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RESEARCH ARTICLE

Discrete modulation of anti-predatory and agonistic behaviors by sensory communication signals in juvenile crayfish

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ABSTRACT

We investigated how the exchange of sensory signals modulates the individual behaviors of juvenile crayfish in an anti-predatory context as well as during intraspecific agonistic encounters. We first compared crayfish housed in total sensory isolation or in pairs with access to chemical and visual cues. After 1 week of housing, we analysed their individual responses to a visual danger signal while they were foraging. We found that crayfish previously housed in pairs with exchange of sensory signals responded to a simulated predator attack predominantly with freezing behavior, whereas animals deprived of all sensory communication mostly responded by performing escape tail-flips. Next, we used the same housing conditions in between repeated fights in pairs of crayfish. Aggressive and submissive behaviors increased in subsequent fights both after total isolation and after exchange of olfactory and visual signals. Thus, unlike responses to simulated predator attacks, intraspecific agonistic behavior was not modulated by exposure to the same sensory signals. However, when we tested the effects of olfactory or visual communication independently, aggression increased dramatically after the exchange of olfactory signals, which also led to a high number of rank reversals in second fights, suggesting a destabilization of the original dominance relationship. Exposure to visual cues during the 1-week separation, however, produced the opposite effect, reducing agonistic behaviors and rank reversals. These findings demonstrate that exchange of sensory signals modulates future anti-predatory decision-making and intraspecific agonistic behaviors discretely, suggesting that the effect of these signals on shared neural circuitry is context dependent.

KEY WORDS: Aggression, Olfaction, Decision making, Isolation, Vision

INTRODUCTION

Successfully escaping a predator attack is crucial for survival, and the most adaptive decisions integrate the costs and benefits of different behavioral choices (e.g. Lima and Dill, 1990). Animals searching for food should favor options that keep them close to the food if the food quality or their nutritional needs are high (Kavaliers and Choleris, 2001; Evans et al., 2019). We have previously shown that juvenile crayfish that foraged towards a food odor release point in a laboratory setting produced binary, incompatible behavioral choices when facing a simulated predator attack. When exposed to a fast-approaching shadow, animals either froze in place, which kept them close to the expected food reward, or they activated their medial giant (MG) tail-flip escape circuit that propelled them backwards and away from the approaching shadow as well as the food reward (Liden and Herberholz, 2008). If the perceived food quality was high or the animals were hungry, most of the animals defaulted to freezing, whereas lower food value and satiated animals predominantly exhibited tail-flipping behavior (Schadegg and Herberholz, 2017). Thus, crayfish are able to make 'economic' decisions that calculate the costs and benefits of different behavioral options, and select the most adaptive choices according to internal and external conditions (Liden et al., 2010). How these anti-predatory decisions are affected by prior exposure to sensory communication signals has not been determined.

Here we tested how access to chemical and visual signals from a conspecific affected subsequent decision making in response to predatory threat when compared with complete sensory deprivation. We hypothesized that the exchange of sensory information during 1 week of physical separation would allow crayfish to recognize the presence of another crayfish. Thus, we predicted that crayfish would primarily freeze in response to a predator signal as the expected competition over food would increase the value of staying close to the expected food reward, despite the higher risk of being preyed upon. We found evidence that supported our hypothesis because crayfish that had access to visual and olfactory signals displayed freezing in response to the predator signal much more frequently than animals that were deprived of sensory signals, which predominantly tail-flipped away from the danger signal.

Dominance hierarchies are present in all social animals, including crayfish. Aggressive behaviors are used to establish dominance, and the most dominant animals gain first access to valuable resources (Issa et al., 1999; Edwards and Herberholz, 2005; Herberholz et al., 2007). Visual, tactile and olfactory sensory cues have been shown to play important roles in the formation and maintenance of social dominance hierarchies across species ranging from insects to primates (e.g. Drickamer, 2001; Pryke et al., 2001; Ghazanfar and Santos, 2004; Tibbetts and Dale, 2004; Green and Patek, 2015).

Extensive literature on this topic exists for crustaceans, including crayfish. Callaghan et al. (2012) looked at pairs and triads of crayfish (*Orconectes rusticus*) with impaired vision, smell, touch, and combinations of those impairments. They concluded that olfaction was the most important sensory modality for communicating social status; vision and touch were able to modulate fights primarily by reducing risk-taking, but olfaction alone was sufficient for establishing dominant–subordinate relationships. A series of experiments in a different species of crayfish (*Procambarus clarkii*) measured the effects of vision, olfaction and touch on agonistic behaviors (Delgado-Morales et al., 2004). The study found that ablating vision or smell delayed the formation of dominance hierarchies, while touch was less important. Once established, only one sense (smell or vision) was needed to

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maintain the existing hierarchy. Olfactory signals contained in the urine have been shown to affect the outcome of fights in blindfolded crayfish, but these signals were less frequently exchanged and thus of lower importance for the maintenance of established dominance relationships. Urine release was tightly linked to aggressive behaviors, and losers of fights reduced signaling after subordinate status had been obtained (Breithaupt and Eger, 2002).

Although these studies emphasize the importance of olfactory signals (and to a lesser degree also visual signals) as mediators of hierarchy formation rather than maintenance, other studies have shown a significant role of olfaction in modulating agonistic behaviors and maintaining the stability of established dominance relationships. Atema and Karavanich (1998) showed that in pairs of American lobsters with the main olfactory receptors removed, the duration of subsequent fights (i.e. after 48 h) was much higher than in controls with intact olfactory systems. A similar result was obtained when urine release was blocked in otherwise intact lobsters, suggesting that olfactory signals were required to recognize the status of the familiar opponents. In *O. rusticus*, blocking urine release also increased fight duration compared with control fights, both in initial fights and in subsequent encounters with both familiar and unfamiliar opponents (Zulandt Schneider et al., 2001). Ablation of olfactory receptors in *P. clarkii* also increased the duration of fights in subsequent pairings compared with controls, while established dominance relationships remained mostly stable (Horner et al., 2008). In addition, it has been shown that the same crayfish recognize odors released into the water by both familiar and unfamiliar dominants, suggesting that they use smell to recognize the social status of a conspecific (Schneider et al., 1999). Interestingly, the 'social memory' of an opponent appears to be limited to 1 week in both lobsters and crayfish. If familiar pairs are reunited for a second fight 1 week after individual isolation, their agonistic behaviors are similar to the first fight, indicating that recognition of the familiar opponent has been lost during the isolation period (Karavanich and Atema, 1998; Zulandt Schneider et al., 2001). However, memory about past encounters can last longer than 1 week in some crayfish species (e.g. *Cherax destructor*; Hemsworth et al., 2007).

