

## Duration of socialization influences responses to a mirror: responses of dominant and subordinate crayfish diverge with time of pairing

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### Summary

Reflective surfaces have been shown previously to modify behaviour in socialized crayfish. Socializing crayfish by pairing them for two weeks established a hierarchy with one dominant and one subordinate crayfish per pair. Dominant crayfish exhibited specific behaviours, such as cornering, turning and crossing, more frequently in a reflective environment than in a non-reflective environment. After 2 weeks of pairing, subordinate crayfish did not respond in this manner but, instead, performed more reverse walking in a reflective environment. The present study investigated how the length of social pairing affects the response to mirrors. Crayfish from a communal tank were paired for 30 min or for 3 days, and their activity was videotaped for 20 min in a test aquarium lined with mirrors on one half and a non-

reflective matte lining on the other half. Crayfish housed in the communal tank were used as a comparison group. After 30 min of pairing, dominant and subordinate crayfish responded similarly to the reflection, showing essentially the same pattern for seven of nine behaviours examined. After 3 days of pairing, dominant crayfish continued to respond to the reflection in essentially the same way, but subordinate crayfish behaved differently, showing differences in seven out of nine behaviours. Thus, the pattern of responses of dominant and subordinate crayfish to reflection diverged with time of pairing.

**Key words:** crayfish, dominance, agonistic behaviour, mirror image, *Procambarus clarkii*.

### Introduction

Aggression exhibited by crayfish as well as other crustacean species has been well documented (Heckenlively, 1970; Edwards and Kravitz, 1997; Kravitz and Huber, 2003). When two crayfish are housed together, fighting between them begins almost immediately. Fights include pushing, lunging, grasping and striking (Bovbjerg, 1953; Bovbjerg, 1956; Hayes, 1975; Tierney et al., 2000). A fight will escalate until one member of the pair retreats and is deemed the loser of the encounter. After a crayfish wins the first fight, it is more likely to win successive fights, and the losing crayfish is less likely to win successive fights. After a number of losses, the retreating crayfish will alter its behaviour and begin to avoid the winning crayfish (Issa et al., 1999; Herberholz et al., 2001). It is often at this point that an observer deems the winning crayfish as dominant and the losing crayfish as subordinate. If more than two crayfish are housed together, a linear dominance hierarchy is established, which is stable over time (Lowe, 1956; Issa et al., 1999; Gherardi and Daniels, 2003).

Dominant and subordinate crayfish behave differently and have different physiological characteristics (Yeh et al., 1996; Yeh et al., 1997; Krasne et al., 1997; Edwards et al., 2003). Dominant crayfish gain first access to shelter, food and mates (Zulandt Schneider et al., 2001; Herberholz et al., 2003). Dominant crayfish frequently exhibit a threat display, raising

the body, extending the abdomen off the substrate and performing a meral spread, in response to a conspecific (Krasne et al., 1997; Listerman et al., 2000). By contrast, subordinate crayfish retreat, often *via* an escape tail flip, in response to certain stimuli such as the presence of an opponent (Huber et al., 1997; Krasne et al., 1997; Edwards et al., 2003). Dominant crayfish also approach a conspecific more frequently than do subordinate crayfish (Copp, 1986; Blank and Figler, 1996). When space is limited, burrowing, which creates a shelter, is important and dominant crayfish burrow significantly more than do subordinate crayfish (Herberholz et al., 2003). Subordinate crayfish exhibit reduced excitability in the lateral giant escape mechanism than do dominant crayfish (Krasne et al., 1997). The lateral giant tail flip escape is likely inhibited as a result of numerous escapes performed during socialization.

Agonistic encounters between crayfish depend on visual, tactile and chemoreceptive input (Rubenstein and Hazlett, 1974; Bruski and Dunham, 1987; Delgado-Morales et al., 2004). Vision appears to play an important role in fighting behaviours such as following and lunging (Bruski and Dunham, 1987); however, fighting occurs in the absence of vision (Kellie et al., 2001; Li and Cooper, 2002). Taction appears to be important for striking, pushing and antennae tapping (Bruski and Dunham, 1987). Agonistic encounters also involve chemical cues (Tierney and Dunham, 1982; Hazlett, 1999; Zulandt Schneider

et al., 1999; Zulantz Schneider and Moore, 2000; Zulantz Schneider et al., 2001; Breithaupt and Eger, 2002), which play an important role in the intensity and outcome of fights (Bergman et al., 2003; Delgado-Morales et al., 2004).

Responses to a reflective environment have been studied in many vertebrate species (Schusterman et al., 1967; Gallup, 1970; Pepperberg et al., 1995; Craft et al., 2003), but few studies have investigated reactions of invertebrates to mirrors. The only crustaceans that have been reported to respond to mirrors are hermit crabs (Dunham et al., 1986), fiddler crabs (McLean and Pratt, 2007) and crayfish (Drozd et al., 2006; May and Mercier, 2006). Specific behaviours, such as turning and remaining in a corner, are enhanced in crayfish by mirrors placed in an aquarium and even by the reflection provided by the aquarium glass, but only in socialized crayfish (Drozd et al., 2006). Further work has revealed that responses of crayfish to a reflection depend on dominance rank (May and Mercier, 2006). In the latter study, crayfish were either paired or isolated for two weeks and were subsequently observed in a test tank with mirrors lining one half of the aquarium and a matte plastic lining the other half. Dominant crayfish performed more cornering, turning and crossing on the reflective side than on the non-reflective side. Subordinate crayfish did not show differences with respect to these behaviours but performed more reverse walking on the reflective side. Isolated crayfish exhibited no behavioural differences between the two environments. In that investigation, crayfish were paired for two weeks. It is possible, however, that the responses to the reflective environment might develop sooner and that they might change over time. Others have demonstrated that certain behaviours change with the length of time during which crayfish are socialized (Issa et al., 1999; Kellie et al., 2001; Gherardi and Daniels, 2004).

