maximum aggression levels of losers during the fight. Aggression levels of antennectomized pairs are not shown because the numbers were too small (see Results). The significance of differences among the groups under different conditions was examined by the Kruskal–Wallis test and Scheffe's *post hoc* test (n.s., not significant; * P <0.05, ** P <0.01).

antennectomized crickets in the light (Fig. 1C, NA/light *versus* NA/NP/light, $P=0.5765$, χ^2 test), suggesting that removal of both antennae and palpi did not cause any severe damage to behavior. Taken together, these data indicated that neither visual nor palpal sensory inputs are necessary to elicit aggressive behavior in antennectomized crickets. Interestingly, in the dark, the antennectomized crickets showed a higher percentage of aggressive behavior than the antennectomized–palpectomized crickets (Fig. 1C, NA/dark *versus* NA/NP/light, $P=0.0249$, χ^2 test), suggesting that visual information may have a suppressive effect against aggressive behavior (see below). Fig. 1D summarizes the aggression levels of fighting behavior between intact and antennectomized or intact and antennectomized–palpectomized crickets, in cases where both crickets showed aggressive behavior. The aggression levels of the fights were significantly different among the three groups (P <0.0001, Kruskal–Wallis test). The pairs under the dark conditions showed significantly higher levels of aggression than those in the other two groups (Fig. 1D, NA/dark *versus* NA/light, P <0.05; NA/NP/light, P <0.01, Scheffe's test). These data suggest that visual information has an inhibitory effect on aggressiveness (see also Fig. 1C, NA groups).

Pairing an intact with an antennectomized cricket resulted in fights with high levels of aggression, which was as high as that observed with two intact crickets (Fig. 1D, NA/light *versus* Fig. 1B, intact/light, $P=0.2259$). However, a significantly larger number of the intact crickets approached (24.4%, $N=45$, P <0.0001, χ^2 test) or showed the threat posture towards (31.1%, $N=45$, $P=0.0007$, χ^2 test) the antennectomized crickets. These data support the suggestion that

the antennae are necessary to initiate fighting towards a non-aggressive opponent (see above). In fights between an antennectomized cricket and an intact cricket, the intact crickets won significantly more often (70.5%, $N=44$, $P=0.0003$, χ^2 test), suggesting that antennectomy somehow depressed motivation for fighting in crickets. We further examined the detailed behavioral sequence of fighting behavior between intact and antennectomized crickets by analyzing each individual's behavior every 100 ms. Fig. 2 shows ethograms for aggressive behavior of intact losers (Fig. 2A) and antennectomized losers (Fig. 2B) against intact winners. For the antennectomized crickets, displaying threat posture towards their opponent (Fig. 2B, TP) was considered comparable to antennal fencing in intact crickets (Fig. 2A, AF); the behavioral sequence of antennectomized losers was quite similar to that of intact losers (Fig. 2). The occurrence probability of a high level of aggression, including mandible flare and/or engagement, was relatively low for antennectomized crickets (Fig. 2B, MF, ME). One remarkable feature is that most antennectomized crickets (52 of 54 crickets) did not approach the opponent but were approached by the intact opponent at the start of the fight (compare Ap with bAp in Fig. 2B). These results indicate that antennectomy did not impair expression of the escalation of fighting behavior towards the attacking opponent but prevented recognition of the opponent to initiate fighting. In contrast, intact winners showed a similar behavioral sequence irrespective of the condition of the opponent, i.e. intact or antennectomized (supplementary material Fig. S1), suggesting that the crickets' aggressive behavior was unaffected by changes in the opponent's status.