Taken together, the literature suggests that among the sensory modalities tested, olfaction seems to play the most important role in shaping agonistic behaviors in crayfish (and closely related species); it affects both the formation of dominant–subordinate relationships and the maintenance of established hierarchies. While generally considered somewhat less important, vision contributes to hierarchy formation in crustaceans and might also be used for recognition of opponents (Van der Velden et al., 2008; Gherardi et al., 2010; Bruce et al., 2018).

Based on these previous findings, we hypothesized that established dominance relationships would be maintained by the exchange of visual and olfactory signals, and agonistic behaviors would be reduced in repeated fights. However, our results did not support the hypothesis. Instead, we found that access to a combination of visual and olfactory signals increased agonistic behaviors in second fights similar to that observed in isolated animals. Surprisingly, when we separated access to chemical and visual signals, we observed that olfaction alone increased agonistic behaviors substantially and destabilized dominance relationships in subsequent fights, whereas vision produced the opposite effect. The implications of these results are discussed.

MATERIALS AND METHODS

Juvenile crayfish, *Procambarus clarkii* (Girard 1852), were obtained from a commercial supplier (Atchafalaya Biological Supply, Lafourche, LA, USA) and housed in large aquaria (height 31 cm, length 76 cm, width 32 cm) in groups of 30–35 animals at controlled temperature (23°C) and light:dark cycle (12 h:12 h). Animals were fed *ad libitum* twice weekly. Individuals were removed from communal tanks, sized and sexed, and inspected for intactness and molt status. Only animals of good physical condition and at inter-molt stages were used in experiments. Two animals were placed together in a small two-compartment isolation tank (height 10 cm, length 15 cm, width 8 cm). Each member of the pair was marked with one or two small dots on the carapace using a non-toxic silver marker pen. The isolation tank was painted white on the outside with non-toxic acrylic paint, contained dividers according to the conditions described below, and was filled to three-quarters of its height with water. On the day of isolation, each animal received a single medium-sized shrimp-based protein pellet (Formula One Pellets; Ocean Nutrition, Newark, CA, USA) and was not fed any other food until completion of the experiment.

Anti-predator behavior

Two isolation protocols were used: (1) total isolation (TI): pairs were housed in tanks that contained two compartments divided by a white barrier to ensure that no physical, visual or chemical signals could be exchanged between the two animals; and (2) chemo-visual (CV): the two compartments were divided by a clear transparent divider with small holes allowing both visual and chemical signals to be exchanged between the two conspecifics.

We tested the effectiveness of tanks designed for complete isolation (TI) by adding a few drops of food coloring (McCormick) to one side. Only the tanks where no food coloring crossed over to the other side were used. When using transparent dividers with small holes (CV), the same test was applied. Only tanks where food coloring moved quickly through the holes to the other side and distributed equally on both sides of the tank after a few minutes were used.

One week after the start of isolation, the animals were individually tested in an apparatus that allowed us to quantify their responses to a visual danger signal. The procedures used for predator simulation followed previously published protocols (Liden and Herberholz, 2008; Liden et al., 2010; Schadegg and Herberholz, 2017). In short, an experimental tank (height 21 cm, length 31 cm, width 17 cm) was filled with distilled water and bath recording electrodes made from copper wire were attached to the sides of a narrow tunnel in the middle of the tank. The beam of a goose-neck illuminator was directed towards the long side of the tank, which was covered with white translucent paper. A shadow propagating towards the animal was created by moving a rectangular piece of opaque plastic (18 cm×9 cm) on a single-axis linear stepper forcer (model STPM-SL-05-36-R; Optimal Engineering Systems, Van Nuys, CA, USA) through the light beam. The brightness inside the tank was monitored using a lux light meter (SM 700; Milwaukee Instruments, Rocky Mount, NC, USA) and was reduced by ∼95% when the shadow fully covered the tank. A reservoir that distributed the food odor to the tank was filled with solution made from 1.0 g of medium-sized shrimp pellets (Formula One; Ocean Nutrition), filtering the extract dissolved in 1 liter of distilled water, and further dilution (200 ml of stock solution in 4800 ml of distilled water). A tube dispensing the food odor mixture was attached to the end of the tunnel opposite the starting chamber. The flow of food odor into the tank (190 ml min^{-1}) was regulated by a flow meter (Cole-Parmer Instrument Company, St Neots, UK). Photodiodes were attached to the outside of the apparatus to measure the first appearance of the shadow at the side of the tank opposite the starting chamber, as well

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as at the location of the bath electrodes. Signals from both the photodiodes and bath electrodes were connected to an extracellular amplifier (A-M Systems, Sequim, WA, USA), filtered and digitized (Molecular Devices, San Jose, CA, USA). Recordings were stored on a computer with specialized software (Axoscope; Molecular Devices) for later analysis.

Animals were placed in the starting chamber of the tank near the entrance to the tunnel. After 10 min of acclimation, the video camera was turned on and the food odor tube was opened to release the food mixture into the tunnel. After the door of the starting chamber was removed, the experimenter observed the behavior of the animal on a television monitor (Sony WEGA). When the crayfish entered the tunnel from the starting chamber and started foraging towards the food odor release point, recordings of the diodes and bath electrodes were started. Once the crayfish reached the bath electrodes (8.0 cm from the entrance point and 17.5 cm from the end of the tunnel), the shadow was triggered using the programmable stepper motor control system (Allegra-1-10; Optimal Engineering Systems) that moved the sail mounted onto the motorized rail through the light beam. The velocity of the shadow was fixed and kept constant at 2 m s^{-1} throughout all experiments.

A total of 114 juvenile crayfish of 3.0–3.6 cm in length (mean± s.d. 3.44±0.15 cm; size measured from rostrum to telson) were used for this part of the study. No animal was used more than once and each animal was only exposed to one shadow signal. Fifteen animals were later excluded because they did not meet experimental criteria (Liden et al., 2010); i.e. when the shadow was released, they were not moving, they were past the electrodes by more than 10 mm, or they had turned more than 45 deg inside the tunnel. This resulted in 99 animals for data analysis (50 for TI, and 49 for CV).

Agonistic behaviors

Animals were first isolated for 1 week in individual tanks to eliminate social histories related to previous fight experiences in the communal aquaria. After 1 week, the animals were transferred into new, equally sized tanks with a removable divider. After a 5 min acclimation period, the divider was lifted and the animals were allowed to interact for 30 min (‘Fight 1’). Behaviors were recorded with a digital video camera and stored. Next, animals were housed under different conditions for another week. In addition to the housing protocols described above for anti-predator behavior, two more conditions were studied: (1) visual (V): a clear transparent divider was used, which allowed visual signals to be exchanged, but prevented physical and chemical communication and (2) chemical (C): an opaque divider contained small holes to allow exchange of olfactory signals, but prevented physical and visual communication.

After 1 week of housing in one of these four conditions (TI, CV, C, V), animals were transferred back into the ‘fight tank’ for another 30 min of unrestricted agonistic interactions (‘Fight 2’).