In the present work, crayfish were paired for 30 min, which is sufficient to produce dominance ranks (Lowe, 1956; Herberholz et al., 2001). Dominant and subordinate crayfish were observed for 30 min independently in a test tank that consisted of an aquarium with one half lined with mirrors and the other half lined with non-reflective plastic. A separate group of crayfish was housed in pairs for 3 days, and a comparison group consisting of crayfish housed in a large community tank were also observed in the test tank. The frequency and duration of behaviours previously shown to be enhanced by reflection were calculated for each crayfish group (Drozd et al., 2006; May and Mercier, 2006). Results indicated that responses of dominant crayfish paired for 30 min were similar to those of dominant crayfish paired for two weeks. By contrast, subordinate crayfish required 3 days of socialization for behaviour to resemble that of subordinate crayfish paired for two weeks.

### Materials and methods

Adult male crayfish (*Procambarus clarkii* L.), obtained from Atchafalaya Biological Supply, Co., Raceland, LA, USA, were used for this investigation. All crayfish were intact, and none moulted during the experiment. Crayfish masses ranged from 18.4 to 50.3 g ( $34.4 \pm 9.7$  g; mean  $\pm$  s.d.), and the body length, measured from rostrum to telson, ranged from 8.1 to 11.0 cm ( $9.4 \pm 0.6$ ; mean  $\pm$  s.d.).

All crayfish were initially housed for at least one week in a large round community tank with a depth of 70 cm and a diameter of 120 cm. This tank typically housed 30 crayfish at one time and contained ample rocks and PVC tubing for shelter. Three groups were used in this study. One group consisted of 40 crayfish that were paired for only 30 min. The second group consisted of 40 crayfish that were paired for 3 days. The third group served as a control and included 20 crayfish that came directly from the same community tank as all the other crayfish and were treated in exactly the same way but were never paired.

All crayfish were fed *ad libitum* three times weekly with artificial crab meat obtained from local grocers. All containers were maintained in a controlled environment with a 12 h:12 h light:dark photoperiod with both water and room temperature from 18 to 21°C.

### Dominance testing

For the 30 min group, 40 crayfish were taken randomly from the large community tank, each placed into a separate plastic container (30 cm  $\times$  17.5 cm  $\times$  13 cm) and transported from the housing facility to a testing room. The crayfish were then left isolated in containers for 30 min prior to testing. Two crayfish of approximately the same size were moved, using a plastic flower pot to reduce handling, into a new plastic container of the same size, containing filtered, aerated water. Crayfish in each pair were matched to within 10% of rostrum-to-telson length. There were no significant differences between masses or lengths of the eventual dominant (mass  $38.9 \pm 8.7$  g, length  $9.6 \pm 0.6$  cm) and subordinate crayfish (mass  $37.3 \pm 8.0$  g, length  $9.4 \pm 0.6$  cm) paired for 30 min or between dominant (mass  $37.4 \pm 8.8$  g, length  $9.6 \pm 0.6$  cm) and subordinate crayfish (mass  $35.1 \pm 8.3$  g, length  $9.2 \pm 0.7$  cm) paired for 3 days. Each pair remained together for 30 min and were observed by the researcher during this period to determine the dominance rank. The small size of the container encouraged contact between the crayfish, and fighting began almost immediately. Typically, the encounters were initiated by one crayfish approaching with raised chelae. The encounters escalated to include pushing, lunging and striking. This behaviour led to retreat of the losing crayfish by means of walking backwards and tail-flip escape behaviour, and eventually the loser avoided the winning crayfish. These behaviours have previously been described in detail (Bovbjerg, 1953; Copp, 1986; Bruski and Dunham, 1987; Huber and Delago, 1998; Lundberg, 2004). A crayfish that retreated from the first fight often retreated from subsequent fights. After retreating from a number of fights, the losing crayfish always changed its behaviour by no longer engaging in contact and by avoiding the other crayfish. Such avoidance behaviour by the losing crayfish always appeared within 30 min, and this crayfish was deemed subordinate; the winning crayfish was deemed dominant. This method of determining dominance rank has been used reliably and repeatedly (Guíasu and Dunham, 1997; Goessmann et al., 2000; Bergman et al., 2003) and resulted in 20 dominant and 20 subordinate crayfish.

