

RESEARCH ARTICLE

Dominance hierarchy-dependent behavioural plasticity of crayfish avoidance reactions

Sawako Fujimoto¹, Bunpei Hirata¹ and Toshiki Nagayama^{2,*}

¹Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan and

²Department of Biology, Faculty of Science, Yamagata University, Yamagata 990-8560, Japan

*Author for correspondence (nagayama@sci.kj.yamagata-u.ac.jp)

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SUMMARY

Crayfish showed avoidance reactions when mechanical stimulation was applied to their tailfan. The response pattern of the avoidance reaction was dependent on crayfish size. Small crayfish showed an escape-like dart response while larger crayfish displayed a defensive-like turn response. We show that the response pattern to the same sensory stimulus changed with social status in the small crayfish. Crayfish are territorial animals and a dominant–subordinate relationship was determined when two previously unacquainted animals were paired. This winner–loser hierarchy was observed in pairs of small crayfish. Before fighting, all crayfish showed a dart response following mechanical stimulation of the tailfan. However, the probability of occurrence of a turn response increased significantly in the crayfish that won a fight. This behavioural transition from dart to turn response occurred immediately and was maintained for at least 3 days even if a dominant crayfish was isolated again with no opponent.

Key words: crayfish, dominance hierarchy, behaviour, plasticity, memory.

INTRODUCTION

Arthropods, and in particular insects and crayfish, frequently show reflex-like stereotyped behaviour. Their central nervous systems are relatively simple and many neurones are identifiable (Nagayama et al., 1994). Such characteristics make arthropods particularly suitable for neuroethological analyses of behaviour. Even in these animals, however, the behavioural process is often complex. Depending on the external environment, internal physiological state, ageing and learning, the same sensory signal may trigger different behaviours in the same animal (Davis, 1979; Ritzmann and Tobias, 1980; Bellman and Krasne, 1983). For example, when one crayfish approaches from the rear and touches the tailfan of another crayfish of the species *Procambarus clarkii*, that crayfish exhibits an avoidance reaction in which either one of two alternative responses occurs: a dart or a turn, depending upon the size of the crayfish (Nagayama et al., 1986). In smaller crayfish (less than 8 cm in length from rostrum to telson), a gentle mechanical tactile stimulus to the exopodite usually evokes a dart response in which the crayfish rapidly closes both uropods and walks forward away from the stimulus. With increasing body size, the turn response becomes more probable, with crayfish showing a turning movement towards the stimulus source with a flexion or extension of the abdomen. Similar age-dependent behavioural transitions have also been observed between the escape and defensive behaviours of the lobster (Lang et al., 1977).

When two previously unacquainted crayfish encounter each other, fighting occurs immediately and a dominant–subordinate relationship is determined after a few contacts (Bovbjerg, 1953; Bovbjerg, 1956; Lowe, 1956; Fiedler, 1965). Although larger crayfish usually win fights (Bovbjerg, 1953; Rubenstein and Hazlett, 1974; Pavey and Fielder, 1996), a social hierarchy is formed between

juvenile crayfish of similar size (Issa et al., 1999; Herberholz et al., 2007). Because some stereotypical behaviours of crayfish change in their response patterns depending on social status (Yeh et al., 1997; Herberholz et al., 2003; Song et al., 2006), in this study we analysed whether response patterns of the avoidance reaction change depending on social status.

MATERIALS AND METHODS

Adult male crayfish *Procambarus clarkii* (Girard 1852), 6–8 cm in length from rostrum to telson, were obtained from a commercial supplier and placed individually in separate containers of 19×33×15 cm (width×length×height) filled with water to a depth of 10 cm for at least 30 days. This period of isolation was enough to prevent the effects of hysteresis (Hemsworth et al., 2007), as the winning crayfish was more likely to win subsequent conflicts (Daws et al., 2002; Bergman et al., 2003; Seebacher and Wilson, 2007).

