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## SHORT COMMUNICATION

# Alteration of size perception: serotonin has opposite effects on the aggressiveness of crayfish confronting either a smaller or a larger rival

Julien Bacqué-Cazenave, Daniel Cattaert, Jean Paul Delbecque\* and Pascal Fossat\*<sup>‡</sup>

## ABSTRACT

We injected serotonin (5-HT) into adult male crayfish before pairing them with size-matched non-injected competitors, and observed dyadic agonistic interactions. Paradoxically, 5-HT elicited opposite behavioral responses if the injected animal was opposed by a smaller or larger rival: the level of aggressiveness of the injected crayfish was higher when facing a larger rival but lower when facing a smaller rival. Our results indicate that the effects of 5-HT on aggressiveness are dependent on the perception of the relative size difference of the opponent. In both cases, however, 5-HT significantly delayed the decision to retreat. We conclude that 5-HT does not primarily act on aggressiveness but rather on the brain centers that integrate risk assessment and/or decision making, which then modulate the aggressive response. Our findings support a reinterpretation of the role of 5-HT in crustacean agonistic behavior that may be of interest for studies of other animals.

**KEY WORDS:** Aggression, Agonistic context, Opponent perception, 5-HT

## INTRODUCTION

Animals generally compete for resources such as food, territory, shelter or mates, and conflicts between two conspecific individuals often involve aggression and fights, i.e. behaviors that escalate from intimidation to aggressive confrontations with the purpose of repelling, hurting or eliminating the opponent. The decision to initiate, continue or stop a fight depends not only on the social context and the respective strengths of the antagonists but also on their hostile motivation, which is generally defined as aggressiveness.

Among the numerous neuromodulators and signaling molecules that exert multifactorial control over aggressiveness, serotonin (5-HT) is generally considered one of the most important (Nelson and Chiavegatto, 2001; Nelson and Trainor, 2007). However, the effects of 5-HT often appear contradictory in different animal species or even in similar animals studied using different experimental approaches. In humans and other mammals, a higher level of aggression is frequently associated with a deficiency in 5-HT activity, but this interpretation has only taken precedence after decades of conflicting observations (de Boer and Koolhaas, 2005; Olivier and van Oorschot,

2005; Duke et al., 2013). By contrast, the inverse interpretation generally prevails in crustaceans, as it has been demonstrated that 5-HT increases aggressive motivation and delays the decision to stop fighting (Huber et al., 1997; Huber and Delago, 1998; Herberholz, 2014). However, conflicting observations have also been described in these animals (Peeke et al., 2000; Panksepp et al., 2003; Tricarico and Gherardi, 2007). Moreover, in insects, the effects of 5-HT appear to vary in different species: in crickets, 5-HT is able to decrease aggression and favor escape behavior (Stevenson and Rillich, 2012; Stevenson and Schildberger, 2013), but in ants, aggressiveness is weakly affected by exogenous 5-HT (Szczuka et al., 2013). In flies, aggressiveness is increased by the administration of a 5-HT precursor (Bubak et al., 2014) or by selective 5-HT neuron activation (Hoopfer, 2016). Interestingly, recent observations in flies have also shown that 5-HT has a much more complex role in aggressiveness by modulating the perception of a rival (Bubak et al., 2015).

Our previous investigations in the crayfish *Procambarus clarkii* have also led us to hypothesize that the effects of 5-HT are more complex than a simple stimulatory role in aggressiveness. Indeed, 5-HT is involved in the response to stress that controls anxiety-like behavior (Fossat et al., 2014), which also plays a role during agonistic interactions (Bacqué-Cazenave et al., 2017). Moreover, we have observed that, during dyadic encounters, the concentrations of 5-HT significantly increase in the brains of both opponents, reaching higher concentrations in the losers than in the winners (Bacqué-Cazenave et al., 2017).

To study whether these increased concentrations of 5-HT have similar effects on the aggressiveness of antagonists, we designated pairs of size-matched adult male crayfish and injected one animal in each pair with either 5-HT or saline solution. We then observed the interactions of each pair during a time-limited confrontation. Surprisingly, we found that 5-HT had different effects on aggressiveness depending on the relative difference in size of the opponents. These results led us to seek alternative interpretations of the role of 5-HT in the control of social interactions in crayfish.