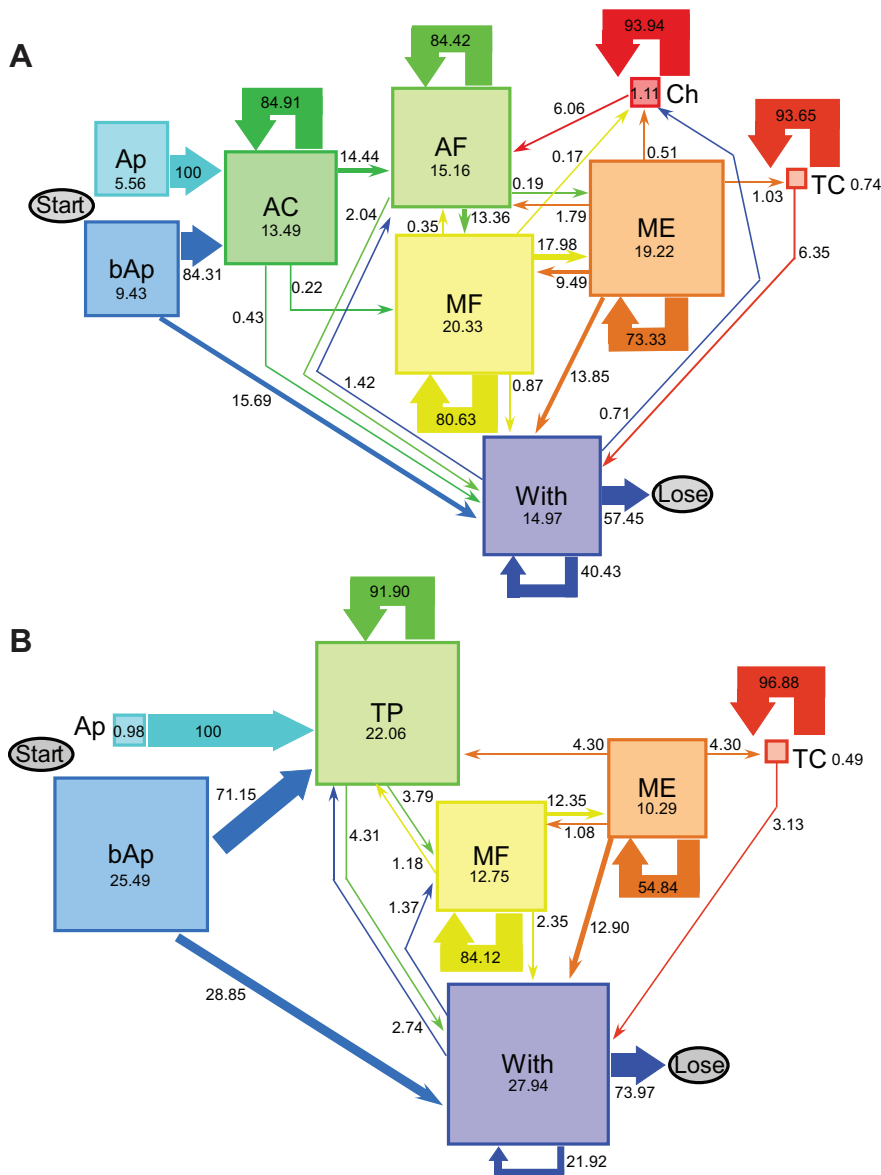


Fig. 2. Ethograms for fighting behavior of intact (A; $N=81$) and antennectomized losers (B; $N=54$) towards intact winners. The size of the squares indicates the occurrence probability of each behavioral category, and the thickness of the arrows indicates the transition probability between each behavioral category. Each number indicates the exact value (%) of the occurrence or transition probability. Ap, approach; bAp, being approached; AC, antennal contact; AF, antennal fencing; TP, threat posture; MF, mandible flare; ME, mandible engagement; TC, tactile combat; Ch, chase; With, withdrawal. Behavioral variation was defined based on a previous study (Stevenson et al., 2000).

Second encounter between antennectomized and intact crickets

Next, we observed a second encounter (15 min after the first; see Materials and methods) between intact and antennectomized crickets. To determine the effects of antennectomy on the behavior of the loser, the levels of fighting between intact crickets were compared with those between intact and antennectomized crickets in cases where the antennectomized cricket had lost in the first encounter. In intact pairs, the losers in the initial bout showed reduced levels of aggression in their second encounter compared with their first encounter (Fig. 3A, intact/light, $P<0.0001$, Wilcoxon's test). The majority of losers showed avoidance behavior instead of aggressive behavior (92.0%, $N=26$). The situation was markedly different with antennectomized crickets. In these crickets, even though they were losers in the initial bout, they exhibited significantly higher levels of aggression in the second encounter compared with the intact losers (Fig. 3A, 2nd, intact/light versus NA/light, $P=0.0056$, Mann-Whitney U -test). Under dark conditions, the antennectomized losers also showed significantly higher levels of aggression in their second encounter than the intact losers (Fig. 3B,