Experimental trials were carried out in a dimly lit laboratory at room temperature (~23°C). Two crayfish of the same sex and similar size (length difference <5%) were selected and paired in a new container of 26×38×24 cm (width×length×height) filled with water to 5 cm depth. Prior to pairing (on the day before experiments started), the response of each crayfish to mechanical stimulation of the uropods was examined in 10–20 trials. The uropods are the terminal appendages of the tailfan and lie on either side of the medial telson. Each uropod was biramous, consisting of a basal protopodite bearing a blade-like inner endopodite and outer exopodite (Field et al., 1990). Avoidance reactions were elicited by touching the exopodite on either side with a long-handled (30 cm) fine brush in the water. The brush was moved slowly towards the uropod from behind when a crayfish was in a stationary resting position or as it proceeded slowly forward with a passive extension of the abdomen.

Before touching the crayfish, the brush was waved in the air to confirm that visual input did not affect the responsiveness of the crayfish. If an animal showed any response before contact, the stimulus was postponed; the same procedure was then performed again after several minutes. Behavioural acts that occurred in response to the mechanical stimulation were categorized as one of four types: dart, turn, tailflip or 'other', including backward walking, pause and no response (Nagayama et al., 1986). Stimulation was delivered at intervals of at least 5 min. After two crayfish were paired in the new container, the animals started fighting and soon a dominance hierarchy was determined ($N=57$ pairs). The winner and loser relationship was determined when the subordinate crayfish showed a retreat or tailflip response following the dominant's approach with rising claws at least three times in succession. One hour after pairing, the response pattern of the dominant and subordinate crayfish to mechanical stimulation of the uropods was examined again in 10–20 trials. Stimulation was delivered at intervals of at least 5 min. In some cases, dominant and subordinate crayfish were then re-isolated separately. We used 24 dominant crayfish after the first fight in this experiment. The following day, the dominant crayfish was paired with another dominant crayfish to form a new hierarchy ($N=12$ pairs). The response of each crayfish to mechanical stimulation of the uropods was examined again in 10–20 trials before and after the second fight.

In some preparations, the responses of the dominant and subordinate crayfish to mechanosensory stimulation were recorded using a Sony digital video camera (DCR-TRV950). The angle of the exopodite relative to the telson was measured from video frames and compared before and after mechanical stimulation. In the turn response, asymmetrical movement of the uropods and a postural change in the abdomen occurred sequentially. The maximum angle of the exopodite relative to the telson was determined by measuring video frames before the abdomen was flexed. The spike activities of the uropod closer and opener motor neurones, and abdominal slow extensor and flexor motor neurones were monitored extracellularly using two suction electrodes. After behavioural observation, the nerve chain from the second to sixth (terminal) abdominal ganglion with relevant nerve roots was isolated from the abdomen, and pinned dorsal-side up in a Sylgard-lined small chamber, containing cooled van Harreveld's solution (van Harreveld, 1936). The spike activity of the closer motor neurones was recorded extracellularly from the right nerve root 2 motor bundle of the terminal abdominal ganglion at the bifurcation to the reductor and adductor muscles, while that of the opener motor neurones was recorded from the right nerve root 3 motor bundle of the terminal ganglion at the bifurcation to the ventral rotator and the abductor exopodite muscles. The spike activity of the slow extensor motor neurones was recorded extracellularly from the right nerve root 2 motor bundle of the third or fourth abdominal ganglion, while that of the slow flexor motor neurones was recorded from the nerve root 3 motor bundle of the third or fourth abdominal ganglion on the same side. Another suction electrode was placed over the cut end of the right nerve root 2 sensory bundle of the terminal ganglion to stimulate the sensory neurones innervating hairs on the surface of the exopodite.