Videos from Fight 1 and Fight 2 were analysed according to previously established protocols (Herberholz et al., 2003, 2007, 2016). This was done by a member of the laboratory who did not conduct the experiments and, to the best extent possible, was blind to the conditions of the animals. We counted the frequency of aggressive (attack and approach) and submissive (escape and retreat) behavioral acts for each animal. An ‘attack’ (AT) was defined as a swift movement towards the opponent with an elevated body posture and raised claws, while an ‘approach’ (AP) was defined as slower walking movements towards the other animal. An ‘escape’ (ES) was defined as a tail-flip, a rapid flexion of the abdomen that thrusts the animal away from its opponent, whereas a ‘retreat’ (RT) was defined as walking (and/or turning) away from the other animal. The dominance relationship between the two members of the pair was determined by calculating a dominance index (DI) for each animal (Graham and Herberholz, 2009). Because attacks are considered more intense than approaches, and escapes are considered more intense than retreats, these behaviors were multiplied by two {DI=(AP+2AT)/[(AP+2AT)+(RT+2ES)]×100}. The animal with the higher DI was identified as dominant, and the animal with the lower DI as subordinate. The stability of dominance relationships was determined by comparing DIs of both members of each pair for the first and second fights.

A total of 134 juvenile crayfish of 2.9–4.1 cm in length (3.29± 0.28 cm; size measured from rostrum to telson) were used in 67 different pairs in our study. Ten pairs (20 animals) were excluded from the final dataset because of experimental error, molting, or death of one or both animals during the testing period. This resulted in 57 complete pairs (114 animals) for data analysis (13–16 pairs in each of the four conditions).

Statistical analysis

All descriptive and analytical statistics were performed using IBM SPSS Statistics 23. All values are presented as means±s.e.m., except animal sizes, which are presented as means±s.d. Non-parametric statistical tests were used (due to non-normality of some data), and each type is identified in the text and figure legends. All files containing raw data collections and statistical results will be made available by the authors upon request.

RESULTS

Behavioral responses to shadow signals

We compared juvenile crayfish that were housed for 1 week in a shared tank in total isolation (TI; N=50; size 3.42±0.17 cm) with equally sized animals that were housed for 1 week in a shared tank with a divider that allowed the exchange of olfactory and visual signals (CV; N=49; size 3.43±0.15 cm). There was no significant difference in sizes between the two groups (Mann–Whitney U-test, P=0.827). To rule out other differences that could have affected the behavioral choices of the animals, we measured distances from the bath electrodes and orientations (angles) of the animals inside the tunnel of the tank when the shadow was released. We found no significant differences between the two groups for average distance from the bath electrodes (TI: 6.78±0.28 mm; CV: 6.06±0.28 mm; Mann–Whitney U-test, P=0.082) or average orientation angles (TI: 8.18±1.09 deg; CV: 8.78±1.08 deg; Mann–Whitney U-test, P=0.516). Next, we analysed differences within groups between animals that froze and animals that tail-flipped. The sizes of TI animals that froze (3.45±0.14 cm) and TI animals that tail-flipped (3.38±0.19 cm) was not significantly different (Mann–Whitney U-test, P=0.269). In addition, there were no differences in distance from the bath electrodes for TI animals that froze (7.28±0.41 mm) and TI animals that tail-flipped (6.28±0.38 mm; Mann–Whitney U-test, P=0.086), nor was there a difference in average orientation for TI animals that froze (8.04±1.82 deg) and TI animals that tail-flipped (8.32±1.25 deg; Mann–Whitney U-test, P=0.289). Similar results were obtained for CV animals that froze or tail-flipped. The size of CV animals that froze (3.43±0.16 cm) was no different from CV animals that tail-flipped (3.43±0.13 cm; Mann–Whitney U-test, P=0.294). We also found no differences in average distance from the bath electrodes for CV animals that froze (6.27±0.35 mm) and VC animals that tail-flipped (5.42±0.26 mm; Mann–Whitney U-test, P=0.257), and no significant difference in average orientation for CV animals that froze (9.65±1.35 deg) and VC animals that tail-flipped (6.08±1.16 deg; Mann–Whitney U-test, P=0.278).

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As shown in Fig. 1, TI animals produced an equal number of tail-flips (50%) and freezes (50%) when responding to the predatory threat. CV animals, on the other hand, produced fewer tail-flips (24%) and more freezes (75%). The difference between the two groups housed with or without exchange of sensory signals was statistically significant (Fisher's exact χ^2 test, P=0.012).

We also found that in both groups, crayfish that froze in response to the shadow reached the food odor release point at the end of the tunnel earlier than animals that tail-flipped, confirming prior research using a similar design (Liden et al., 2010; Schadegg and Herberholz, 2017), and supporting the notion that a quantifiable cost is associated with tail-flipping. TI animals that froze reached the expected food reward after 201.68±27.03 s, whereas animals that tail-flipped needed 300.72±37.25 s to reach this point in the tank (Mann–Whitney U-test, P=0.023). Similarly, CV animals that froze reached the expected food reward after 161.51±17.86 s, whereas animals that tail-flipped needed 354.33±57.10 s to reach this point in the tank (Mann–Whitney U-test, P=0.001).

Interestingly, as shown in Fig. 2, we also found that CV animals that froze in response to the shadow entered the tank more quickly (90.62±17.00 s) than CV animals that tail-flipped (143.92±30.54 s), a significant difference (Mann–Whitney U-test, P=0.047), which was not observed between TI animals that froze (102.76±17.62 s) and TI animals that tail-flipped (124.48±22.97 s; Mann–Whitney U-test, P=0.560). CV animals that froze also moved more quickly towards the food odor release point compared with animals that tail-flipped as documented by a significantly shorter time period between entering the tunnel in the tank and reaching the bath electrodes, a distance of 8 cm, where the shadow was perceived (23.49±1.63 versus 36.50±4.69 s; Mann–Whitney U-test, P=0.008). In TI animals, no significant difference (Mann–Whitney U-test, P=0.236) was observed between animals that froze (37.48±10.12 s) and animals that tail-flipped (40.12±7.94 s).

We also measured response latencies (i.e. how quickly the animals tail-flipped after the shadow became visible) for TI and CV animals using the signal of the bath electrodes (see Materials and Methods). TI animals and CV animals initiated their escapes with very similar latencies (TI: 72.89±2.36 ms; CV: 70.79±1.98 ms), and the small difference was not significant (Mann–Whitney U-test, P=0.987). As shadows were always advanced at a constant velocity of 2 m s^{-1}, the danger signals reached the bath electrodes (and approximate location of the animals) 87.5 ms after they first appeared. Thus, both TI and CV animals activated escape tail-flips, on average, before head-on 'collision' with the shadow occurred.