For the 3-day group, 40 crayfish were taken from the community tank, arranged in pairs according to size, and maintained in pairs for 3 days. Each pair was housed in a plastic container measuring 58 cm long  $\times$  30 cm wide  $\times$  35 cm high. Each container was filled with filtered, aerated water and

Table 1. *Description of behaviours analysed*

Behaviour	Description
Cornering	The crayfish faces the corner and remains there for a minimum of 5 s.
Turning	The crayfish turns more than 90°, changing the direction of its walking path from clockwise to counter-clockwise or <i>visa versa</i> . Turns towards the corner take place within one body length of the corner.
Crossing	The crayfish leaves the perimeter of the tank, enters the centre and crosses from one wall to another. The crayfish must be at least one body length from the corner to be considered crossing the tank.
Freezing	The crayfish abruptly ceases all visible movement, including appendage and antennae movements, for at least 5 s.
Reverse walking	The crayfish walks backwards or backs up for a minimum of one crayfish body length.
Crossing at the midline	The crayfish crosses the vertical midline of the aquarium, leaving one environment and entering the other.

contained one PVC pipe, measuring 10–15 cm in length, to serve as a shelter. Paper towels were placed between tanks to prevent visualization of other crayfish pairs. Each pair was observed for the first 30 min of socialization. Fights followed the same pattern, and dominance rank was determined as described above. Each pair was subsequently observed for the following two days to ensure that the rank remained stable. Dominant crayfish always occupied the shelter and gained first access to food. No rank reversals were observed during the course of this experiment.

#### *Reflection testing*

Each crayfish was tested for responses to reflective surfaces in a specially constructed glass aquarium measuring 52 cm long  $\times$  25 cm wide  $\times$  30 cm deep. Half of the tank's perimeter was lined with mirrors. This included one end wall and half of each of the adjoining walls, including two corners, which provided a reflective environment on one side of the aquarium. A non-reflective environment was created by lining the other half of the aquarium with a semi-transparent matte plastic. White paper was placed underneath the tank to provide stronger contrast for videotaping. The aquarium was filled approximately 15 cm deep with filtered, aerated water that was replaced between trials.

Animals paired for 30 min were tested for responses to reflection immediately following the socialization period. Animals paired for three days were placed in a plastic container (30 cm  $\times$  17.5 cm  $\times$  13 cm) together and were transported from the housing facility to the testing room. They remained paired for 30 min to reduce the effects of any stress created during transportation and were tested immediately thereafter. Animals housed in the community tank were transported in the same manner and were given the same 30 min acclimation period before being tested.

Each crayfish was placed gently, using a flower pot to minimize contact, into the centre of the test aquarium, facing one of the midlines separating the two environments. The dominant or subordinate member of each pair was alternatively chosen to be tested first; thus, 50% of each group were tested first, and 50% were tested 30 min later. The experiment was also counterbalanced to remove the effects of any preference for one side of the room. The aquarium was turned between trials so that half of all crayfish tested experienced the mirrored environment on the left, and the others experienced it on the right.

Crayfish activity was videotaped for 20 min using a webcam (Logitech, Fremont, CA, USA) mounted 30 cm above the aquarium. Video files were acquired using Windows Movie Maker and burned to CD for later analysis. Table 1 provides a full description of all behaviours analyzed in the present report. Cornering, turning towards corners, crossing and reverse walking have been described in earlier reports on responses of *P. clarkii* to reflective environments and were examined here because they have been shown to be enhanced by reflection (Drozdz et al., 2006; May and Mercier, 2006). These behaviours were also examined because we thought the results might provide some insight into whether or not crayfish respond to the mirror image as they would to a conspecific (see Discussion). Freezing, defined as ceasing all visible movement (including all appendages) for a minimum of 5 s, was not examined in earlier studies of reflective environments but has been described in other reports (Gherardi and Peracchini, 2004; Lundberg, 2004).

## **Results**

### *Cornering*

Socialized crayfish performed cornering more frequently than any other identified behaviour while exploring the test aquarium. After 30 min of pairing, both dominant (Fig. 1A) (paired *t*-test,  $P < 0.0001$ ) and subordinate ( $P < 0.005$ ) crayfish cornered significantly more on the mirrored side of the tank than on the matte side. After 3 days of socialization, only dominant crayfish cornered more frequently on the mirrored side of the aquarium ( $P < 0.0001$ ). Group-socialized crayfish also cornered more frequently on the reflective side of the aquarium than on the non-reflective side ( $P < 0.05$ ).

Because crayfish remained in corners for many seconds at a time, the total time each crayfish spent cornering was measured. Fig. 1B depicts the time each crayfish group spent in reflective and non-reflective corners. Both dominant and subordinate crayfish paired for 30 min spent more time cornering in the reflective environment than in the non-reflective environment (Fig. 1B) (paired *t*-test,  $P < 0.0001$  and  $P < 0.05$ , respectively). After 3 days of pairing, only dominant crayfish cornered longer on the reflective side of the aquarium ( $P < 0.005$ ). There was no significant difference between cornering times on the two sides for subordinate crayfish paired for 3 days ( $P = 0.35$ ) or for group-socialized crayfish ( $P = 0.06$ ), although the results approached significance in the latter case.