## MATERIALS AND METHODS

### Animals

Crayfish, *Procambarus clarkii* (Girard 1852), were caught in swamps near Bordeaux (in the Réserve Naturelle de Bruges) and stored inside an animal house specialized for crayfish at 20°C on a 12 h:12 h light:dark cycle in individual tanks (50×30×30 cm) equipped with re-circulating water. The animals were provided with food pellets regularly. We used a total of 184 adult male crayfish ranging from 11.3 to 56.9 g (mean±s.e.m. mass, 28.6±0.7 g). Consistent with most other studies of crustaceans, we isolated the males before any experiment (for at least 3 weeks under our conditions) to erase past social histories. Crayfish that showed signs of molting (such as a soft cuticle) were excluded from the study.

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We then separated the crayfish into 92 dyads (13 dyads in the control group, 25 dyads in the saline-injected smaller mass group, 8 dyads in the low 5-HT-injected smaller mass group, 8 dyads in the high 5-HT-injected smaller mass group, 13 dyads in the saline-injected larger mass group, 11 dyads in the low 5-HT-injected larger mass group and 14 dyads in the high 5-HT-injected larger mass group). The mass of the smaller crayfish overlapped that of the larger individuals: 14.5–56.9 g for the larger crayfish (mean±s.e.m., 31±0.9 g) and 11.3–46.1 g for the smaller crayfish (mean±s.e.m., 26±0.2 g), with a mean difference in dyad mass of 12.3±0.7%. All experiments were performed in accordance with the guidelines for animal care recommended by the French National Center for Scientific Research (CNRS).

### Treatments

Fifteen minutes before the actual confrontation, crayfish (only one per dyad) were injected intramuscularly between two abdominal segments with a saline solution (195 mmol l<sup>-1</sup> NaCl, 5 mmol l<sup>-1</sup> KCl, 13 mmol l<sup>-1</sup> CaCl<sub>2</sub>, 2 mmol l<sup>-1</sup> MgCl<sub>2</sub> and 3 mmol l<sup>-1</sup> Hepes pH 7.65) either alone or supplemented with 5-HT (Sigma Aldrich, St Louis, MO, USA). Two concentrations of 5-HT were used to check for possible dose-dependent effects: 0.05 µg g<sup>-1</sup> (in a single low dose using 0.05 µg µl<sup>-1</sup> 5-HT) or 5 µg g<sup>-1</sup> (in a single high dose using 5 µg µl<sup>-1</sup> 5-HT). Intramuscular injections were used to limit animal manipulation and moderate the delivery of exogenous substances into the hemolymph. Our previous investigations have demonstrated that such injections lead to significant increases in 5-HT content in crayfish brains (Fossat et al., 2015).

### Social interactions

Social interactions between two animals were observed only once they had been placed in a fighting arena aquarium (33×23×20 cm L×W×H) illuminated from above; interactions were recorded for 20 min with a laterally positioned video camera. During the experiment, several variables directly related to the aggressiveness of the injected crayfish were measured. We specifically examined four time variables: (1) the time between the beginning of a bout and the first approach made by each individual, in which an approach was defined as a series of steps toward the opponent that ended with no or minimal touching; (2) the time between the beginning of the bout and the first attack, in which an attack was defined as a sudden acceleration with elevated claws until the opponent was firmly touched or grasped; (3) the time of retreat of one of the opponents; (4) the duration of the fight period, calculated as the time interval between the first approach and the retreat (or to the end of the 20 min experimental period in the absence of retreat). We also counted the number of approaches and the number of attacks during the fight period, and to facilitate comparisons between experimental groups, we expressed the approaches and attacks as the number per minute by dividing the total number by the fight duration.

### Statistical analyses

#### Between-group comparisons

Analyses were performed using Prism software (GraphPad v5). A normal distribution was verified for most samples, and thus the results were expressed as means±s.e.m. However, to compare the effect of 5-HT between groups, we performed non-parametric tests, specifically Kruskal–Wallis tests, followed by Dunn's multiple comparison tests, which are more convenient with small samples. Fisher's test was also used to compare the proportions of the fight outcomes in the different groups. In all tests,  $P \leq 0.05$  was considered significant.