2nd, intact/dark versus NA/dark, $P=0.0179$). Taken together, these data suggest that antennectomy impairs the behavioral modulation of losers irrespective of visual information from the opponent. However, the level of aggression in the second encounter was significantly lower than that in the first encounter (Fig. 3A, 1st versus 2nd, $P=0.0001$, Wilcoxon's test), suggesting that the experience-dependent behavioral decision was made somewhere in the CNS and modulated by the sensory input from the antennae.

In 14 of 63 pairs, an antennectomized cricket won in the first encounter with an intact cricket. In these cases, fighting did not occur in the second encounter within the experimental period (six of seven pairs, data not shown). In these cases, the intact losers only moved enough to avoid the antennectomized winner. Thus, contact between the winner and the loser did not occur and the winner did not chase the loser. A clear fighting behavior was observed in the second encounter in only one of the seven cases, in which the intact loser approached the antennectomized winner from behind and then they started to fight. These findings add further support to our suggestion that the antennae are necessary to recognize the attacking opponent to initiate fighting behavior.

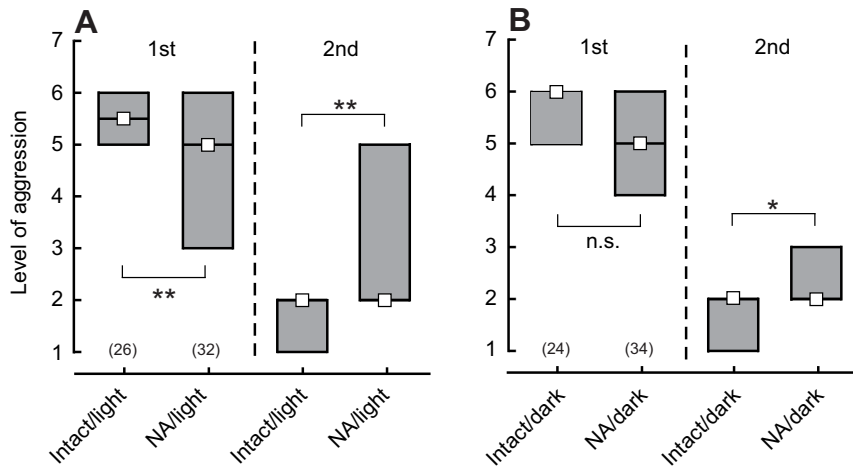


Fig. 3. Bar graphs (median \pm interquartile range) of maximum aggression levels of losers during the first and second fights. Experiments were performed under light (A) and dark (B) conditions. Treatments for the experimental crickets are shown under each column. NP, no palpi (palpectomized); NA, no antennae (antennectomized). Numbers in parentheses indicate the number of experimental pairs. The significance of differences between intact and antennectomized losers was examined by the Mann–Whitney *U*-test (n.s., not significant; * P <0.05, ** P <0.01).

Behavioral responses to male cuticular substances

The data obtained from the antennectomy experiments strongly suggested a crucial role of the antenna in recognition of a fighting opponent (Figs 1, 2) and behavioral modulation based on their fighting experience (Fig. 3). The cuticular substances on the body surface represent a plausible candidate for mediating information about the opponent. Therefore, we examined the response of crickets to the chloroform extract of male forewings to determine whether they use cuticular substances as a signal for recognition of another male. Fig. 4 summarizes the behavioral responses of male crickets to the forewing extract before and after a fight. Before the fight, nearly 60% of crickets showed aggressive behavior towards filter paper soaked with the forewing extract, whereas over 60% did not show any behavioral responses to filter paper soaked with the pure chloroform control stimulus (Fig. 4, naive). After a fight, most winners still showed aggressive behavior towards the forewing extract (Fig. 4, winner). In contrast, about 50% of the losers showed avoidance behavior towards the forewing extract (Fig. 4, loser). A significantly lower percentage of losers showed aggressive behavior towards the forewing extract than did naive crickets and winners (Fig. 4, naive *versus* loser, $P=0.0001$; winner *versus* loser, $P<0.0001$, χ^2 test). These data indicate that crickets change their behavioral response to male cuticular substances from aggressiveness to avoidance after losing a fight.

