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## Biogenic amines influence aggressiveness in crayfish but not their force or hierarchical rank

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This study aimed at understanding the influence of biogenic amines on the agonistic behaviour of the crayfish *Procambarus clarkii*, a model organism for crustacean decapods. Specifically, we investigated whether the hierarchical rank of fighting individuals might be altered by injecting solutions of either serotonin or octopamine into their haemolymph. First, we assessed the effect and duration of the bioamines on the behaviour, posture, and chelar force of 60 adult males paired for size. Then, we examined their potential to modify dominance hierarchies by observing, for 2 h after the treatment, the behaviour of three categories of familiar size-matched pairs: (1) 20 'control pairs' (both individuals injected with a physiological solution), (2) 20 'reinforced pairs' (the dominant individual, alpha, injected with serotonin, and the subordinate individual, beta, with octopamine), and (3) 20 'inverted pairs' (alpha injected with octopamine and beta with serotonin). The results clearly show that the two bioamines were able to alter the posture and aggressiveness of the treated individuals in opposite directions without affecting their chelar force. However, the large majority of the 'inverted pairs' retained their former position in the hierarchy. The role that intrinsic characteristics and prior social experience play in maintaining dominance hierarchies in crayfish is discussed.

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**Keywords:** crayfish; dominance hierarchies; intrinsic characteristics; octopamine; prior experience; *Procambarus clarkii*; serotonin

The biogenic amines serotonin (5-HT, 5-hydroxytryptamine) and octopamine (OA, DL-octopamine) are recognized to play an important role in controlling the aggression of crustacean decapods (Livingstone et al. 1980; Kravitz 1988, 2000; Fingerman et al. 1994; Huber et al. 1997a, b; Weiger 1997). Both were found to exert their effects by acting in three basic modes: as classical neurotransmitters, as neuromodulators or as neurohormones (Orchard 1982; Weiger 1997).

Serotonin has been extensively studied in several vertebrate and invertebrate species, being involved in a plethora of behavioural effects (Weiger 1997; Lucki 1998). Serotonergic neurons were found to be among the earliest neurons to develop in the nervous system of many animals, including crustaceans (Benton et al. 1997). Particularly in decapods, serotonin takes a prominent part in a number

of physiological processes, such as feeding, walking, reproduction, heart rate, and aggression (e.g. Huber et al. 1997a, b; Yeh et al. 1997; Listerman et al. 2000; Teshiba et al. 2001; Tierney & Mangiamele 2001). Contrary to its mode of action in vertebrates (e.g. *Apteronotus leptorhynchus*, Maler & Ellis 1987; *Columba livia*, Ison et al. 1996; *Anolis carolinensis*, Larson & Summers 2001), serotonin activity increase in invertebrates is generally accompanied by heightened levels of aggression (Weiger 1997). So, in decapods an enhancement in 5-HT neuron function is closely associated with aggressive or dominant behaviour (Huber et al. 1997b); after fighting, the winners show higher concentrations of 5-HT in the haemolymph than the losers (Sneddon et al. 2000).

Similarly to other decapods (the lobster *Homarus americanus*, Kravitz et al. 1980; Livingstone et al. 1980; Harris-Warrick & Kravitz 1984; Kravitz 2000; the shore crab *Carcinus maenas*, Benvegut & Clarac 1982; the crab *Chasmagnathus granulatus*, Aggio et al. 1996; the squat lobster *Munida quadrispina*, Antonsen & Paul 1997), crayfish with serotonin injected into their haemolymph increase

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their aggressive motivation (*Procambarus clarkii*, Livingstone et al. 1980; *Astacus astacus*, Huber et al. 1997b; Huber & Delago 1998) and assume a dominant posture: their chelipeds are spread out and raised ('meral spread') and the abdomen is semi-flexed. Subordinates treated with serotonin show a reduced tendency to retreat and fight for longer and more strongly against dominant crayfish (Huber et al. 1997b; Huber & Delago 1998).

Octopamine has the opposite effects on decapod aggression. Together with the bioamine tyramine, octopamine is the only neuroactive nonpeptide transmitter, whose physiological role is restricted to invertebrates, being homologous to the noradrenergic system of vertebrates (Roeder 1999). Although octopamine has been thought to exert a widespread action, its role has been explored in detail only in insects (Orchard 1982; Leitch et al. 2003), where it is partly responsible for the social cohesion between nest mates in ant colonies (Boulay et al. 2000) and for the division of labour in honey bees (Schulz et al. 2002). In crayfish, the injection of octopamine leads to a decrease in aggression (*Procambarus clarkii*, Livingstone et al. 1980) and to the acquisition of a posture typical of subordinate individuals, that is, tonic extension of the extremities with the chelipeds lowered.