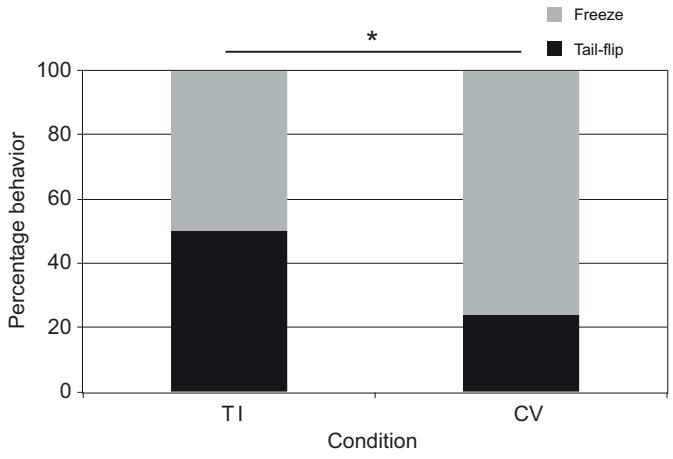

Fig. 1. Ratios of freezing and tail-flipping in response to the visual danger signal in *Procambarus clarkii*. Animals kept in total isolation (TI; N=50) for 1 week produced significantly more tail-flips and fewer freezes than animals housed with exchange of chemical and visual cues (CV; N=49) which exhibited fewer tail-flips and more freezes. Fisher's exact χ^2 test: *P≤0.05.

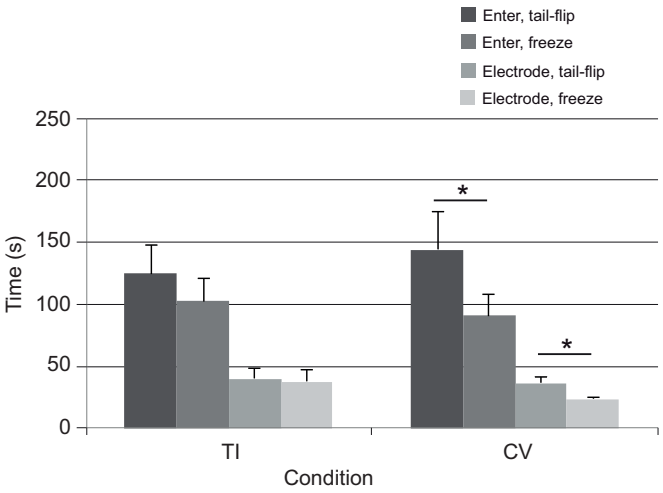

Fig. 2. Comparison of 'time to enter' and 'time to reach the bath electrodes' (after entering) between 50 animals kept in total isolation (TI) and 49 animals with exchange of chemical and visual cues (CV). CV animals that tail-flipped in response to the danger signal (N=12) took significantly longer to enter the tunnel and walked significantly more slowly after entering the tunnel leading to the food odor release point compared with CV animals that froze (N=37). No significant differences were found for TI animals that tail-flipped (N=25) or froze (N=25). Mann–Whitney U-test: *P≤0.05.

Taken together, these results suggest that animals that had access to chemical and visual signals from a conspecific during 1 week of shared housing (CV) predominantly selected freezing, the riskier behavioral response to the shadow, which kept them closer to the expected food reward. Moreover, among these animals, those that responded by freezing to the predatory threat signal were more motivated to approach the anticipated food reward compared with those that tail-flipped. Animals that were isolated from other conspecifics during housing (TI), however, produced an equal number of freezes and tail-flips in response to the shadow and displayed no difference in motivation to approach the food. Lastly, we found no difference for escape latencies between the TI and CV animals that tail-flipped.

Agonistic behaviors in repeated fights

We first identified all aggressive and submissive behaviors that occurred in Fight 1 and Fight 2 of TI and CV groups (Fig. 3). However, as we did not discover any significant differences between these two groups (see below), we added two more groups: one where *only* visual signals (V) could be exchanged during the 1-week separation period, and one where *only* chemical (C) signals could be exchanged.

In the TI group (16 pairs, N=32; size 3.29±0.26 cm), animals produced on average 33.4±9.3 aggressive acts and 30.4±9.4 submissive acts during Fight 1. The number of agonistic behaviors increased in Fight 2, where on average 40.1±20.6 aggressive acts and 38.1±17.3 submissive acts were displayed by the animals. The increase in aggressive behaviors between Fight 1 and Fight 2 was not significant (Wilcoxon signed-rank test, P=0.301), but it was for submissive behaviors (Wilcoxon signed-rank test, P=0.034).

In the CV group (14 pairs, N=28; size 3.30±0.27 cm), animals produced on average 39.0±22.0 aggressive acts and 42.1±26.5

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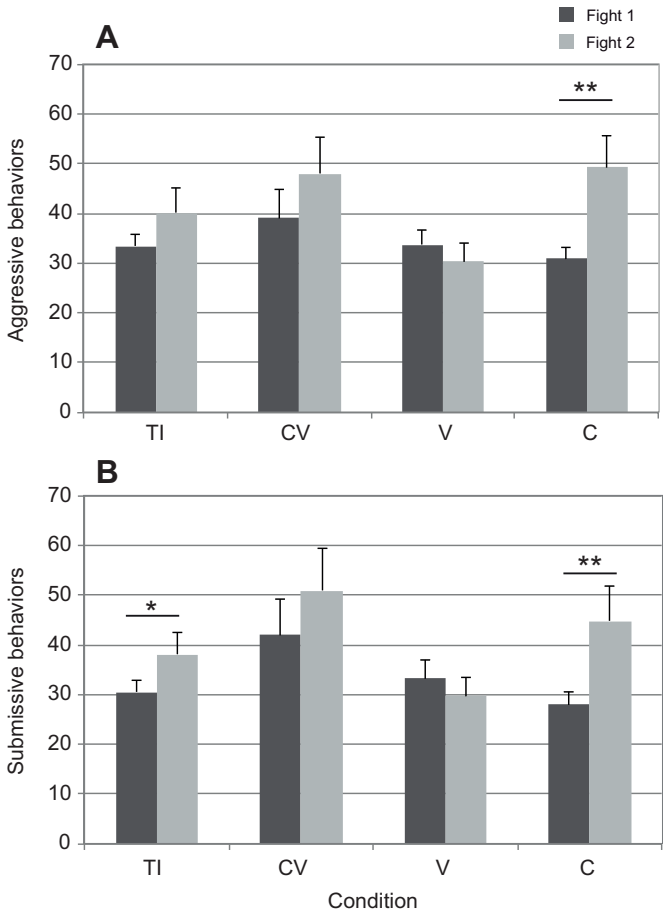

Fig. 3. Total number of aggressive (attacks and approaches) and submissive behaviors (escapes and retreats) displayed in first and second fights across all 57 tested groups. Pairs with exchange of chemical cues (C; *N*=14) produced significantly more aggressive behaviors (A) and significantly more submissive behaviors (B) during second fights compared with first fights. Pairs kept in total isolation (TI; *N*=16) produced significantly more submissive behaviors in second fights compared with first fights. No difference was observed for pairs with exchange of chemical and visual cues (CV; *N*=14) or visual cues alone (V; *N*=13). Wilcoxon signed-ranks test: **P*≤0.05, ***P*≤0.01.

submissive acts during Fight 1. As in the TI group, the number of agonistic behaviors increased in Fight 2. Animals produced on average 48.0±27.1 aggressive acts and 50.9±31.4 submissive acts during Fight 2. Neither the increase in aggressive behaviors (Wilcoxon signed-rank test, *P*=0.158) nor the increase for submissive behaviors (Wilcoxon signed-rank test, *P*=0.187) between Fight 1 and Fight 2 was significant.