DISCUSSION

Antennal sensory input for recognition of the opponent

In this study, we separately observed aggressive behavior of antennectomized crickets towards another intact or antennectomized cricket. This allowed us to observe ‘offensive’ (i.e. against a non-attacking opponent) and ‘defensive’ (i.e. against an attacking opponent) aggressive behavior separately; our data clearly indicated that defensive aggression does not rely on antennal sensory input.

Our observation that most antennectomized pairs would not initiate fighting behavior (Fig. 1A,B) is consistent with earlier reports (Hofmann and Schildberger, 2001; Murakami and Itoh, 2003). In both of those previous studies, typical fighting behavior was rarely seen; instead, the crickets often showed courtship behavior towards their opponent. Hofmann and Schildberger reported that fighting escalates only when the frequency of antennal fencing is high enough in both animals (Hofmann and Schildberger, 2001). This suggests that crickets obtain information about their opponent’s willingness to fight *via* antennal sensory input (probably both mechanical and chemical). Thus, if antennal input is absent, crickets revert to escape or courtship behavior. Here, we report that

antennectomized crickets showed typical fighting behavior when paired with an intact cricket (Figs 1, 2), clearly indicating that antennal input is not crucial to elicit aggressive behavior itself. In the fight, intact and antennectomized crickets exhibited a similar behavioral sequence towards their opponent (Fig. 2). In addition, the behavioral sequence of the intact crickets towards antennectomized crickets was not different from that towards intact crickets (supplementary material Fig.S1). These observations indicate that neither the cricket’s own antennal input nor input from the opponent’s antennae during the fight is necessary for normal sequentially escalating fighting behavior. However, antennectomy strongly affected the start situation of the fight. In the encounters between intact and antennectomized crickets, a significantly larger number of the intact crickets were the first to show aggressive displays. Moreover, in cases where the antennectomized cricket was the winner, they would often not give chase to the intact loser in the second encounter. The most likely reason for this is that because they lack antennae, they cannot sense the opponent unless the opponent physically approaches them. Taken together, these results

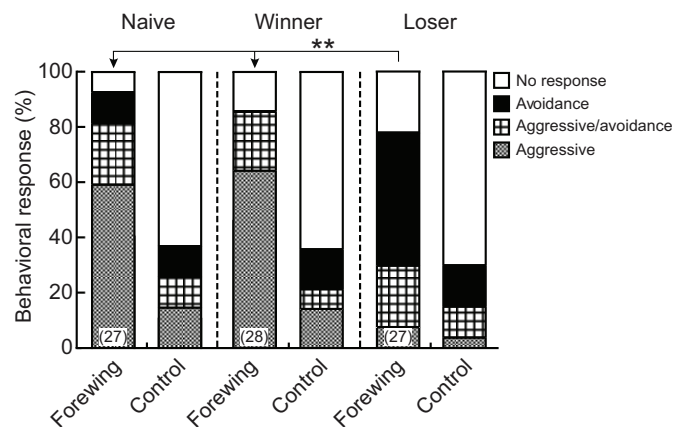


Fig. 4. Behavioral responses of male crickets to filter paper soaked with chloroform extract of male forewings or pure chloroform (as a control) before (naive) and after (winner, loser) the fight. Numbers in parentheses indicate the number of experimental individuals. The behavior of individual crickets was categorized into three classes: aggressive, avoidance and no response. Individuals showing both aggressive and avoidance behaviors repeatedly during the experiments were treated as a separate category (aggressive/avoidance). The significance of differences among the groups was examined by the χ^2 test (** P <0.01).

suggest that sensory antennal input is crucial for recognition of the non-aggressive opponent at the initiation of the fight.