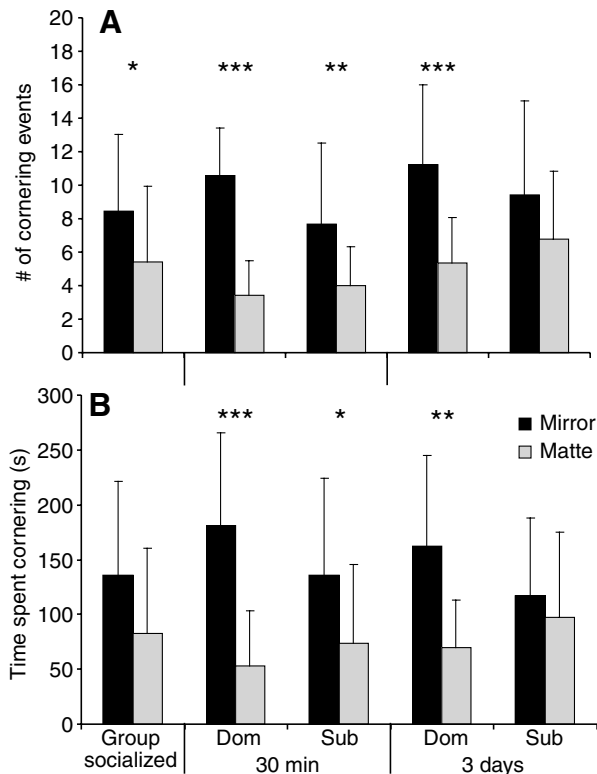


Fig. 1. Cornering observed during 20 min of observation. (A) Mean frequency of cornering events in the reflective and non-reflective (matte) environments. Dominant (Dom) and subordinate (Sub) crayfish paired for 30 min cornered more frequently on the mirrored side of the tank. After 3 days of pairing, dominant crayfish cornered more frequently on the reflective side. Group-socialized crayfish also cornered more on the reflective side of the tank compared with the non-reflective side. (B) Mean time each crayfish group spent cornering on the reflective and non-reflective (matte) sides of the test aquarium. After 30 min of pairing, both dominant and subordinate crayfish cornered for a longer time on the mirrored side of the tank, but after 3 days of pairing only dominant crayfish did (paired *t*-test: \*\*\* $P < 0.0001$ , \*\* $P < 0.005$ , \* $P < 0.05$ ;  $N = 20$ ). Error bars depict standard deviation.

#### Turning towards corners

Turns can occur at a number of locations in the aquarium but commonly occur at or near the corners. Turns towards the corner occurred when the crayfish was within one body length of the corner and changed its direction to turn towards a corner. Crayfish that were paired for 30 min turned more frequently towards reflective corners than non-reflective corners (Fig. 2) (paired *t*-test; dominant,  $P < 0.005$ ; subordinate,  $P < 0.005$ ). After 3 days of pairing, only dominant crayfish turned towards corners more on the reflective side of the tank than on the non-reflective side ( $P < 0.05$ ). Subordinate crayfish paired for 3 days ( $P = 0.17$ ) and group-socialized crayfish ( $P = 0.6$ ) showed no preference for turning towards reflective corners.

#### Crossing

Crayfish sometimes left the perimeter of the test tank, entered the centre and crossed to another wall. While in the mirrored

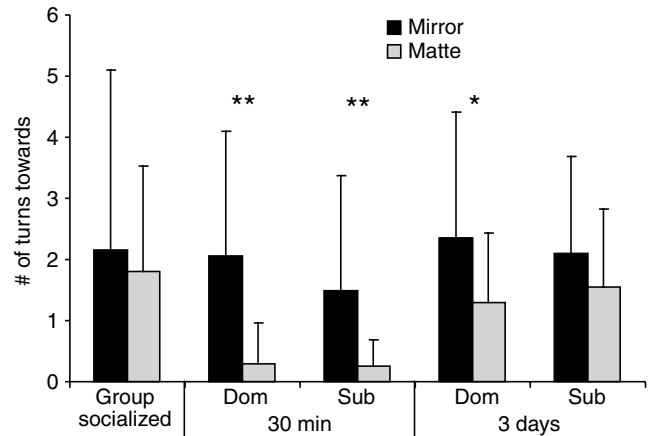


Fig. 2. Turning behaviour of crayfish observed for 20 min. Mean frequency of turns towards reflective and non-reflective (matte) corners. Dominant and subordinate crayfish that were paired for 30 min both turned more frequently towards reflective corners. After 3 days of pairing, only dominant crayfish turned more often towards corners on the reflective side (paired *t*-test: \*\* $P < 0.005$ , \* $P < 0.05$ ;  $N = 20$ ). Error bars depict standard deviation.

environment, a crayfish can cross to another mirrored wall, or it can enter the non-reflective environment and cross towards a non-reflective wall; while in the non-reflective environment, the converse is true. Thus, as in an earlier report (May and Mercier, 2006), the following outcomes were considered: crossing towards a reflective wall *vs* a non-reflective wall, and crossing away from a reflective wall *vs* a non-reflective wall. Group-socialized crayfish did not cross towards reflective walls any more often than towards non-reflective walls (Fig. 3A) (paired *t*-test,  $P = 0.42$ ). Following 30 min of socialization, dominant crayfish crossed more often towards reflective walls ( $P < 0.005$ ), but subordinate crayfish did not ( $P = 0.11$ ). This pattern remained the same after 3 days of pairing, when again only dominant crayfish crossed towards reflective walls more often than non-reflective walls ( $P < 0.05$ ), and subordinates showed no preference ( $P = 0.24$ ).

Crossing away from reflective *versus* non-reflective walls was also quantified. Group-socialized crayfish crossed away from reflective walls more than non-reflective walls (Fig. 3B) (paired *t*-test,  $P < 0.05$ ). After 30 min of pairing, both dominant ( $P < 0.005$ ) and subordinate ( $P < 0.05$ ) crayfish also crossed away from reflective walls more than non-reflective walls. After 3 days of pairing, this pattern changed. Although dominant crayfish continued to cross away from non-reflective walls more frequently than reflective walls ( $P < 0.005$ ), subordinate crayfish did not ( $P = 0.24$ ).