### Principal component analysis (PCA)

PCA was performed as previously described (Fossat et al., 2014) using R software (Ade4 package) and based on the behavioral variables. In the first step, the contribution of each variable to the variance in the first and second components of the PCA was calculated. We only used those variables that made a larger contribution to the variance of a given component than the mean contribution of all variables. The separation between pairs of groups was evaluated by calculating the inertia, which was defined as the ratio of the between-group variance to the global variance. The statistical significance of the inertia was estimated using a Monte Carlo permutation test (1000 runs) and fixed to  $P < 0.05$ . The simulated inertia was calculated in each run, and the distribution of the simulated values was compared with that of the real inertia. A  $P$ -value was then calculated as the ratio of the number of simulations in which the simulated inertia was larger than the real inertia to the total number of runs.

## RESULTS AND DISCUSSION

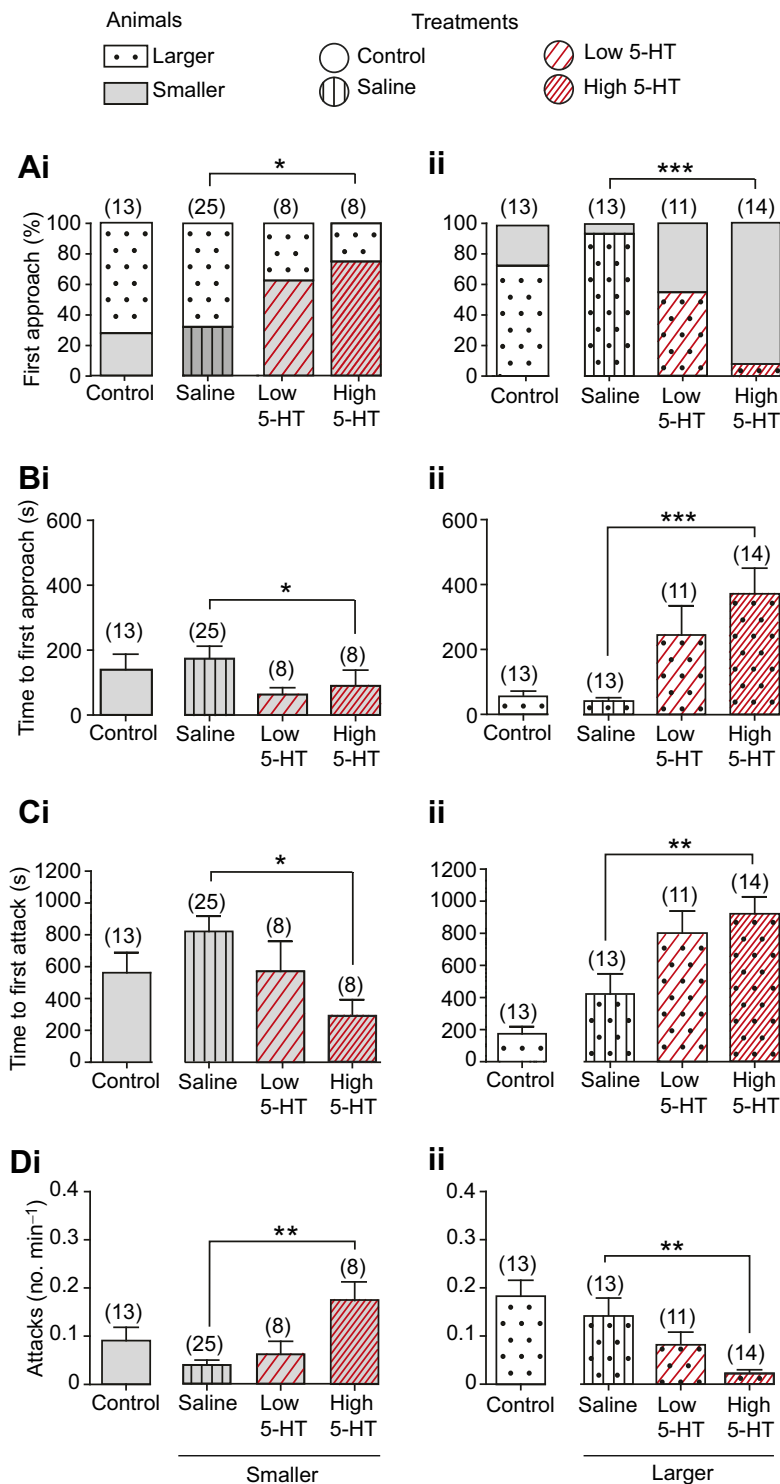
### Effects of 5-HT on approaches and aggressive acts