Chemical and tactile information are two major inputs from the antennae, and these sensory cues appear to determine the type of agonistic behavior adopted by a male cricket (Adamo and Hoy, 1995). In some cricket species, it has been suggested that male individuals use chemical signals emitted from the cuticle for conspecific sex recognition (Rence and Loher, 1977; Hardy and Shaw, 1983; Nagamoto et al., 2005; Iwasaki and Katagiri, 2008). Here, we found that the chloroform extract of the male forewings induced aggressive behavior by male individuals, clearly indicating that the males use sensory cues emitted from the cuticle. However, the aggressive behavior elicited by male cuticular substances is often much weaker than that elicited by active conspecific males (Fig. 4) (Hardy and Shaw, 1983; Adamo and Hoy, 1995). This implies that expression of sequential aggressive behavior requires multimodal sensory processes and the chemical signals may be necessary only at the very beginning stage of the fight, i.e. recognition of the opponent. The roles of the tactile cue from the antennae in fighting behavior have not been studied in detail. A previous report suggested that the tactile cue from the opponent is not used for opponent recognition but for evaluation of an attacking opponent during the fight (Hofmann and Schildberger, 2001) (see above). As we did not observe strong aggressiveness in male crickets elicited by cuticular substances (Fig. 4), it is possible that the chemical and tactile stimuli from the antennae have synergetic effects, and the male crickets would exhibit proper aggressive behavior by receiving both cues.

Normally, once they lose a fight, the crickets show avoidance behavior instead of aggressiveness towards the winner (Adamo and Hoy, 1995; Hofmann and Stevenson, 2000; Iwasaki et al., 2006). Here, we found that the antennectomized losers showed significantly higher levels of aggression in the second encounter compared with intact losers (Fig. 3). The most convincing explanation for this phenomenon is that behavioral changes in the antennectomized losers could be inhibited by interrupting the antennal sensory pathway. The chemically induced behavior elicited by male cuticular substances was strongly dependent on their former experience, i.e. motivation for the fight (Fig. 4). When the cricket was well motivated to fight, such as when it was a winner, it showed clear aggressive behavior towards the cuticular substances of the male forewings (e.g. showing the threat posture and/or antennal fencing). In contrast, the cricket showed avoidance behavior (e.g. a quick retreat from the stimulus) when it was not motivated to fight, such as when it was a loser. The cricket probably recognizes another conspecific male by its cuticular substances and decides whether to fight or flee depending on its former experiences and on its own motivation to fight. It has been reported that the behavioral changes in losers are also suppressed when the nitric oxide (NO) signal in the brain is pharmacologically inhibited (Iwasaki et al., 2007). Some previous reports suggested that NO is required for proper chemical information processing in the antennal lobe (Collmann et al., 2004; Wilson et al., 2007). It has also been reported that antennal mechanosensory neurons contain the target of NO, soluble guanylyl cyclase (Elphick and Jones, 1998), suggesting that the NO signal may modulate the sensitivities of the mechanosensory neurons in the antennae. Thus, it is also possible that the NO signal of the antennal sensory pathway is interrupted by antennectomy, and suppression of the loser's aggressiveness is partly impaired. Another possible explanation for the higher levels of aggression in antennectomized losers is that they were not completely defeated because they lost track of the opponent during fights. Further biochemical analysis of the cuticular components and

electrophysiological studies of the antennal sensillae are necessary to understand the neural mechanisms underlying recognition of conspecific animals in the cricket.

Sensory input to elicit aggressive behavior

Shutting off the visual or palpal sensory input of the antennectomized crickets during the fight was not critically involved in the expression of aggressive behavior. Rather, lack of visual input had a facilitatory effect on aggressiveness (Fig. 1). This observation indicates that crickets would receive some information about the opponent visually even though they are nocturnal and fight under conditions of poor visibility in nature. Our results are in agreement with previous reports that visual cues from the opponent are not necessary for escalating fighting behavior but rather suppress the opponent's aggressiveness (Hofmann and Schildberger, 2001; Rillich et al., 2007). These authors also suggested that visual cues supply information concerning the opponent's size and willingness to fight. Stimulation of the palpi by a male or female antenna elicits aggressive or courtship behavior by the male, showing that they recognize the opponent's sex not only *via* the antennae but also *via* the palpi (Nagamoto et al., 2005). However, behavioral responses towards the female antenna are much stronger than those towards the male antenna. As palpectomy did not cause any serious problems in fighting, the palpi may be used mainly for courtship behavior, i.e. female recognition.