In the V group (13 pairs, *N*=26; size 3.29±0.30 cm), animals produced on average 33.7±10.6 aggressive acts and 33.2±13.0 submissive acts during Fight 1. Contrary to the TI and CV groups, the number of agonistic behaviors decreased in Fight 2. Animals produced on average 30.3±13.5 aggressive acts and 29.7±13.8 submissive acts during Fight 2. The decrease in aggressive behaviors (Wilcoxon signed-rank test, *P*=0.350) and submissive behaviors (Wilcoxon signed-rank test, *P*=0.289) was not significant.

In the C group (14 pairs, *N*=28; size 3.31±0.33 cm), animals produced on average 30.9±8.1 aggressive acts and 27.9±9.9 submissive acts during Fight 1. The number of agonistic behaviors increased substantially in Fight 2. Animals produced on average 49.3±23.3 aggressive acts and 44.8±25.9 submissive acts during Fight 2. The increase in aggressive (Wilcoxon signed-rank test, *P*=0.009) and submissive behaviors was significant (Wilcoxon signed-rank test, *P*=0.019).

Next, we determined the change in average agonistic behaviors (i.e. combined aggressive and submissive acts) for all groups between Fight 1 and Fight 2 (Fig. 4). For the TI group, agonistic behaviors increased in Fight 2 (14.4±7.8). For CV animals, we found a similar increase in average agonistic behaviors (17.9±17.2). However, for animals of the V group, agonistic behaviors actually decreased in Fight 2 (−6.9±8.4), whereas they substantially increased for animals in the C group (35.3±11.5).

Statistical comparison of the changes in agonistic behaviors between Fight 1 and Fight 2 in all tested groups revealed no significant overall difference (Kruskal–Wallis test, *P*=0.059). However, as the test across all groups approached statistical significance, we followed up with a pairwise comparison using the Mann–Whitney *U*-test, which showed a significant difference between the V group and the C group (*P*=0.009), whereas no statistical significance was found for any other comparison (TI versus CV: *P*=0.580; TI versus V: *P*=0.075; TI versus C: *P*=0.166; CV versus V: *P*=0.094; CV versus C: *P*=0.603).

Dominance index and reversals

As a measure of the stability of dominance relationships, we calculated DI for both the original winner (dominant) and loser (subordinate) of Fight 1 (Fig. 5; see Materials and Methods for description of DI). We then determined DI for both animals again for Fight 2. An increase in DI of the original dominant and a decrease for the subordinate signifies stability of the social relationship, whereas opposite trends suggest destabilization (Graham and Herberholz, 2009). In addition, we measured rank reversals between the original dominant and subordinate in Fight 2 as another proxy for estimating the stability of the relationship. The two measures are related as more rank reversals will be reflected in higher DIs of former subordinates and lower DIs of former dominants.

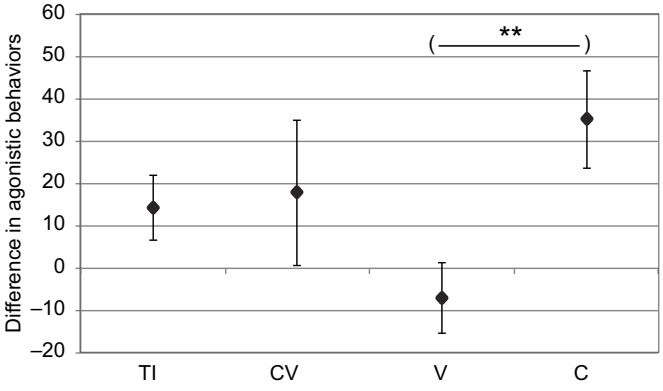

Fig. 4. Differences of all agonistic behaviors (aggressive and submissive) between first and second fights for all tested groups. Agonistic behaviors were reduced in second fights for pairs with exchange of visual cues (V; *N*=13), but were increased for pairs with exchange of chemical cues (C; *N*=14), a significant difference between these two groups. Agonistic behaviors increased slightly for pairs kept in total isolation (TI; *N*=16) and pairs with exchange of chemical and visual cues (CV; *N*=14), but they were not significantly different from the other tested groups. Pairwise comparison was performed after a Kruskal–Wallis test approached significance for all groups (*P*=0.059). Mann–Whitney *U*-test: ***P*≤0.01.

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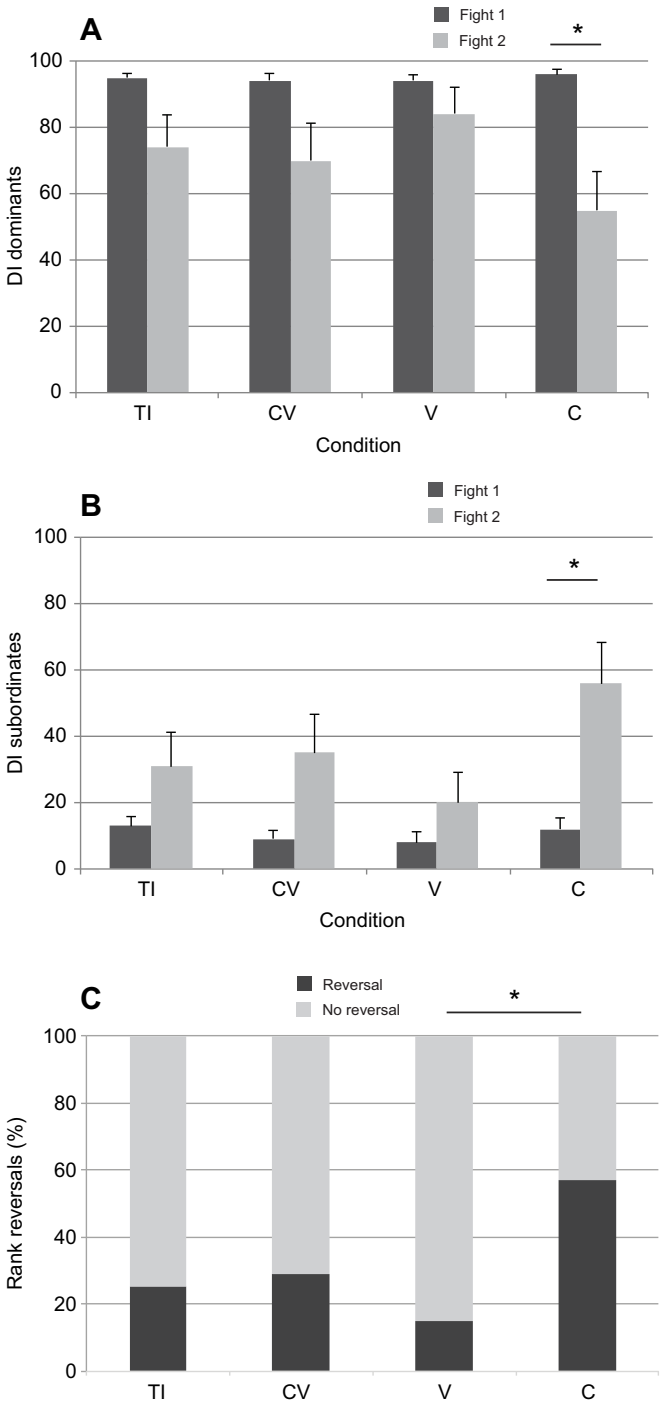