To characterize the behavioural changes of the avoidance reaction in the dominant crayfish, their response to sensory stimulation were continuously tested within three different groups. In the first group ($N=17$), the dominant and subordinate crayfish were kept as a pair in the same container for a further 7 days. Dominant crayfish of the second group ($N=18$) were re-isolated from the subordinates immediately after the test on the first day. To prevent the effect of chemical signals between dominants and subordinates (e.g. Breithaupt and Eger, 2002), the tank water was changed when

dominants were re-isolated. In the third group ($N=14$), the dominant and subordinate crayfish were separated after the test on the first day. From the next day onwards, the two crayfish were paired together for 1 h per day in a new container, and then dominant and subordinate crayfish were again re-isolated. In all three groups, mechanical stimulation of the uropod was applied 10–20 times and the responses of the dominant crayfish were characterized into four types: dart, turn, tailflip and 'other'.

Statistical analysis was conducted with SigmaPlot v11. A change of occurrence probability of the dart or turn response was compared before and after they became dominant or subordinate using Mann–Whitney rank-sum test. Daily changes in the performance between the dart and the turn responses of the dominant crayfish were statistically analysed using Student's *t*-test if data were normally distributed, or using Mann–Whitney rank-sum test if they were not.

RESULTS

After two crayfish were paired in a new container, they raised both chelipeds and attacked each other. The period of each fight was variable from several seconds to a few minutes. The probability of fighting declined gradually over time and the relationship between dominant and subordinate was usually determined within 35 min of pairing. The subordinate crayfish were then usually observed to move rapidly away from the dominant crayfish as they approached.

Social status-dependent behavioural plasticity of avoidance reaction

Before a fight, crayfish showed a dart response in more than 90% of trials (Fig. 1, black bar). When the exopodite was lightly touched,

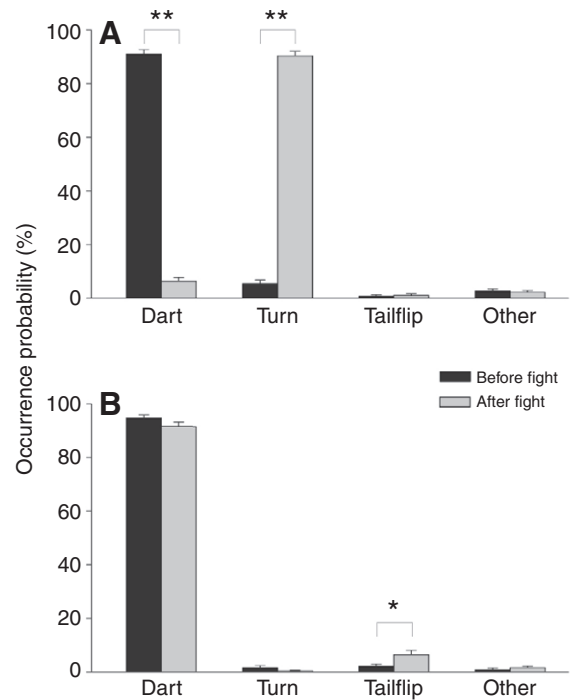


Fig. 1. Response patterns of dominant (A) and subordinate (B) crayfish (57 animals each) before (black bars) and after (grey bars) a fight. The response to mechanical stimulation of the tailfan was categorized as a dart, turn, tailflip or 'other', and the probability of occurrence of each response is shown. Bars represent means + s.e.m. Asterisks indicate that the occurrence probability of the response differed significantly before and after fighting (* $P<0.05$, ** $P<0.001$, Mann–Whitney rank-sum test).