What is the key stimulus necessary to elicit aggressive behavior in antennectomized crickets? An intriguing possibility is that mechanical stimulation of the body surface, such as the head and wings, by contact with the opponent's antennae is the key stimulus. When a male cricket approaches an opponent for a fight, it comes close to the opponent with a threatening posture and frequent antennal movements. We have also observed that stimulation of antennectomized crickets with an artificial antenna, such as the fine hair of a brush, can sometimes induce weak aggressive behavior (data not shown). It is possible that the cricket recognizes mechanical contact with the attacking opponent's antennae by the intensity and frequency of the contact. The present results indicate that there are at least two steps in the aggressive behavior of crickets: threat behavior triggered by chemical recognition of a male individual ('offensive' aggressive behavior), and aggressive response against an attacking opponent, which is mediated by physical contact by the opponent's antennae ('defensive' aggressive behavior). Normally, these two steps must occur simultaneously at the beginning of the fight. In the case of antennectomized crickets, because they are unable to sense chemical signals from the antennae, they do not show any threat behavior voluntarily but exhibit typical aggressive behavior in response to a physical stimulus from the opponent.

It is still unclear where in the CNS these sensory inputs are processed and integrated. At present, no information is available regarding the tactile sensors on the body surface. With regard to antennal sensory integration, mechanosensory neurons in the antennae are considered to project into the ventral area of flagellar afferents (*vfa*), which is located in the posterior deutocerebrum in the cricket (Staudacher and Schildberger, 2000). In the carpenter ant, the female-specific basiconic sensilla on the antennae that are responsible for detecting cuticular hydrocarbons project to specific glomeruli in the dorsomedial region of the antennal lobe (Nakanishi et al., 2010). A recent anatomical study indicated that the chemical and tactile information pathways from the antenna are definitely dissociated at least at the level of second-order neurons (Yoritsune and Aonuma, 2012), i.e. the projection neurons from the antennal

lobe and vfa terminate into segregated areas in the protocerebrum. Together, these findings suggest that it is very likely that the chemical and tactile information from the antennae are integrated at the second-order or higher centers in the brain and processed as motor commands sent to the thoracic ganglia through descending neurons. As octopamine is thought to represent a motivational component of aggressive behavior (Stevenson et al., 2005), and the NO/cGMP signal is involved in the experience-dependent behavioral change of losers (Iwasaki et al., 2007), investigation of the localization of these neuromodulators would provide further insight into the neural mechanisms underlying aggressive behavior.

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M.S. designed and performed the research; M.S. and H.A. wrote the paper.

COMPETING INTERESTS

No competing interests declared.