Fig. 5. Dominance indexes for dominants and subordinates in first and second fights, and percentage of rank reversals in second fights for all tested groups. (A) Dominants; (B) subordinates; (C) rank reversals. Dominance indexes (DI) of dominants were significantly lower and DIs of subordinate were significantly higher in second fights for animals with exchange of chemical cues (C; *N*=14). No significant changes were observed for animals that were kept in total isolation (TI; *N*=16), animals with exchange of chemical and visual cues (CV; *N*=14), or exchange of visual cues only (V; *N*=13). The percentage of rank reversals was lowest in pairs with exchange of visual cues (V; 2/13) and highest for pairs with exchange of chemical cues (C; 8/14), a significant difference between these two groups. Although rank reversals were also observed for pairs kept in total isolation (TI; 4/16) and for pairs with exchange of chemical and visual cues (CV; 4/14), they were not significantly different from the other tested groups. Wilcoxon signed-ranks test (A,B) and Fisher's exact χ^2 test (C): *$P \leq 0.05$.

For animals in the TI group, DIs for the dominant decreased between Fight 1 and Fight 2 (Fight 1, 95.1±1.3%; Fight 2, 73.9±9.7%) and increased for subordinates (Fight 1, 12.8±2.8%; Fight 2, 30.9±10.4%). Accordingly, during Fight 2, the original relationship between the dominant and subordinate of Fight 1 reversed in 25% of all pairs (4/16).

For animals in the CV group, DIs for the dominant decreased between Fight 1 and Fight 2 (Fight 1, 93.9±2.1%; Fight 2, 69.8±11.4%) and increased for subordinates (Fight 1, 8.9±2.8%; Fight 2, 34.6±11.5%). During Fight 2, the original dominance relationship between the winner and loser of Fight 1 in the VC group was reversed in 29% of all pairs (4/14).

For animals in the V group, DIs for the dominant slightly decreased between Fight 1 and Fight 2 (Fight 1, 94.4±1.9%; Fight 2, 84.0±8.1%) and increased for subordinates (Fight 1, 7.9±3.4%; Fight 2, 20.1±9.0%). During Fight 2, the original dominance relationship between the winner and loser of Fight 1 in the V group was reversed in only 15% of all pairs (2/13).

For animals in the C group, DIs for the dominant decreased substantially between Fight 1 and Fight 2 (Fight 1, 96.3±1.5%; Fight 2, 55.2±11.8%) and increased substantially for subordinates (Fight 1, 12.3±3.3%; Fight 2, 55.9±12.4%). Not surprisingly, during Fight 2, the original dominance relationship between the winner and loser of Fight 1 was reversed in 57% of all pairs (8/14).

Statistical analysis revealed that the DIs of dominants and subordinates in the C group differed significantly between the first and second fights (Fig. 5A,B). For dominants, the DI decreased (Wilcoxon signed-rank test, P=0.012) whereas it increased significantly for subordinates (Wilcoxon signed-rank test, P=0.026). None of the differences observed in all other groups was statistically significant (Wilcoxon signed-rank test: TI dominants, P=0.182; TI subordinates, P=0.679; CV dominants, P=0.480; CV subordinates, P=0.300; V dominants, P=0.484; V subordinates, P=0.583).

In addition, the number of status reversals between original dominants and subordinates in Fight 2 was significantly higher in the C group compared with the V group [Fisher's exact test (two-sided), P=0.046], but not different for any of the other groups [Fisher's exact test (two-sided): TI versus CV, P=1.000; TI versus V, P=0.663; TI versus C, P=0.135; CV versus V, P=0.648; CV versus C, P=0.252; Fig. 5C].

In summary, allowing the exchange of olfactory signals between two juvenile crayfish during 1 week of physical separation increased agonistic behaviors in a subsequent fight and led to a large number of rank reversals, i.e. more than half of the original dominants became subordinates (and vice versa) in their second fights. The smallest effect was observed in animals that exchanged only visual signals between fights, both in terms of agonistic behaviors and in terms of dominance stability. Interestingly, there was no difference between TI and VC animals, contrary to what was observed in our first experiment that measured decision-making.

DISCUSSION

Behavioral responses to shadow signals

We found that access to sensory communication signals modulated decision-making and behavioral choices in crayfish. Animals that were exposed to chemical and visual cues from a single conspecific responded to predatory threat mostly with freezing, whereas animals that were kept in total isolation during the same time period used tail-flip escapes more frequently.

Using a similar experimental design, we have previously shown that crayfish calculate the costs and benefits of different behavioral

choices and select the most desirable option. This decision-making process is affected by both external and internal conditions. For example, if the velocity of the predator signal (approaching shadow) renders successful tail-flip escape impossible, crayfish tend to freeze (Liden and Herberholz, 2008). If the quality of the food increases, crayfish also default to freezing, presumably due to an increased value of this behavioral option despite this being the riskier choice (Liden et al., 2010). This is further supported by the finding that hungry crayfish predominantly freeze, whereas satiated animals tail-flip away from the approaching danger signal (Schadegg and Herberholz, 2017). We have also shown that freezing and escape have distinct advantages and disadvantages; tail-flip escape moves animals further away from the food, a limited resource, but if executed quickly enough it also moves the animal away from the approaching attack, which might be the safer choice (Liden and Herberholz, 2008).