When placed for the first time together in the experimental aquarium, two adult male crayfish generally spent some time exploring this new environment before assessing their antagonist and deciding whether to approach. An approach was defined here as a series of movements towards the opponent, with no or minimal touching. Under control conditions (non-injected animals), the smaller crayfish of a dyad generally did not make the first approach, and the same was observed for the saline-injected smaller animals. However, when injected with 5-HT and then opposed by a larger untreated animal (Fig. 1Ai), the smaller crayfish frequently approached first (in 63% of pairings after low 5-HT injection and 75% after high 5-HT injection). By contrast, 5-HT-injected animals opposed by a smaller, untreated opponent (Fig. 1Aii) frequently did not make the first approach (50% after low 5-HT injection and 7% after high 5-HT injection), whereas larger control or saline-injected animals generally approached first (72% and 75%, respectively).

These observations were confirmed by analyzing the time to the first approach, which decreased for the smaller animal of a dyad injected with high-dose 5-HT (Fig. 1Bi), but increased for the larger animal of a dyad receiving the same treatment (Fig. 1Bii).

Similar paradoxical observations were obtained for attacks, defined here as sudden accelerations with elevated claws until the opponent was grasped or firmly touched. The time to the first attack was significantly reduced in smaller animals treated with the high 5-HT dose (Fig. 1Ci) but increased in larger animals injected with the same dose (Fig. 1Cii). The number of attacks (expressed as the number per minute during the fight period to allow better comparison) by the smaller crayfish increased with 5-HT injection (Fig. 1Di), and the number of attacks by the larger crayfish decreased with 5-HT injection (Fig. 1Dii).

These results surprisingly indicate that 5-HT injections have opposite effects on crayfish depending on whether the opponent is a smaller or a larger animal. These opposite effects were not related to the absolute size of the animals but to their relative size difference, as suggested by the following two arguments: (1) the distribution of the larger animals overlapped that of the smaller ones (Fig. 2A); and (2) we verified that for the same mass class, 5-HT increased the aggressiveness of animals facing a larger rival but decreased that of individuals facing a smaller one (attacks per minute shown in Fig. 2B; similar observations for approaches are not shown).