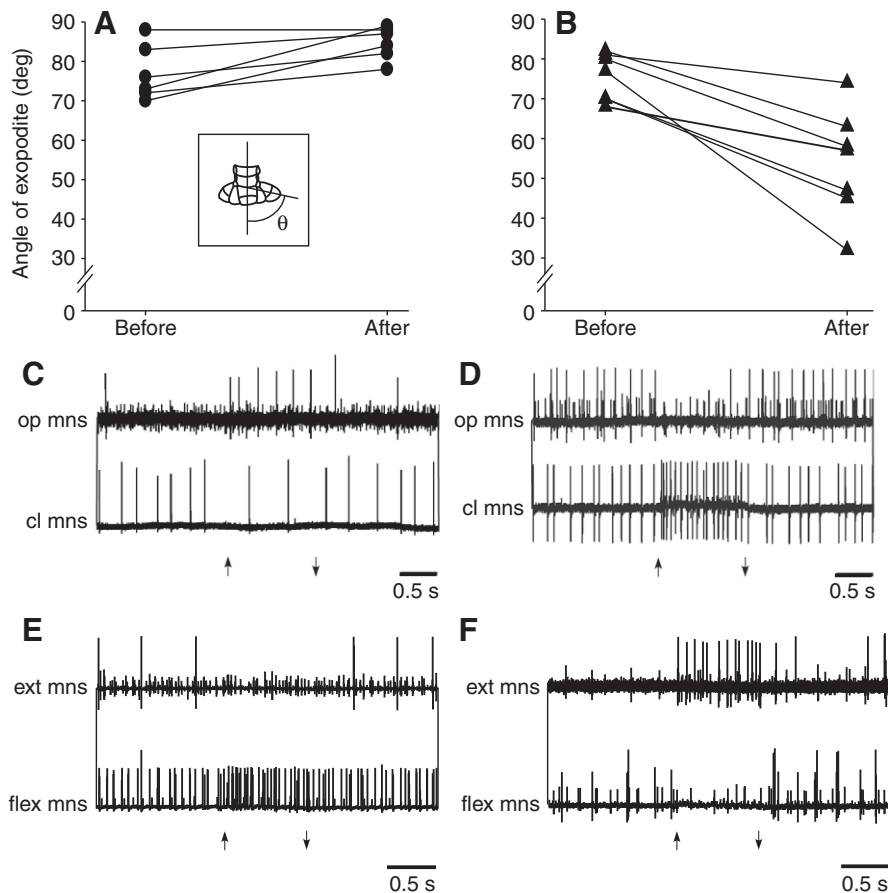


Fig. 2. (A,B) The angle (θ) of the exopodite relative to the telson before and after mechanical stimulation of the exopodite, measured from video frames for (A) six dominant crayfish and (B) seven subordinate crayfish. (C,D) Pattern of activity of uropod motor neurones in response to sensory stimulation of the exopodite. Dominant crayfish showed an opening-like motor pattern (C), while subordinate crayfish showed a closing-like motor pattern (D). op mns, opener motor neurones; cl mns, closer motor neurones. Sensory stimulation (arrows) was applied to the nerve root 2 sensory bundle of the terminal ganglion at 20 Hz for 11 electrical pulses (indicated by arrows). (E,F) Pattern of activity of abdominal postural motor neurones in response to sensory stimulation of the exopodite. Dominant crayfish showed a flexion-like motor pattern (E), while subordinate crayfish showed an extension-like motor pattern (F). ext mns, extensor motor neurones; flex mns, flexor motor neurones. Sensory stimulation (arrows) was applied to the nerve root 2 sensory bundle of the terminal ganglion at 20 Hz for 11 electrical pulses (indicated by arrows).

both uropods were closed and the crayfish walked forward with an extended abdomen. No statistical difference was observed between dominant and subordinate crayfish (means \pm s.e.m.: $91.1 \pm 1.6\%$ from 57 dominant crayfish and $94.7 \pm 1.2\%$ from 57 subordinate crayfish). After a fight, the response pattern of the dominant crayfish changed considerably (Fig. 1A). While they typically showed a dart response before a fight, mechanical stimulation predominantly elicited a turn response after they became dominant ($90.4 \pm 1.7\%$). Crayfish turned towards the stimulus source, mainly with a flexed abdomen. In only 6.4% of trials did dominant crayfish show a dart response. Statistical analysis showed that the probability of occurrence of the dart response and that of the turn response before and after a fight were significantly different in dominant crayfish ($P < 0.001$ for both: Mann–Whitney rank-sum test). In contrast, the response of the subordinate crayfish to mechanical stimulation was unchanged after a fight (Fig. 1B). Touching the exopodite still elicited a dart response ($91.6 \pm 1.6\%$). Although the probability of occurrence of the non-giant mediated tailflip of the subordinate animals was considerably lower than that of the dart response, it significantly increased ($P < 0.05$: Mann–Whitney rank-sum test) after a fight ($2.1 \pm 0.8\%$ before fight and $6.5 \pm 1.6\%$ after fight).