Our current data provide important new insight into this process. For the first time, we have now documented that exposure to sensory signals affects future anti-predatory decision-making. One possible explanation for the observed shift towards freezing in animals that had access to signals from a conspecific is a change in internal state related to the expected competition over food. Thus, by recognizing the presence of a potential competitor, freezing (i.e. staying close to the food) becomes the more valuable option. This is further supported by our finding that animals that had access to sensory signals from the conspecifics entered the tank more quickly and moved more quickly towards the food odor release point, especially when the eventual decision was to freeze. This suggests a change in motivation related to foraging activity based on the perception of conspecific signals during the prior week.

It is quite remarkable that exchange of sensory signals alone, and in the absence of any physical interactions, was able to produce these effects. However, prior work has shown that adult crayfish (*O. rusticus*) exposed to the odors of dominant or subordinate crayfish for 5 days behaved analogous to subordinates or dominants, respectively, in subsequent fights against isolated crayfish (Bergman and Moore, 2005). More recently, Wee et al. (2020 preprint) showed that exposure to conspecific odors, especially from siblings, reduced defensive behaviors to aversive stimuli in isolated zebrafish. In addition, physiological changes (i.e. increases in heart and ventilation rates) have been observed in crayfish dyads as a response to visual signals before the opponents engaged in fighting (Listerman et al., 2000; Schapker et al., 2002), and olfactory cues from a conspecific were able to increase heart rate for an extended period of time in blind cave crayfish (Li et al., 2000). Thus, it is reasonable to suggest that a combination of visual and olfactory signals is capable of modulating the intrinsic state, motivation and future behavior of the receiver.

Another recent paper reported that zebrafish larvae that were reared in isolation produced stronger avoidance responses compared with group-reared conspecifics (Groneberg et al., 2020 preprint). This is in line with a large body of literature showing that social isolation (both chronic and acute) increases sensitivity to threat (e.g. increased predator evasion) in a number of species, including humans (e.g. Cacioppo et al., 2011). Thus, an intriguing alternative explanation for our finding is that the change took place in the isolated animal rather than the one with access to sensory signals. One avenue for future experiments would therefore include testing animals of different satiation levels. If crayfish are fed to satiation before testing their response to a danger signal, and the observed behavioral differences (freezing versus tail-flipping) are maintained between isolated and communicating animals, these differences are likely to be based on isolation rather than competition over food. Moreover, given the accessibility of the crayfish nervous system, and the large number of identified neurons, future experiments could be aimed at uncovering the interplay between sensory signals and social experience, and its consequences for neurobehavioral function (see below).

Agonistic behaviors

Our second project investigated the effects of sensory communication signals on agonistic behaviors. Agonistic behaviors are a combined measure of aggressive and submissive behaviors, which are interlinked. For example, an attack will likely evoke an escape, an approach will likely elicit retreat, and accordingly, we did find these behaviors to be firmly correlated across the tested conditions.

The intensity of agonistic interactions increased slightly in second fights after both chemical and visual cues were accessible during the 1-week separation period. A similar increase in aggression was found for animals that were deprived of all sensory communication between fights. This is in contrast to the results from our first study on anti-predatory responses, suggesting that the effects of communication signals did not generalize across behaviors. Compared with sensory deprivation, access to olfactory and visual signals from a conspecific modified anti-predator behavior, whereas intraspecific aggression was mostly unaffected. However, when we separated olfactory and visual signals, we found that they modified agonistic behaviors in opposite directions.

Existing literature led us to predict that exchange of olfactory signals would be likely to strengthen existing dominance relationships. The release of chemical cues, most likely contained in the urine, has been shown to provide information about an opponent's dominance status and to reduce aggression in subordinate receivers when paired with familiar or unfamiliar opponents (Schneider et al., 1999; Breithaupt and Eger, 2002). Moreover, blocking chemical signaling increased the intensity and duration of both initial and repeated fights in crayfish (Zulandt Schneider et al., 2001), while blocking olfactory receptors disrupted established dominance relationships and eliminated winner effects (Daws et al., 2003; Moore and Bergman, 2005; Horner et al., 2008). Lastly, crayfish exposed to only dominant odors for several days displayed subordinate status, whereas animals exposed to subordinate odors behaved like dominants in fights with socially naïve opponents (Bergman and Moore, 2005).

We therefore expected that the smell of another animal during the 1-week separation period would keep the original dominance relationship intact and result in less overall aggression when the two combatants met again. There are a few possibilities why this did not happen. First, most of the previous experiments were done in adult crayfish and often involved ablation of olfactory receptors or other manipulations (e.g. blocking urine release) during first and/or second fights. In our experiments, juvenile crayfish were exposed to olfactory signals between fights, and no other manipulations occurred. Second, during the time when signals were exchanged, animals were not able to physically interact with each other. Thus, they may have recognized that a prior opponent remained nearby, but they were unable to physically respond and reinforce their ranks. Third, animals in our experiments always encountered the same opponents in second fights after the separation period.

Although we did not measure urine release in our experiments, it is possible that communicating social status without physical reinforcement may have produced this result. Clearly, animals did not simply stop signaling when they were separated for 1 week with the perforated divider because the observed behavioral changes

were significantly different from complete sensory isolation. In addition, the change in dominance index and the high number of rank reversals after chemical cues were exchanged further suggest that the subordinates from the first fight were motivated to challenge the original dominants during the second encounter, and they were often successful in defeating them. Future experiments could be aimed at identifying the urine signaling pattern of the original dominant and subordinate during the period of separation to gain a better understanding of the mechanisms that led to disruption of established dominance relationships. Similar destabilization of existing dominance relationships has previously been reported in crayfish when a larger intruder crayfish was temporarily added to a dominant and subordinate; however, the roles of sensory signals in this process have not been identified (Graham and Herberholz, 2009; Herberholz et al., 2016).

We also found that exchange of visual signals slightly reduced agonistic behaviors in second fights compared with first fights and produced the smallest number of rank reversals, thus creating the opposite outcome compared with chemical communication. Together, with the result for olfactory signals, this might suggest that visual cues *without* chemical cues are able to stabilize dominance relationships. Bruski and Dunham (1987) reported that crayfish (*O. rusticus*) produced fewer agonistic interactions in dim light and in darkness, suggesting that exchange of visual signals reduces the intensity of fights. In American lobsters, it has previously been demonstrated that intact vision reduced aggression during second fights between familiar opponents, while blocking vision eliminated this effect (Bruce et al., 2018). Thus, exchange of visual signals has been shown to decrease agonistic behaviors in encounters of different crustaceans, and these findings are in line with our observation. It is interesting to note that crayfish are responsive to their own reflection when facing a mirror or the walls of a glass aquarium, and the type and intensity of the responses depend on social status (Drozdz et al., 2006; May and Mercier, 2007). As we used a transparent plastic divider in our experiments, it is less likely, but not impossible, that the reflection of the animals' own images might have contributed to the observed effects.

However, it is important to reiterate that in our experiments the exchange of visual signals (and all other sensory signals) occurred only during the 1 week between two fights, and none of the sensory modalities was impaired during agonistic interactions. Thus, similar to our anti-predator experiment, the animals must have experienced modifications to their internal states as a result of the signal exchanges, which then shaped future behavior.