#### Freezing

'Freezing' behaviour commonly occurred at or near the mid-line of the aquarium but was observed anywhere in the tank. Both dominant and subordinate crayfish paired for 30 min froze more frequently in the reflective environment than in the non-reflective environment (Fig. 4) (paired *t*-test; dominant,  $P < 0.0001$ ; subordinate,  $P < 0.005$ ). After 3 days of pairing, both dominant ( $P < 0.05$ ) and subordinate ( $P < 0.005$ ) crayfish



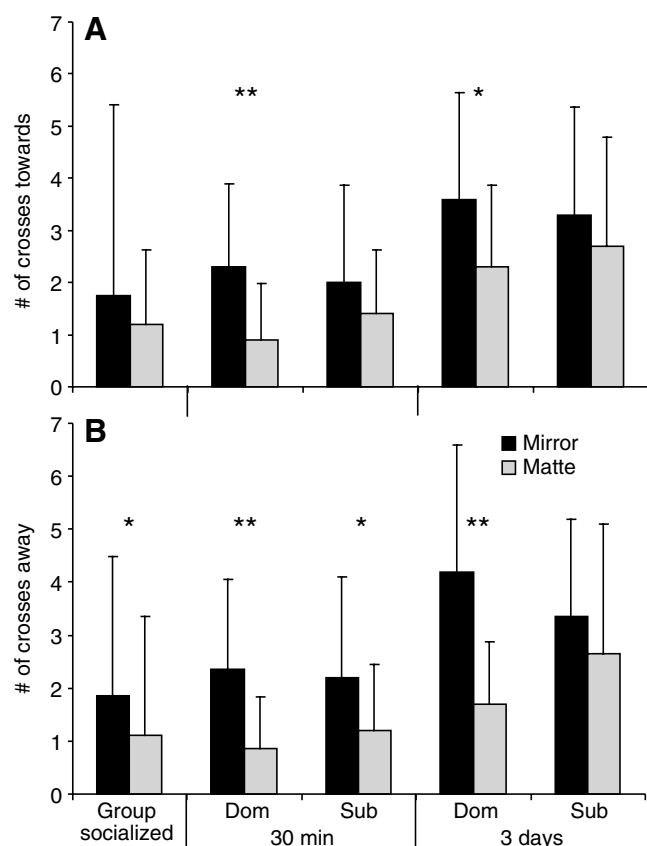


Fig. 3. Crossing behaviour of crayfish recorded during 20 min of observation. (A) Mean frequency of crosses towards reflective and non-reflective (matte) walls. Group-socialized crayfish crossed towards reflective walls more than non-reflective walls. Dominant crayfish paired for 30 min and 3 days both crossed towards reflective walls more frequently. (B) The mean frequency of crosses away from reflective and non-reflective (matte) walls. Group-socialized crayfish crossed away from reflective walls more frequently than matte walls. After 30 min of pairing, both dominant and subordinate crayfish crossed away from reflective walls more than non-reflective walls. Dominant crayfish paired for 3 days also crossed away from reflective walls more frequently (paired *t*-test: \*\* $P < 0.005$ , \* $P < 0.05$ ;  $N = 25$ ). Error bars depict standard deviation.

exhibited freezing more often on the reflective side of the tank than on the non-reflective side. Group-socialized crayfish did not show a preference for freezing in either environment ( $P = 0.06$ ), but the results approached statistical significance, with a trend suggesting more freezing on the reflective side.

#### Reverse walking

Reverse walking occurred infrequently but was not associated with any external disturbance. Group-socialized crayfish performed reverse walking more often on the mirrored side of the aquarium than on the non-reflective side (Fig. 5) (paired *t*-test,  $P < 0.005$ ). Neither dominant nor subordinate crayfish paired for 30 min exhibited a preference for reverse walking on either side of the aquarium (dominant,  $P = 1.0$ ; subordinate,  $P = 1.0$ ). By contrast, after 3 days of pairing, both dominant ( $P < 0.05$ ) and subordinate ( $P < 0.05$ ) crayfish reverse walked more frequently on the mirrored side of the tank.

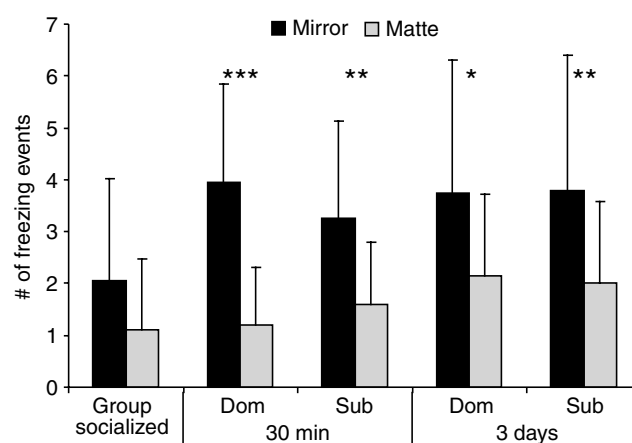


Fig. 4. The mean frequency of freezing behaviour recorded during 20 min of observations on the reflective and non-reflective sides (matte) of the test aquarium. Dominant and subordinate crayfish paired for 3 days froze more frequently on the mirrored side of the tank. After 3 days of pairing, dominant and subordinate crayfish also froze more in the reflective environment as opposed to the non-reflective environment (paired *t*-test: \*\*\* $P < 0.0001$ , \*\* $P < 0.005$ , \* $P < 0.05$ ;  $N = 20$ ). Error bars depict standard deviation.