Video and electrophysiological analyses supported the change in the pattern of the avoidance reaction of dominant crayfish (Fig. 2). The angle of the exopodite against the telson on the stimulated side was measured from video frames before and after mechanical stimulation of the exopodite. Before stimulation, the exopodite was usually held at an angle of 70–80 deg in both dominant and subordinate crayfish. Dominant crayfish that showed a turn response (Fig. 1A) opened their exopodite on the stimulated side and closed the exopodite on the contralateral side. The angle of the exopodite

against the telson was therefore slightly increased (Fig. 2A). In contrast, in subordinate animals that showed a dart response (Fig. 1B), the uropods on both sides closed and the angle of the exopodite against the telson decreased to approximately 40–70 deg (Fig. 2B). Changes in the angle of the exopodite before and after stimulation were significantly different between dart and turn responses ($P < 0.001$: *t*-test). Furthermore, the spike activity of antagonistic closer and opener motor neurones in response to electrical stimulation of sensory afferents innervating hairs of the exopodite was reversed (Fig. 2C,D). Spikes of the opener motor neurones increased and those of the closer motor neurones decreased in the dominant crayfish (Fig. 2C). In the subordinates, sensory stimulation elicited a closing-like motor pattern of the uropod. The closer motor neurones were excited and the opener motor neurones were inhibited (Fig. 2D).

In the dart response, crayfish scuttled forwards, away from the mechanical stimulation using walking legs with an extended abdomen. In the turn response, in contrast, crayfish turned toward the stimulus source with a flexed abdomen (in over 75% of observations). As a final form of response, an animal lifted both chelipeds in a defensive posture. In response to electrical stimulation of the sensory bundle innervating sensory hairs on the exopodite, dominant crayfish showed a flexion-like activity pattern of the abdominal postural motor neurones (Fig. 2E). Flexor motor neurones were excited and the spike discharge of the extensor motor neurones was inhibited. In contrast, flexor motor neurones were inhibited and extensor motor neurones were excited in the subordinate crayfish, showing an extension-like reciprocal motor pattern (Fig. 2F).

To further examine whether the behavioural plasticity of the avoidance reaction was depended on social status, crayfish that

became dominant after the first fight were paired with another dominant crayfish the following day. Upon pairing, they started fighting immediately, and a new dominant and subordinate hierarchy was soon established (the second pairing). Before a fight, both crayfish showed mainly a turn response. The winners ($N=12$) of the fights of the second pairing (Fig. 3A) still occupied a dominant status, and touching the uropods continued to elicit a turn response ($83.0\pm 4.5\%$ before the second pairing and $93.0\pm 3.6\%$ after the second pairing). For the losers ($N=12$) after the second pairing (Fig. 3B), the pattern of response reversed from a turn ($83.0\pm 4.5\%$ before the second pairing) to a dart response ($83.0\pm 4.5\%$ after the second pairing). For the winners, the occurrence probability of the dart or turn response before and after the second pairing was not changed. In contrast, that of the dominant which became subordinate after the second pairing was significantly different ($P<0.001$ for both: Mann–Whitney rank-sum test).