**Fig. 1. Opposite effects of 5-HT on approaches and aggressive acts.** (Ai) Smaller crayfish injected with 5-HT were more likely to make the first approach (5/8 and 6/8 for low and high 5-HT groups, respectively) than those in the control (no injection) and saline-injected groups (3/13 and 8/25, respectively; \* $P < 0.05$ , Fisher's exact test between saline and high 5-HT groups). (Aii) Larger crayfish injected with 5-HT made the first approach (6/11 and 1/14 for low and high 5-HT groups, respectively) less frequently than larger control and saline-injected animals (10/13 and 13/14, respectively; \*\*\* $P < 0.001$ , Fisher's exact test). (Bi) Time to the first approach by the smaller crayfish to the larger crayfish was decreased by 5-HT injection ( $85 \pm 26$  s for low 5-HT and  $120 \pm 65$  s for high 5-HT groups) compared with that in the control and saline-injected groups ( $187 \pm 64$  and  $233 \pm 51$  s, respectively; \* $P < 0.05$ , Dunn's multiple comparison test for saline versus high 5-HT groups). (Bii) Time to the first approach by the larger crayfish to the smaller crayfish was increased by 5-HT injection ( $329 \pm 121$  s for low 5-HT and  $500 \pm 103$  s for high 5-HT groups) compared with control and saline-injected groups ( $76 \pm 21$  and  $58 \pm 13$  s, respectively; significant difference between saline and high 5-HT groups, \*\*\* $P < 0.001$ , Dunn's multiple comparison test). (Ci) Time to the first attack by the smaller crayfish on the larger crayfish was decreased by 5-HT injection ( $591 \pm 188$  s for low 5-HT and  $296 \pm 96$  s for high 5-HT groups) compared with the control and saline groups ( $562 \pm 126$  and  $821 \pm 96$  s, respectively; \* $P < 0.05$ , Dunn's multiple comparison test for saline versus high 5-HT groups). (Cii) Time to the first attack by the larger crayfish on the smaller crayfish was increased by 5-HT injection ( $794 \pm 144$  s for low 5-HT and  $922 \pm 105$  s for high 5-HT groups) compared with the control and saline groups ( $174 \pm 45$  and  $423 \pm 124$  s, respectively; significant difference between saline and high 5-HT groups, \*\* $P < 0.01$ , Dunn's multiple comparison test). (Di) Injection of a high 5-HT dose induced a higher number of attacks by smaller crayfish ( $0.17 \pm 0.04$  attacks  $\text{min}^{-1}$ ) than was seen in the other groups ( $0.09 \pm 0.03$ ,  $0.04 \pm 0.01$  and  $0.06 \pm 0.03$  attacks  $\text{min}^{-1}$  for control, saline-injected and low 5-HT groups, respectively; significant difference between saline and high 5-HT groups, \* $P < 0.01$ , Dunn's multiple comparison test). (Dii) Injection of 5-HT induced a decrease in the number of attacks by larger crayfish ( $0.08 \pm 0.03$  attacks  $\text{min}^{-1}$  for low 5-HT and  $0.02 \pm 0.01$  attacks  $\text{min}^{-1}$  for high 5-HT groups) compared with that in the control and saline-injected groups ( $0.18 \pm 0.03$  and  $0.14 \pm 0.04$  attacks  $\text{min}^{-1}$ , respectively; significant difference between saline and high 5-HT groups, \*\* $P < 0.01$ , Dunn's multiple comparison test). Number of crayfish is shown in parentheses.

**5-HT differentially modifies aggression and decision making**

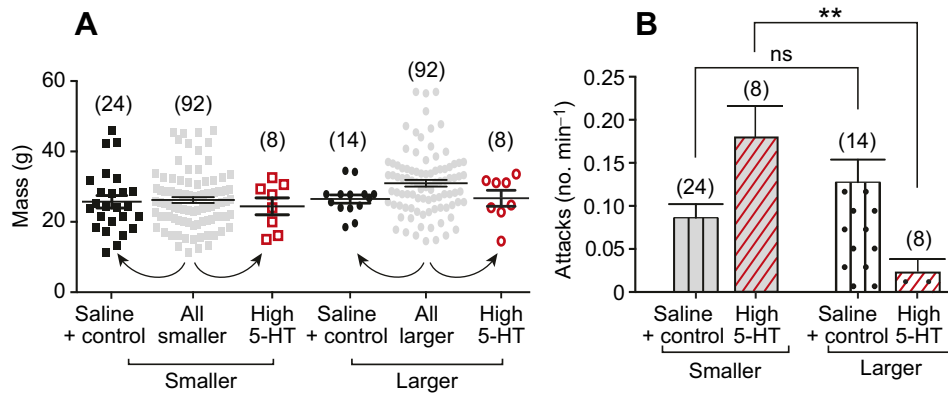
The decision to retreat by one of the opponents, frequently associated with a tail flip, generally marked the end of the fight period (Huber et al., 1997; Bacqué-Cazenave et al., 2017). The injection of a high dose of 5-HT into the smaller animals of the dyads significantly increased the fight duration (Fig. 3Ai). Similarly, the injection of 5-HT into the larger animals significantly increased the fight duration regardless of the dose (Fig. 3Aii).

Because we chose to limit the experiment time to 20 min (1200 s), injection of 5-HT into smaller animals (Fig. 3Bi) and larger animals

(Fig. 3Bii) significantly increased the number of fights where the outcome was indecisive, to the detriment of determining the probability of either winning or losing a fight.