#### Time spent in reflective environment

During the 20 min observation period, group-socialized crayfish spent more time on the reflective side of the aquarium than on the matte side (Fig. 6) (paired *t*-test,  $P < 0.05$ ). After 30 min of pairing, both dominant ( $P < 0.005$ ) and subordinate ( $P < 0.05$ ) crayfish preferred the reflective side of the tank over the non-reflective side. Dominant crayfish that had been paired for 3 days also spent more time on the reflective side of the tank compared with the non-reflective side ( $P < 0.05$ ), but subordinate crayfish paired for 3 days showed no preference for any side ( $P = 0.84$ ).

#### Overall activity

Overall activity level was assessed in two ways. First, the number of times each crayfish crossed the midline of the tank, leaving one environment and entering another, was measured. Second, the number of occurrences of all behaviours examined (cornering, turning, crossing and reverse walking) were combined and were used to determine if there was a difference between crayfish groups with regard to overall activity level. An ANOVA revealed a difference between groups for the number of times crayfish crossed the aquarium midline (Fig. 7) ( $P = 0.012$ ). A Tukey HSD *post-hoc* analysis revealed that dominant crayfish paired for 3 days crossed the midline more frequently than did dominant crayfish paired for 30 min ( $P < 0.05$ ) and subordinate crayfish paired for 30 min ( $P < 0.05$ ). When behaviours on both sides of the tank were combined, an ANOVA found no difference in activity level between crayfish groups, but the results approached statistical significance ( $P = 0.06$ ). The total behavioural events for each crayfish group were as follows: group-socialized,  $33.2 \pm 18.1$ ; 30 min dominant,  $29.4 \pm 9.5$ ; 30 min subordinate,  $28.5 \pm 15.5$ ; 3-day dominant,  $40.0 \pm 14.6$ ; and 3-day subordinate,  $38.2 \pm 14.9$ .

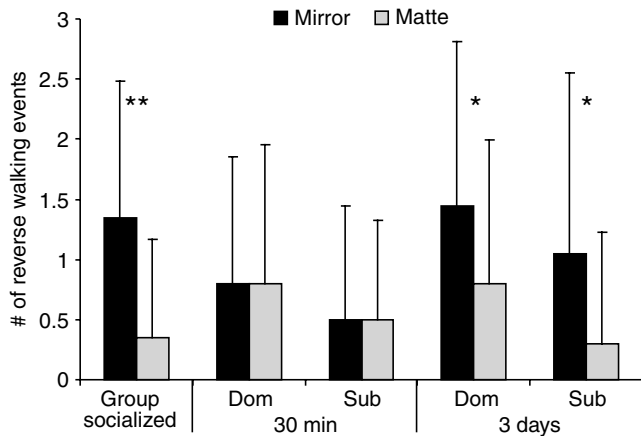


Fig. 5. The mean frequency of reverse walking behaviour observed in crayfish for 20 min on the reflective or non-reflective (matte) sides of the test tank. Group-socialized crayfish reverse walked more on the mirrored side of the tank. Both dominant and subordinate crayfish paired for 3 days also reverse walked more on the reflective side compared with the non-reflective side of the aquarium (paired *t*-test: \*\* $P < 0.005$ , \* $P < 0.05$ ;  $N = 20$ ). Error bars depict standard deviation.

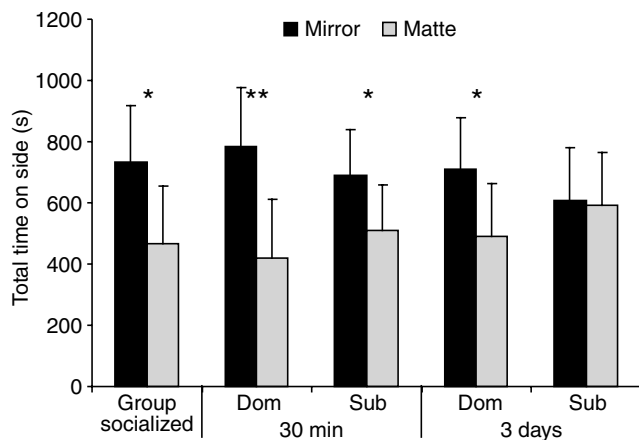


Fig. 6. The mean total time each crayfish group spent on the reflective and non-reflective (matte) sides of the aquarium during 20 min of observation. Group-socialized crayfish spent more time overall on the reflective side of the tank. Both dominant and subordinate crayfish paired for 30 min spent more time in the reflective environment compared with the non-reflective environment. After 3 days of pairing, only dominant crayfish spent more time on the reflective side of the test tank (paired *t*-test: \*\* $P < 0.005$ , \* $P < 0.05$ ;  $N = 20$ ). Error bars depict standard deviation.

### Discussion

The present study provides new information about how the behaviour of dominant and subordinate crayfish changes during the socialization period. A summary of statistically significant differences for all crayfish groups and all behaviours was constructed (Table 2), and data previously published by May and Mercier (May and Mercier, 2006) are included for comparison. After 30 min of pairing, when social rank is first emerging, dominant and subordinate crayfish respond with remarkable similarity to a reflective environment. Their