Memory of behavioural plasticity of avoidance reaction

When dominant and subordinate crayfish were paired in the same tank, the dominant crayfish frequently tried to attack, and the subordinate retreated when the dominant crayfish approached. Although attacks by the dominant crayfish diminished in probability day by day, more than half of the subordinate crayfish were killed within 4 days of pairing. Dominant crayfish consistently preferred to perform a turn response when a mechanical stimulus was applied (Fig. 4A). One day after pairing (day 1), the occurrence probability of the turn response of the dominant crayfish was almost the same as that of the dominant on the pairing day (day 0). In a test 1 h after pairing, $91.8\pm 2.6\%$ of dominant crayfish showed a turn response to touching the exopodite. On day 1, $92.5\pm 3.5\%$ of dominant crayfish showed a turn response. Two or 3 days after pairing (day 2 or 3), more than 80% of dominant crayfish showed a turn response ($80.0\pm 4.1\%$ on day 2 and $85.6\pm 4.4\%$ on day 3). The occurrence probability of the turn response declined gradually day by day ($74.3\pm 3.0\%$ on day 4 and $65.0\pm 5.0\%$ in day 7), but it was still significantly higher than that of the dart response ($P<0.001$: *t*-test) after the seventh day of pairing.

To examine how long this behavioural plasticity of the dominant crayfish was maintained, dominant and subordinate

crayfish were re-isolated 2 h after pairing on the experimental day (day 0 in Fig. 4B). As the days passed, the probability of occurrence of the turn response in the re-isolated dominant crayfish decreased and that of the dart response increased. The occurrence probability of the turn response was still statistically higher than that of the dart response at day 1 (turn response $83.5\pm 3.2\%$ and dart response $12.1\pm 3.2\%$) and day 2 (turn response is $75.6\pm 4.0\%$ and dart response is $19.2\pm 4.0\%$) after re-isolation ($P<0.001$: *t*-test). At day 3 after re-isolation, the occurrence probability of the turn response was $52.9\pm 5.5\%$ while that of the dart response was $41.4\pm 5.9\%$; there was no significant difference between turn and dart responses ($P=0.159$: *t*-test). On the fourth day (day 4), the dart response was observed more frequently; the occurrence probability of the dart and turn responses was reversed (turn response $28\pm 3.9\%$ and dart response $67.0\pm 4.1\%$) and this difference was statistically significant ($P<0.001$: *t*-test). Subsequently, the probability of occurrence of the dart response increased and that of the turn response decreased. On the seventh day from re-isolation (day 7), dominant crayfish showed the dart response in more than 80% of trials ($82.5\pm 4.0\%$). This was similar to the probability for the dominant crayfish before pairing (day 0 before; $88.4\pm 3.1\%$).

Fig. 4C shows the response of dominant crayfish that were re-isolated 2 h after pairing on the experimental day, but paired for 1 h per day from the next day onwards before application of a mechanical stimulus. During re-pairing on every following day, dominant animals were observed to approach subordinate animals at least once. As subordinate animals moved away to avoid the approach of the dominant crayfish, no contact or fight was observed between these animals. In this group, the dominant animal almost exclusively performed the turn response during the test stimulation. A gradual decline in the probability of occurrence of the turn response was evident in the continuously paired group, but was not observed in re-isolated but subsequently paired for 1 h per day group even after day 7 (cf. Fig. 4A,C). In each group, subordinate crayfish continued to perform the dart response to sensory stimulation (not shown).

DISCUSSION

Small crayfish of 6–8 cm in body length usually showed a dart response during mechanosensory stimulation of the exopodite (Nagayama et al., 1986). Our study demonstrates that the pattern of response to the same stimulus was changed to a turn response when that crayfish held a dominant status after fighting (Figs 1–3). This behavioural switch from the dart to the turn response occurred immediately after formation of the status hierarchy and was maintained for several days without the existence of an opponent (Fig. 4). Nevertheless, the turn response performed by dominant crayfish reversed quickly to the dart response when that crayfish lost their status after a second pairing with another dominant crayfish (Fig. 3). The performance of the avoidance reaction could be changed plastically depending upon their ongoing social status.

Dominance hierarchy and behavioural plasticity of avoidance reaction

A size difference is a major determining factor for hierarchy formation (Bovbjerg, 1953; Lowe, 1956), with larger animals usually becoming dominant in crustaceans (Pavey and Fielder, 1996). Avoidance reactions of crayfish, indeed, show age-dependent plasticity (Nagayama et al., 1986). As animals become larger, the response is changed from a dart response as a variant form of escape behaviour to a turn response as a variant form of defence behaviour.