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REFERENCES

- Adamo, S. A. and Hoy, R. R. (1995). Agonistic behaviour in male and female field crickets, *G. bimaculatus*, and how behavioural context influences its expression. *Anim. Behav.* **49**, 1491-1501.
- Alexander, R. D. (1961). Aggressiveness, territoriality, and sexual behaviour in field crickets (Orthoptera: Gryllidae). *Behaviour* **17**, 130-223.
- Ashikaga, M., Sakura, M., Kikuchi, M., Hiraguchi, T., Chiba, R., Aonuma, H. and Ota, J. (2009). Establishment of social status without individual discrimination in the cricket. *Adv. Robot.* **23**, 563-578.
- Bagnères, A. G. and Morgan, E. D. (1990). A simple method for analysis of insect cuticular hydrocarbons. *J. Chem. Ecol.* **16**, 3263-3276.
- Collmann, C., Carlsson, M. A., Hansson, B. S. and Nighorn, A. (2004). Odorant-evoked nitric oxide signals in the antennal lobe of *Manduca sexta*. *J. Neurosci.* **24**, 6070-6077.
- Dixon, K. A. and Cade, W. H. (1986). Some factors influencing male-male aggression in the field cricket *Gryllus integer* (time of day, age, weight and sexual maturity). *Anim. Behav.* **34**, 340-346.
- Elphick, M. R. and Jones, I. W. (1998). Localization of soluble guanylyl cyclase alpha-subunit in identified insect neurons. *Brain Res.* **800**, 174-179.
- Hack, M. A. (1997). Assessment strategies in the contests of male crickets, *Acheta domesticus* (L.). *Anim. Behav.* **53**, 733-747.
- Hardy, T. N. and Shaw, K. C. (1983). The role of chemoreception in sex recognition by male crickets: *Acheta domesticus* and *Teleogryllus oceanicus*. *Physiol. Entomol.* **8**, 151-166.
- Hofmann, H. A. and Schildberger, K. (2001). Assessment of strength and willingness to fight during aggressive encounters in crickets. *Anim. Behav.* **62**, 337-348.
- Hofmann, H. A. and Stevenson, P. A. (2000). Flight restores fight in crickets. *Nature* **403**, 613.
- Iwasaki, M. and Katagiri, C. (2008). Cuticular lipids and odors in duce sex-specific behaviors in the male cricket *Gryllus bimaculatus*. *Comp. Biochem. Physiol.* **149A**, 306-313.
- Iwasaki, M., Delago, A., Nishino, H. and Aonuma, H. (2006). Effects of previous experience on the agonistic behaviour of male crickets, *Gryllus bimaculatus*. *Zoolog. Sci.* **23**, 863-872.
- Iwasaki, M., Nishino, H., Delago, A. and Aonuma, H. (2007). Effects of NO/cGMP signaling on behavioral changes in subordinate male crickets, *Gryllus bimaculatus*. *Zoolog. Sci.* **24**, 860-868.
- Murakami, S. and Itoh, M. T. (2003). Removal of both antennae influences the courtship and aggressive behaviors in male crickets. *J. Neurobiol.* **57**, 110-118.
- Nagamoto, J., Aonuma, H. and Hisada, M. (2005). Discrimination of conspecific individuals via cuticular pheromones by males of the cricket *Gryllus bimaculatus*. *Zoolog. Sci.* **22**, 1079-1088.
- Nakanishi, A., Nishino, H., Watanabe, H., Yokohari, F. and Nishikawa, M. (2010). Sex-specific antennal sensory system in the ant *Camponotus japonicus*: glomerular organizations of antennal lobes. *J. Comp. Neurol.* **518**, 2186-2201.
- Phillips, L. H., II and Konishi, M. (1973). Control of aggression by singing in crickets. *Nature* **241**, 64-65.
- Rence, B. and Loher, W. (1977). Contact chemoreceptive sex recognition in the male cricket, *Teleogryllus commodus*. *Physiol. Entomol.* **2**, 225-236.
- Rillich, J., Schildberger, K. and Stevenson, P. A. (2007). Assessment strategy of fighting crickets revealed by manipulating information exchange. *Anim. Behav.* **74**, 823-836.
- Simmons, L. W. (1986). Inter-male competition and mating success in the field cricket, *Gryllus bimaculatus* (De Geer). *Anim. Behav.* **34**, 567-579.
- Staudacher, E. and Schildberger, K. (2000). A newly described neuropile in the deutocerebrum of the cricket: antenna: antennal afferents and descending interneurons. *Zoology* **102**, 212-226.
- Stevenson, P. A., Hofmann, H. A., Schoch, K. and Schildberger, K. (2000). The fight and flight responses of crickets depleted of biogenic amines. *J. Neurobiol.* **43**, 107-120.
- Stevenson, P. A., Dyakonova, V., Rillich, J. and Schildberger, K. (2005). Octopamine and experience-dependent modulation of aggression in crickets. *J. Neurosci.* **25**, 1431-1441.
- Wilson, C. H., Christensen, T. A. and Nighorn, A. J. (2007). Inhibition of nitric oxide and soluble guanylyl cyclase signaling affects olfactory neuron activity in the moth, *Manduca sexta*. *J. Comp. Physiol. A* **193**, 715-728.
- Yoritsune, A. and Aonuma, H. (2012). The anatomical pathways for antennal sensory information in the central nervous system of the cricket, *Gryllus bimaculatus*. *Invert. Neurosci.* **12**, 103-117.