'Bystander effects' and 'eavesdropping' have been reported before in a number of different species, including crayfish (e.g. Earley and Dugatkin, 2002; Grosenick et al., 2007; Wascher et al., 2008; Milner et al., 2010; Clark et al., 2012). For example, Aquiloni et al. (2008) and Aquiloni and Gherardi (2010) found that female crayfish (*P. clarkii*) would predominantly approach a dominant male after they observed two males establish a dominance relationship. Access to both visual and olfactory signals were important for eliciting the discrete behavior in the female when tested in a two-way choice design (with dominant and subordinate males representing these choices). In addition, Zulandt et al. (2008) found that crayfish (*O. rusticus*) that visually observed fights of conspecifics were more likely to be defeated when paired with a naïve opponent afterwards, whereas simple observation of two conspecifics that did not interact was ineffective. Although our study was not designed to measure bystander effects, these findings support the general notion that perception of sensory signals, or lack thereof, produces changes to the neural circuitry that controls behaviors, even if these behaviors are expressed at a later time. It would be interesting to investigate in the future how much time is required in the different housing conditions to produce these effects.

Allowing exchange of sensory signals between two crayfish might represent a more 'natural' social environment than total isolation, and similar to our shadow experiments, we cannot rule out the possibility that the observed changes are partially based on isolation. Our finding that agonistic behaviors (i.e. submissive acts) increased in second fights after complete isolation is not surprising as this has been reported before in various animal species (e.g. Twenge et al., 2001; Miczek et al., 2002; Mumtaz et al., 2018; Agrawal et al., 2020). This increase could be related to the enhanced threat sensitivity produced by social isolation. It is well known that acute and chronic social isolation has profound effects on brain structure and function by inducing changes to several neurotransmitter systems, including serotonergic, dopaminergic and oxytocinergic circuits (Heidbreder et al., 2000; Kiser et al., 2012; Matthews et al., 2016; Wee et al., 2020), a process that is probably linked to stress (e.g. Hall, 1998; Westenbroek et al., 2004; Cacioppo, 2015; Mumtaz et al., 2018). There is mounting evidence that this applies to crayfish as well: agonistic behaviors increased in fights between juvenile crayfish after isolation rearing (Patoka et al., 2019), and impoverished environments, which included social isolation, reduced neurogenesis in adults (Sandeman and Sandeman, 2000; Ayub et al., 2011), while the rate of neurogenesis and cell survival was shaped by different social experience in juveniles (Song et al., 2007). Previously, we have shown that social isolation affected the responses of juvenile crayfish to acute alcohol exposure. When we compared animals from communal housing with animals that were socially isolated for 1 week, we found lower sensitivity to ethanol in socially isolated crayfish, at both the behavioral and single neuron levels (Swierzbinski et al., 2017). This led us to suggest that social isolation shapes the nervous system by modifying the cellular targets for alcohol.

Interactions between sensory signals and neural circuits promoting escape

Although many of the activated neural circuits and corresponding behavioral actions differ between anti-predatory responses and agonistic behaviors, the medial giant (MG) circuit is involved in both. The MG interneuron generates all tail-flip escapes in response to approaching shadows in juvenile crayfish, and the same neuron is also engaged when escaping from natural predators as well as during intraspecific fights when escaping the attacks of an opponent (Herberholz et al., 2001, 2004; Liden et al., 2010).

In our current analysis of repeated fights, we did not measure MG neuron activation because it would have required us to obtain recordings with implanted electrodes or bath electrodes during all interactions (e.g. Herberholz et al., 2001). However, we can certainly speculate that the threshold of the MG neuron, which determines activation of defensive tail-flips during fights, was affected. Given the discrete roles of olfactory and visual signals on agonistic behaviors, it could be hypothesized that exchange of olfactory signals lowers the threshold for MG escape in dominants and increases the threshold in subordinates, whereas visual signals produce the opposite effect. Whether different sensory signals in fact change individual neuronal thresholds in such discrete ways remains to be tested.

In our experiments investigating responses to simulated predator attacks, the activity of the MG neuron and corresponding

frequencies of MG-mediated tail-flips changed. Socially isolated animals tail-flipped significantly more often than animals that exchanged olfactory and visual signals during the 1-week separation period. Although we did not observe differences in the timing of MG neuron activation between the two groups, the neuronal threshold was probably modulated by the presence and/or absence of sensory signals. We have previously shown that MG tail-flips were suppressed in foraging juvenile crayfish when the food odor was more concentrated or the animals were hungry (Liden et al., 2010; Schadegg and Herberholz, 2017). This suggested that both perception of food odor quality as well as the animal's internal state affected the excitability of the MG neuron. This opens up exciting possibilities for future investigation into the underlying neural mechanisms. The MG neuron is accessible for intracellular electrophysiology and neuropharmacological studies (Swierzbinski and Herberholz, 2018; Herberholz et al., 2019). One promising candidate for this investigation is the biogenic amine serotonin. For example, the excitability of the lateral giant interneuron, which controls an escape tail-flip in response to an attack to the rear in crayfish, is modulated by serotonin (Glanzman and Krasne, 1983; Teshiba et al., 2001), and this modulation is dependent on social experiences because it differs between dominant, subordinate and isolated animals (Yeh et al., 1996). However, whether serotonin shapes differences in forming and maintaining social status in freely behaving crayfish is less clear (Listerman et al., 2000; Panksepp and Huber, 2002). Nonetheless, recent literature suggests an interaction between serotonergic functioning and social deprivation in zebrafish (Tunbak et al., 2020), and this has also been reported in other studies ranging from invertebrates to vertebrates (Heidbreder et al., 2000; Muchimapura et al., 2003; Ouellet-Morin, 2013; Bubak et al., 2020). Another idea to explore is that changes in MG threshold are regulated through GABAergic tonic inhibition. The relevance of tonic inhibition in modulating neural excitability and behavior is well documented in crayfish (Vu and Krasne, 1993; Vu et al., 1993), and this is likely to include the MG circuit (Swierzbinski and Herberholz, 2018). Powerful interactions between social isolation and GABAergic function have been uncovered in rodents (e.g. Serra et al., 2007), and recent studies have shed light on the underlying importance of GABAergic inhibition for decisions that lead to either freezing or escape in response to danger stimuli (Tovote et al., 2016; Fadok et al., 2017; Zhou et al., 2019).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.C.E., J.H.; Methodology: A.C.E., J.H.; Validation: L.M.S., J.H.; Formal analysis: A.C.E., L.M.S., J.H.; Investigation: A.C.E., L.M.S.; Writing - original draft: A.C.E., J.H.; Writing - review & editing: A.C.E., L.M.S., J.H.; Supervision: J.H.; Project administration: J.H.; Funding acquisition: J.H.

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