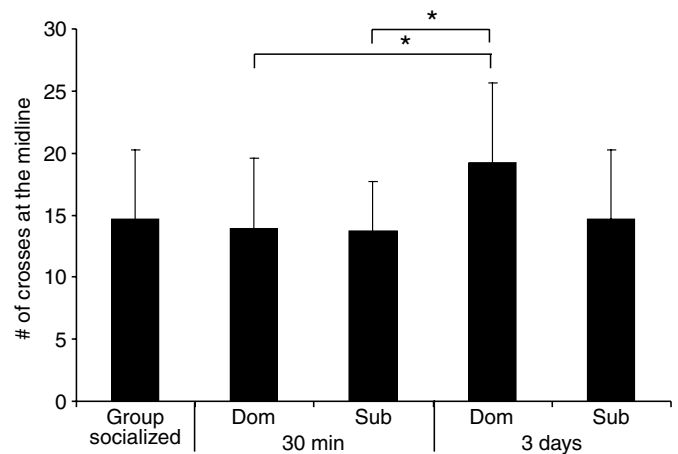


Fig. 7. The mean frequency of crossing at the midline of the aquarium for each crayfish group. Dominant crayfish paired for 3 days crossed the midline significantly more than either dominants or subordinates that were paired for 30 min (ANOVA with Tukey HSD *post-hoc* analysis: \* $P < 0.05$ ;  $N = 20$ ). Error bars depict standard deviation.

responses are essentially the same for seven of the nine behavioural features examined in the present work (Table 2), and their responses are also nearly identical to those of dominant crayfish paired for 2 weeks, reported previously (May and Mercier, 2006). Thus, the first few agonistic encounters appear to produce a similar pattern of behaviours with respect to reflection in all crayfish regardless of whether they win or lose, and this pattern is essentially the same as that exhibited by dominant crayfish after 2 weeks of pairing. After 30 min of pairing, seven of nine responses of dominant and subordinate crayfish to the reflective environment differ from those of group-socialized crayfish (which were not paired). Thus, pairing appears to cause the emergence of a dominant pattern of responses to reflection in all crayfish initially. After 3 days of pairing, several results indicate that responses of dominant and subordinate crayfish diverge (Table 2). First, dominant crayfish show nearly the same pattern as at 30 min of pairing, with eight of nine behaviours the same, and the pattern is also nearly identical to that of dominants after 2 weeks (May and Mercier, 2006). Second, subordinates on day 3 respond differently from subordinates paired for 30 min in eight of nine behaviours. Thus, the behaviour of subordinates changes between 30 min and 3 days. Third, the responses of dominant and subordinate crayfish after 3 days differ in seven of the nine behaviours examined. Thus, the subordinate pattern of responses to a reflective environment develops more slowly than the pattern observed in dominant crayfish (May and Mercier, 2006).

In previous work (Drozd et al., 2006; May and Mercier, 2006), isolated crayfish were used as a comparison group. In those studies, however, the experimental groups were isolated for 2 weeks prior to 2 weeks of pairing, in an attempt to reduce or extinguish effects of socialization in the community tank before pairing occurred. Those paired crayfish responded differently to the reflective environment of the mirror/matte tank than did crayfish that had been isolated for a total of 4 weeks (Drozd et al., 2006; May and Mercier, 2006). In the present

Table 2. Summary of significant differences between behaviours on the reflective vs non-reflective sides of the test tank observed in the present study compared with those reported previously (May and Mercier, 2006) for 14-day pairing

Behaviour	Group socialized	30 min		3 days		14 days <sup>†</sup>	
		D	S	D	S	D	S
Cornering	—	*	*	*	—	*	—
Time cornering	—	*	*	*	—	*	*
Turn towards	—	*	*	*	—	*	—
Turn away	—	*	*	*	—	*	*
Turn at the side	*	*	—	*	*	*	—
Crossing towards	—	*	—	*	—	*	—
Crossing away	*	*	*	*	—	*	—
Freezing	—	*	*	*	*	NR	NR
Reverse walking	*	—	—	*	*	—	*
Total time	*	*	*	*	—	*	—

\* indicates  $P<0.05$  (paired  $t$ -test). — indicates no significance. NR indicates not reported.

<sup>†</sup>Previously reported in May and Mercier (May and Mercier, 2006).

work, no attempt was made to reduce or extinguish effects of socialization in the community tank prior to pairing. Crayfish housed in a community tank were used as a comparison group because their holding conditions and social environment were identical to those of the experimental groups except for the fact that they were not paired. In fact, they came from the same holding tank as the paired groups.

Crayfish housed in large groups naturally develop a stable dominance hierarchy over time (Bovbjerg, 1953; Lowe, 1956). Once the hierarchy is established, fighting behaviour and preludes to fighting behaviour decrease over time (Copp, 1986; Goessman et al., 2000; Bergman et al., 2003). Group-socialized crayfish, therefore, would constitute a mix of varying degrees of dominance states. Results in the present work are consistent with this interpretation. If the different patterns of response to reflection exhibited by dominant and subordinate crayfish after 3 or 14 days (Table 2) represent two extremes, one would predict that a group of crayfish with mixed dominance ranks would behave differently from those two extremes, since they would not all be dominant or subordinate. The group-socialized crayfish differed from 3-day dominant crayfish in six of the 10 behaviours examined and from 3-day subordinate crayfish in four of 10 behaviours (Table 2). Group-socialized crayfish also differed from 14-day dominant crayfish in six of nine behaviours and from 14-day subordinate crayfish in five of nine behaviours (May and Mercier, 2006) (see Table 2).