We analyzed the variables related to the injected animals with PCA (Fig. 3C). The *x*-axis represents variables associated with the aggressiveness of the injected crayfish, from less aggressive (time to first approach and time to first attack) to more aggressive (frequency of approaches and frequency of attacks). The *y*-axis represents the readiness to retreat from a slower (bottom) to a faster (top) decision. It must be emphasized that the vector representing decision making was





**Fig. 2. The opposite effects of 5-HT on aggressiveness depend on the relative size difference.** (A) Mass distribution of crayfish used in this study. Left, mass of the smaller crayfish (gray squares); the saline-injected and control animals (black squares) and high 5-HT-injected animals (red squares) are analyzed separately in B. Right, mass of larger crayfish (gray circles); the saline-injected and control animals (black circles) and high 5-HT-injected animals (red circles) are analyzed separately in B. (B) Attacks (number per minute) in a group of crayfish of the same mass. Crayfish of the same mass from the larger and smaller mass groups (mean±s.e.m.,  $26.2\pm 1.7$  g for smaller and  $26.5\pm 1.2$  g for larger crayfish;  $n=24$  and  $14$ , respectively) exhibited no significant difference in the number of attacks per minute. By contrast, crayfish from the smaller mass group injected with a high 5-HT dose had a significantly higher level of aggression than those from the larger mass group injected with 5-HT ( $25.5\pm 2.3$  and  $26.7\pm 2.3$  g for smaller and larger crayfish,  $n=8$ ; mean number of attacks:  $0.17\pm 0.04$   $\text{min}^{-1}$  for smaller and  $0.02\pm 0.01$   $\text{min}^{-1}$  for larger group,  $**P<0.01$ , Dunn's multiple comparison test). ns, not significant. Number of crayfish is shown in parentheses.

orthogonal to the vectors representing aggressiveness, which suggests that these variables are controlled independently (Fig. 3Cii).

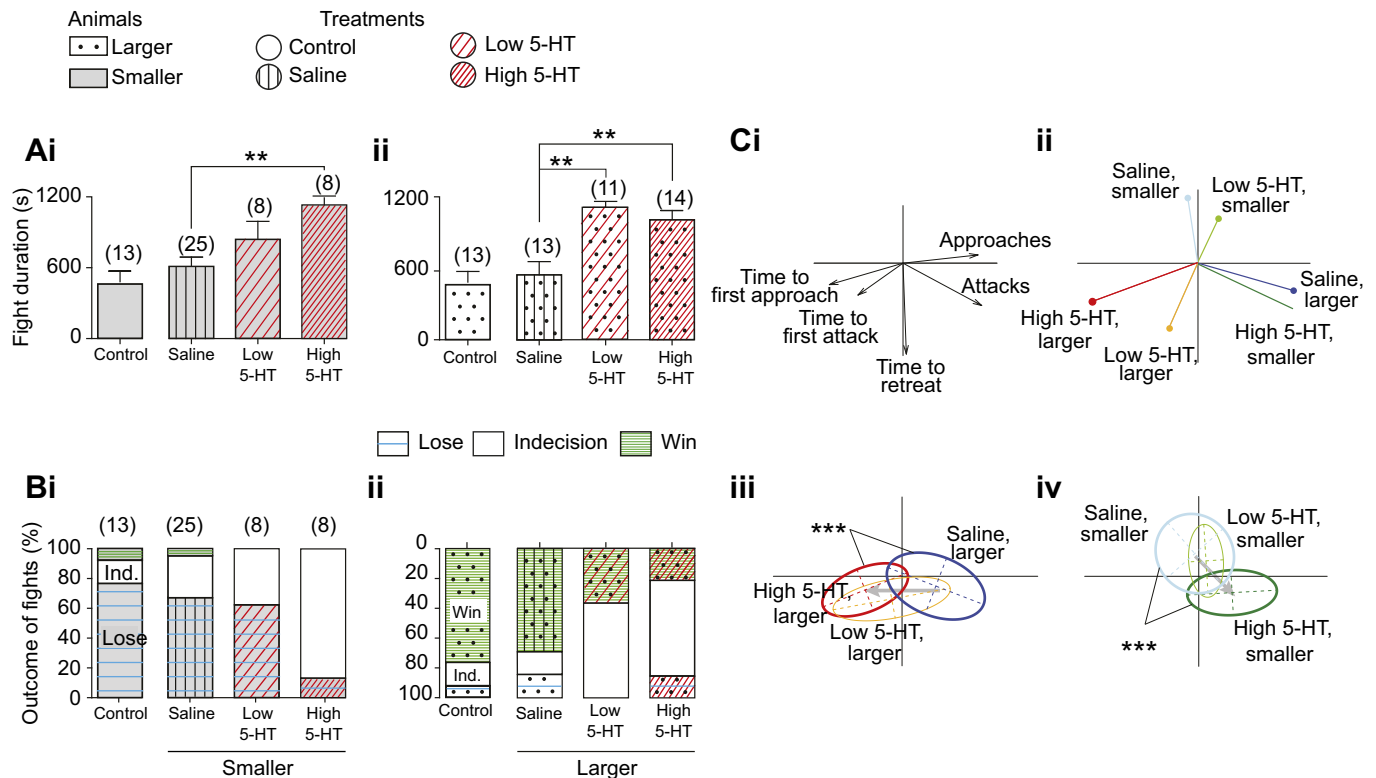
The PCA axes were used to interpret the effects of 5-HT on the smaller and larger animals in the dyads (Fig. 3Cii). The effect of 5-HT injection on larger animals (Fig. 3Ciii) was represented by a shift on only the  $x$ -axis, which corresponded to reduced aggressiveness without a change in the readiness to retreat. By contrast, injection of the smaller animal with 5-HT, particularly at a high dose, was represented by a shift on both axes, corresponding to increased aggressiveness and a delayed decision to retreat (Fig. 3Civ).