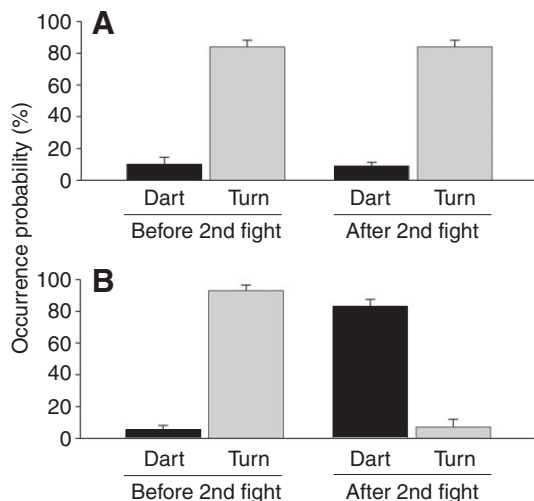


Fig. 3. Response of dominant crayfish before and after a second fight with another dominant crayfish. (A) Response pattern of the dominant winner ($N=12$) after a second fight. (B) Response pattern of the dominant loser ($N=12$) after a second fight. Bars represent means + s.e.m.

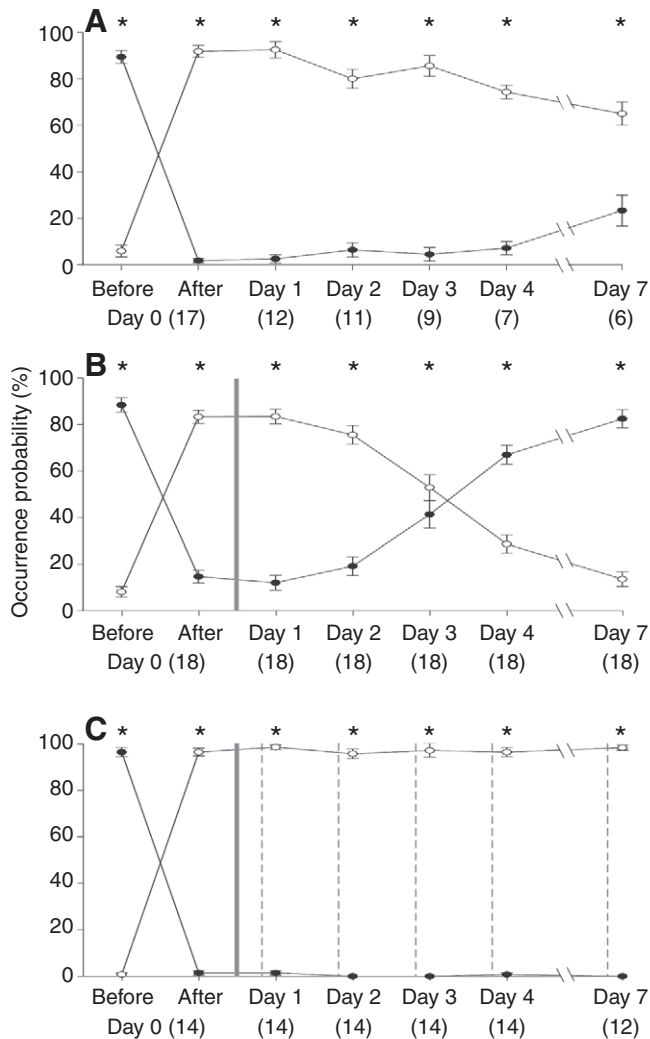


Fig. 4. Daily change in response pattern of the dominant crayfish with and without further pairing. The probability of occurrence of both the dart (filled circles) and the turn (open circles) response in dominant crayfish is plotted as means \pm s.e.m. Number of animals tested is indicated in parentheses for each day. Asterisks indicate that the probability of occurrence of the dart and turn response differs significantly ($P < 0.001$ with *t*-test or Mann-Whitney rank-sum test). In A, a dominant crayfish was paired with a subordinate crayfish in the same tank for 7 days (continuous pairing). In B, a dominant crayfish was isolated from subordinate crayfish soon after the first pairing on the experimental day (day 0). In C, a dominant crayfish was quickly isolated as in B on day 0, but paired again for 1 h per day with a subordinate crayfish on every subsequent day.