The patterns of responses to reflection were very similar between dominant and subordinate crayfish after 30 min of pairing, but they were not identical (Table 2). At this time, dominant and subordinate crayfish both performed more cornering and spent more time cornering in the reflective environment. They both turned towards and away from reflective corners more than non-reflective corners and crossed away from reflective walls more frequently. Both groups also spent more time on the mirrored side of the tank compared with the matte side. All eight of these features are also exhibited by dominant crayfish after 14 days of pairing. Several of these features suggest that the crayfish either seek the reflection or show a preference for it. Such features include more cornering on the mirrored side, more time cornering on the mirrored side,

more turning towards reflective corners than non-reflective corners and more time spent in the reflective environment. The only differences between dominant and subordinate crayfish after 30 min of pairing were that subordinates did not perform more turns at the side in the reflective environment and did not cross more frequently towards reflective walls than non-reflective walls.

After 3 days of pairing, dominant crayfish continued to behave in a manner suggesting preference for the reflective surfaces. They performed more cornering, spent more total time cornering and spent more total time on the reflective side, and they turned more frequently towards reflective sides and crossed more frequently towards reflective sides. At the same time, subordinate crayfish showed no preference for any of these behaviours with regard to reflection. The only features shared by dominant and subordinate crayfish after 3 days were freezing and reverse walking.

Hazlett found that hermit crabs required 3 days of socialization to effectively alter their behaviour (Hazlett, 1966). In those experiments, subordinates moved away or retreated into their shell in response to a conspecific after 3 days. Although the responses to a reflective environment after 3 days of pairing are very similar to those reported previously for crayfish paired for 14 days (May and Mercier, 2006), the patterns are not identical (Table 2). After 3 days, subordinate crayfish turned significantly more at the side of the tank in the reflective environment than in the non-reflective environment, but no such difference occurred for subordinate crayfish after 14 days of pairing. After 14 days of pairing, subordinate crayfish spent more time in reflective corners and turned away more frequently from reflective corners, but after 3 days of pairing, neither of these responses was observed. Although dominant crayfish did not perform more reverse walking in either environment on day 14, they did perform significantly more reverse walking in the reflective environment on day 3. These few differences between responses on days 3 and 14 cannot be attributed to differences in the testing environment because the test tank and testing procedure were identical to those used in the earlier study (May and Mercier, 2006). Some behaviours associated with the reflective environment might be labile and may take more time

to stabilize as dominance rank is established. Dominance rank itself can be labile, as indicated by reversals in rank reported by others (Goessmann et al., 2000; Delgado-Morales et al., 2004; Song et al., 2006). Although no rank reversals occurred in the present investigation, responses to the reflective environment might be more labile than dominance rank. Alternatively, some behaviours (e.g. reverse walking) are infrequent, which might give rise to sampling errors. The current results indicate that many behaviours should be monitored when attempting to distinguish responses to reflection between different groups of crayfish.

It is important to assess overall activity of crayfish reported in this study to determine whether or not differences between dominant and subordinate crayfish merely reflect differences in activity level. For example, did subordinate crayfish fail to respond to reflection simply because they were inactive? Although the data approached statistical significance ( $P=0.06$ ), there was no significant difference in total events for all behaviours when data from reflective and non-reflective sides were combined. The largest differences in activity occurred between different days, and this was true for both dominant and subordinate crayfish. On any given day, however, dominant and subordinate crayfish exhibited very similar activity levels. This observation suggests that dominance rank is associated with changes in the distribution of the various behaviours on the reflective and non-reflective sides rather than a change in the total number of behavioural events in the tank. Dominant crayfish paired for 3 days crossed the midline between reflective and non-reflective environments more frequently than either dominant or subordinate crayfish paired for 30 min (Fig. 7). Occasionally, crayfish would walk back and forth across the midline without travelling far into either side. This type of movement required little locomotion but increased the frequency of midline crossing. Such behaviour might explain why dominant crayfish on day 3 crossed the midline more frequently than other crayfish and yet spent more time in the reflective environment.

Several results suggest but do not prove that crayfish perceive their mirror image as a conspecific. After 3 days of pairing, subordinate crayfish no longer corner more frequently in the reflective environment or spend more time in reflective corners, and they no longer cross more frequently towards reflective walls or turn more frequently towards reflective corners. These observations are consistent with the notion that subordinate crayfish avoid the reflection or no longer seek it out. A crayfish that loses its first agonistic encounter is more likely to lose subsequent encounters and will retreat from and avoid a winning crayfish (Bruski and Dunham, 1987; Huber and Kravitz, 1995; Guisasu and Dunham, 1997; Tierney et al., 2000). This has been referred to as the 'loser effect' (Goessmann et al., 2000). Once rank has been established, subordinate crayfish initiate contact with conspecifics less frequently than do dominant crayfish, and subordinates do not approach dominant crayfish as frequently as they approach naive crayfish (Rubenstein and Hazlett, 1974; Copp, 1986). If crayfish perceive the mirror image as a conspecific, subordinate crayfish should avoid reflective surfaces more frequently the longer they are paired. In the present study, subordinate crayfish spent more time in the reflective

environment after 30 min of pairing but did not do so after 3 days of pairing. However, they did not spend significantly more time in the non-reflective environment.

An alternative explanation for the results reported here is that crayfish respond to movement seen in the mirror and not to the image *per se*. It is possible that dominant crayfish seek out the movement in the mirror and subordinate crayfish do not. This difference may be a result of learned submissiveness in subordinate crayfish. Further work is required to answer the question of whether the crayfish views its mirror image as a conspecific. This question could be addressed by comparing responses to reflection with responses to a live conspecific viewed through a transparent barrier. Under such conditions, one could also test for other known responses to conspecifics such as increased urination (Breithaupt and Eger, 2002) and an increase in heart rate (Listerman et al., 2000).

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