The main results of the present study are that 5-HT has opposite effects on the aggressiveness of crayfish opposed by either a smaller or larger rival but similar effects on delaying the decision to retreat. Our observation of increased aggressiveness by smaller animals injected with 5-HT is in accordance with most previous studies on crustaceans, in which the smaller animal of a dyad was generally treated with 5-HT (Huber et al., 1997; Huber and Delago, 1998; Momohara et al., 2013). By contrast, our data demonstrating that 5-HT injection decreases aggressive acts in the larger animal of a dyad appear paradoxical. Although 5-HT may have opposite effects depending on the dose and the rate and duration of application (Teshiba et al., 2001), our paradoxical observations in smaller and larger animals were obviously not related to the 5-HT doses, which yielded relatively similar results in each case. We assume that these results can only be interpreted by considering that the relative size of the opponents modified the effects of 5-HT. Our experiments were conducted using adult males with relatively small differences in mass, and the group of larger animals overlapped the group of smaller individuals. As 5-HT-injected crayfish of the same mass had opposite behaviors depending on whether they faced either a larger or a smaller opponent, it appears that the effect of 5-HT does not depend on the absolute size of the animal but on the relative size difference perceived by the animals themselves. Thus, our results strongly suggest that crayfish can evaluate the risk of a confrontation with a larger or smaller opponent and assess their strength relative to that of their competitor. More interestingly, serotonin injection was able to disturb this risk assessment in our experiments, suggesting that this bioamine plays a role in risk control. This property is probably

widespread in animals as risk taking is also controlled in higher vertebrates by 5-HT (Macoveanu et al., 2013; Blanchard and Meyza, 2017), which can selectively shape social behavior (Crockett et al., 2010). Previous observations in stalk-eyed flies have shown that 5-HT can increase the aggressiveness of a smaller rival by counteracting the perception of its size discrepancy with a larger opponent (Bubak et al., 2015). Our results confirm and extend these observations in crayfish by showing that 5-HT is also able to decrease the aggressiveness of a larger opponent by affecting its evaluation of a smaller rival. Thus, 5-HT is probably able to exert a fine-tuning on the perception of risks to be taken in response to danger.

Considering the decision to retreat, 5-HT had a similar delaying effect in both smaller and larger crayfish, increasing the duration of the fight period. However, this delay appeared to be differently driven by aggressiveness: the delay in the decision to retreat was clearly due to the 5-HT-induced increase in aggressiveness of the smaller injected animals, as previously observed (Huber et al., 1997); by contrast, in larger injected animals, it was due to the 5-HT-induced decrease in aggressiveness, which postponed the flight of their smaller rivals. Importantly, the reduction by 5-HT of aggressiveness in larger animals was not associated with a rapid withdrawal, suggesting that their lower fighting motivation was not due to increased fear. Their reduced aggressiveness was also not due to a reduced locomotive capacity because they were able to fight normally when attacked by their smaller rival. Consequently, the percentage of lost bouts was not significantly increased after 5-HT injection. Conversely, the percentage of wins was not increased in smaller injected crayfish, as previously observed in another study (Tricarico and Gherardi, 2007). In all cases, 5-HT was able to postpone decision making without significantly inverting the outcome of the bouts.