Thus, some physiological modification is likely to underpin the change in small crayfish during the formation of a dominance hierarchy to perform a more aggressive turn response. The dominant and subordinate status of crayfish is well known to affect their agonistic and non-agonistic behaviours (Herberholz et al., 2003). Song and colleagues have reported that dominants respond to touch on the right or left side of the first abdominal segment with an orienting reaction, while subordinates respond with avoidance reactions (Song et al., 2006). In this study, the occurrence probability of a tailflip was also significantly increased in the subordinate animals, though the frequency was considerably lower than that of the dart response. Thus, the aggressive state could be changed

plastically according to social status. The response pattern of the dominants became more aggressive while that of the subordinates became more timid. At the moment, we know little of the mechanisms that affect neural activity and reverse the output of the neural circuit to elicit opposing effects, e.g. abdominal flexion in dominants and extension in subordinates evoked by mechanosensory signals (Fig. 2). Because the reversal of activity of antagonistic motor neurones occurred in the isolated abdominal preparations, continuous control from the higher centre of the central nervous system (i.e. the brain) is not necessary to affect local centres of the uropod control system and abdominal postural system. Some neuromodulatory factors could contribute to this behavioural plasticity and its underlying local circuit operation. The biogenic amine serotonin is one candidate to affect this plasticity, as the injection of serotonin elicits a dominant-like posture in both lobsters and crayfish (Livingstone et al., 1980; Kravitz, 1988; Kravitz, 2000; Huber et al., 1997) and a dominant-like display in squat lobsters (Antonsen and Paul, 1997). Furthermore, serotonergic modulation of escape circuit and/or dominance hierarchy formation of the crayfish has been analysed physiologically (Edwards and Kravitz, 1997; Yeh et al., 1997; Edwards et al., 2002; Edwards et al., 2003; Araki et al., 2005). A behavioural pharmacological approach combined with neurophysiological analysis could clarify the role of serotonin in the choice of responses of dominant and subordinate crayfish.

Maintenance of dominance hierarchy

The results shown in Fig. 4 suggest that an interaction with opponents is important to maintain a status-dependent plasticity. The probability of occurrence of the turn response declined gradually when the dominant crayfish were re-isolated from the subordinate crayfish. Continuous pairing with the subordinate crayfish was not necessary but, instead, 1 h contact per day was sufficient to maintain the behavioural transition of dominants. As few fights were observed after the formation of a social hierarchy, sensory inputs that recognize subordinates by using visual or chemical cues, or mechanical contact could provide a mechanism for indicating a dominants' physiological state (Hazlett, 1985; Bruski and Dunham, 1987; Karavanich and Atema, 1998; Breithaupt and Eger, 2002; Crook et al., 2004).

On day 7 (Fig. 4), however, the occurrence probability of the turn response in the dominant crayfish with continuous pairing decreased to less than 75% while that of the crayfish with temporary pairing remained at 100%. As the aggressiveness of the dominant crayfish declines to a low level after a break of 2 weeks from pairing (Issa et al., 1999) and that of crayfish lacking antennular sensilla continues at the same level (Horner et al., 2008), a mechanism of sensory adaptation at the receptor level or depression within the central nervous system could occur during subordinate encounters. A further important finding in this study is that dominant crayfish maintain behavioural plasticity for at least 2 days, even if they are re-isolated quickly after fighting. Despite the absence of sensory cues from opponents during the re-isolation period, dominant crayfish from days 1 and 2 showed a turn response more frequently (Fig. 4B). This means that crayfish could learn and memorize their aggressive state. At the moment, the neural mechanisms for this change in aggressiveness and behavioural choice in response to a stimulus are unclear and further neuroethological studies are necessary to clarify this point.

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