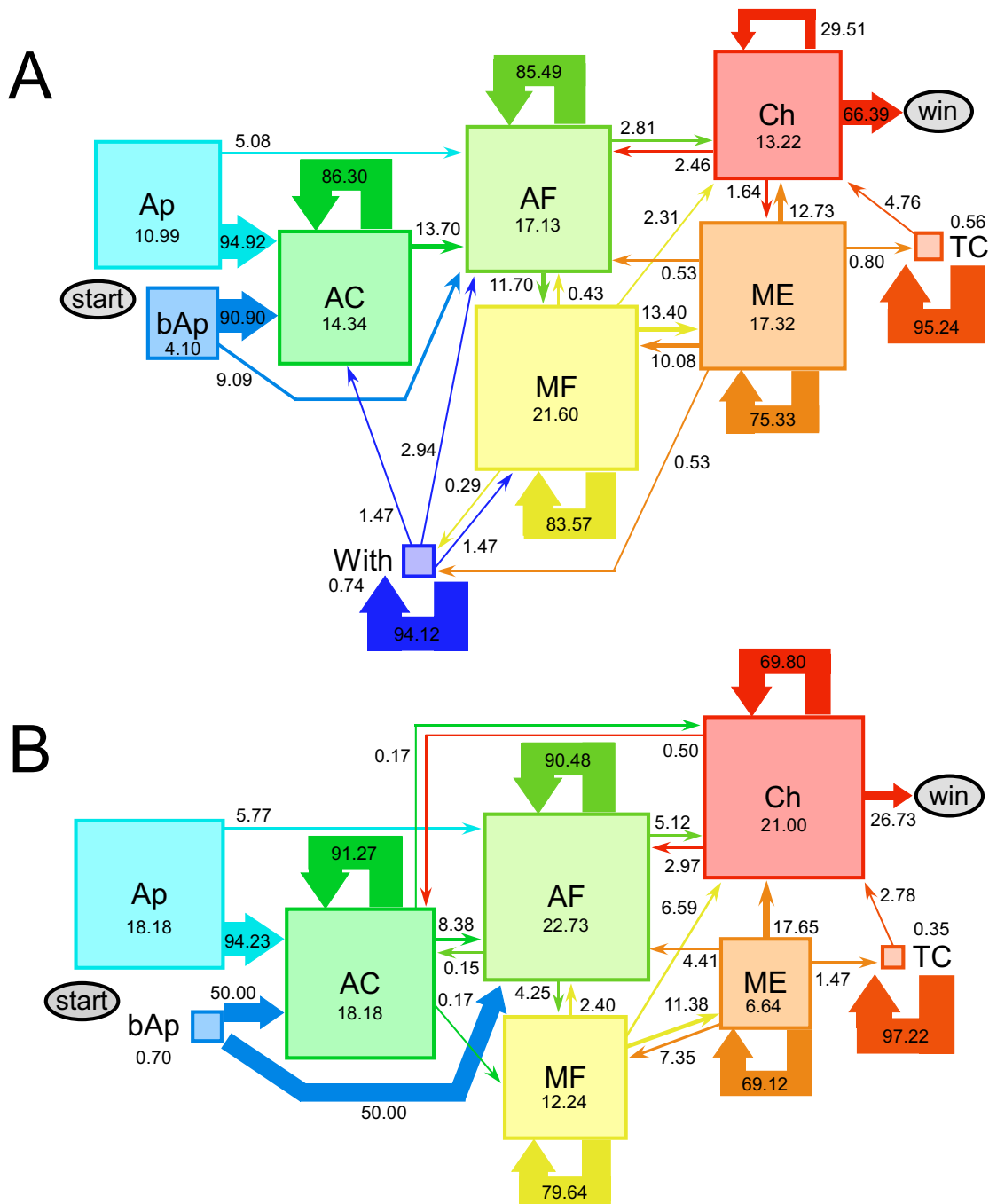


Fig. S1. Ethograms for the fighting behavior of intact winners towards intact (A; $N=81$) and antennectomized losers (B; $N=54$). The size of the squares indicates the occurrence probability of each behavioral category, and the thickness of the arrows indicates the transition probability between each behavioral category. Each number indicates the exact value (%) of the occurrence or transition probability. Ap, approach; bAp, being approached; AC, antennal contact; AF, antennal fencing; MF, mandible flare; ME, mandible engagement; TC, tactile combat; Ch, chase; With, withdrawal. Behavioral variation was defined based on a previous study (Stevenson et al., 2000).