Because of these opposite effects on aggressive behavior in larger and smaller animals as well as the complementary effects on decision making, it can be argued that 5-HT does not act uniformly and directly on aggressiveness. Rather, 5-HT probably modulates neural activities, including risk assessment and decision making, which indirectly influence aggressiveness. This modulation essentially comprises the perception of the size and strength of an opponent, but other inputs could also contribute to the agonistic



**Fig. 3. Serotonin modifies the outcome of fights.** (Ai) 5-HT injection into smaller crayfish increased the duration of the fight period ( $835 \pm 152$  s for low 5-HT and  $1126 \pm 74$  s for high 5-HT groups versus  $458 \pm 110$  s for control and  $608 \pm 81$  s for saline-injected groups; significant difference between saline and high 5-HT groups,  $**P < 0.01$ , Dunn's multiple comparison test). (Aii) 5-HT injection into larger crayfish increased the duration of the fight period ( $1110 \pm 48$  s for low 5-HT and  $1003 \pm 79$  s for high 5-HT groups versus  $458 \pm 110$  s for control and  $542 \pm 111$  s for saline-injected groups; significant difference between saline and both 5-HT groups,  $**P < 0.01$ , Dunn's multiple comparison test). (Bi) 5-HT injection into smaller crayfish reduced the number of fights lost (5/8 and 1/8 for low and high 5-HT groups, respectively, versus 10/13 and 17/25 for control and saline-injected groups; significant difference between saline and high 5-HT groups,  $P < 0.05$ , Fisher's exact test). (Bii) 5-HT injection into larger crayfish reduced the number of fights won (4/11 and 3/14 for low and high 5-HT groups, respectively, versus 10/13 and 9/13 for control and saline groups; significant difference between saline and high 5-HT groups,  $P < 0.05$ , Fisher's exact test). (Ci) Separation of the variables analyzed in the principal component analysis (PCA). The first component (horizontal) was mainly determined by aggressive acts and the second component by the time to retreat, whereas the other component (vertical axis) was interpreted as mainly due to the time to retreat. (Cii) Position of experimental groups. Note that the saline-injected larger crayfish are placed on the right of the PCA diagram, corresponding to higher aggressiveness. (Ciii) Injection of low and high doses of 5-HT shifted the larger crayfish group to the left, indicating a decrease in aggressiveness (saline and low and high 5-HT groups were significantly different,  $***P < 0.001$ ). (Civ) Injection of 5-HT into smaller crayfish shifted the groups to the right, indicating increased aggressiveness, and to the bottom, indicating increased time to retreat (saline and high 5-HT groups were significantly different,  $***P < 0.001$ ).

context perceived by both antagonists. Indeed, it is well known that chemical cues can modulate aggressiveness in crustaceans (Breithaupt and Eger, 2002; Shabani et al., 2009) as well as other invertebrate species (Hoopfer, 2016), and 5-HT is involved in the winner/loser effects that modulate aggressiveness (Momohara et al., 2013).

Altogether, 5-HT probably does not act directly and uniformly on a single center that controls aggressiveness in crayfish but rather plays a role in the modulation of a variety of cognitive events, including previous social experiences, the perception of a new agonistic context, risk evaluation and decision making, that then modulate aggressiveness. This rapid modulation of aggressiveness may be complementary to long-lasting modulation of aggressive behavior by 5-HT, which has also been observed at the level of the motor neurons, implying long-term changes in 5-HT receptors (Teshiba et al., 2001; Yeh et al., 1996; Cattaert et al., 2010).

In conclusion, our paradoxical results may reconcile the views of a positive or negative role of 5-HT in aggressiveness. The two roles may co-exist in crayfish, as well as in other animals, and be expressed depending on the evaluation of risk in an agonistic context.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: D.C., J.P.D., P.F.; Methodology: J.B.-C., J.P.D., P.F.; Validation: P.F.; Formal analysis: D.C., P.F.; Investigation: J.B.-C.; Writing - original draft: J.B.-C., J.P.D., P.F.; Writing - review & editing: D.C.

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