Undoubtedly, an understanding of the role of bioamines in aggression is crucial to help solve the still heated debate around the mechanisms that maintain dominance hierarchies in crayfish. As suggested by Moore & Bergman (2005), extrinsic (e.g. previous history, sensory communication) and intrinsic chemical processes (e.g. the neurochemical state) are determinants not only for the formation of dominance relationships in crayfish, but also for their maintenance. While a number of studies (Copp 1986; Zulantz Schneider et al. 2001; Bergman et al. 2003) support the role that status recognition plays in maintaining hierarchies ('assessment hierarchies', Barnard & Burk 1979), others (Rubenstein & Hazlett 1974; Daws et al. 2002) underline the influence of past social experience, in the form of 'winner and loser effects' ('confidence hierarchies', Goessmann et al. 2000). The two mechanisms may, however, coexist (*Procambarus acutus*, Gherardi & Daniels 2003; *Orconectes* sp., Bergman & Moore 2003; *Austropotamobius italicus*, Tricarico et al. 2005).

Today, the hypothesis prevails that crayfish, as do American lobsters (Karavanich & Atema 1998; Breithaupt et al. 1999), signal their status through the emission of chemical substances in their urine (Zulantz Schneider et al. 1999, 2001; Breithaupt & Eger 2002; Bergman et al. 2003). The perception of those substances may induce responses typical of a 'winner' (or of a 'loser') in the opponent (Bergman et al. 2003). When individuals have been experimentally deprived of the ability to detect chemical cues, by, for example, obstructing their chemoreceptors or preventing urine release, the intensity of aggression increases and fights get longer (Zulantz Schneider et al. 1999, 2001; Bergman et al. 2003). A plausible hypothesis is that these chemical substances are metabolites of serotonin and/or octopamine (Huber et al. 1997a; Breithaupt 2004; The et al. 2004; Moore & Bergman 2005), the concentration of which in the haemolymph

changes in function of the different social states in decapods (Sneddon et al. 2000).

The aim of this study was to test the influence that serotonin and octopamine exert on the maintenance of dominance hierarchies in the red swamp crayfish, *P. clarkii*. Specifically, we hypothesized that bioamines affect crayfish behaviour to the extent of reversing the hierarchical rank in combating pairs. To test this hypothesis, through the injection of serotonin and octopamine into the crayfish haemolymph, we manipulated the aggressive level of male individuals in size-matched pairs, (1) to describe the effects of bioamines on crayfish posture, behaviour, and chelar force, (2) to assess the duration of these possible effects, and (3) to test whether a raised concentration of bioamines in the haemolymph was sufficient to change the rank of an individual in a hierarchy. Particularly, we disguised an alpha as a beta and a beta as an alpha to assess whether an alpha-like (and a beta-like) behaviour was enough to reverse a hierarchy. Our general purpose was to understand the relative importance of different factors, including intrinsic characteristics and prior experience, in maintaining dominance hierarchies in decapods rather than quantify the effects of bioamines on their agonistic behaviour.

## METHODS

### Subjects, Collection and Housing

To eliminate any factor that could induce an obvious bias to our experiments (e.g. mutilations, moult stage), only sexually mature males (form I; Huner 1988) in good condition (no mutilations or visible diseases, symmetric chelae) were collected with traps in the wetland 'Padule di Fucecchio' (40 km from Florence, Italy) between 2004 and 2006. In the laboratory, we measured the carapace length (34–50 mm), the width and length of both chelae, and the weight of each animal. Crayfish were maintained in PVC aquaria (50 × 75 cm) at a constant temperature of 20 ± 1°C, under a natural 14:10 h light:dark cycle regime, and were fed every day with a 0.1 g earthworm. The maintenance phase lasted for a maximum of 2 weeks.

### Experimental Design

We conducted two sets of experiments from 1000 to 1800 hours. In experiment 1, we recorded the effects of the injected bioamines on crayfish behaviour, posture, and chelar force and assessed their duration. In experiment 2, we examined whether the injection of bioamines induced a change in the hierarchy once formed. The two sets of experiments consisted of 3 phases of which the first two were the same for each experiment.

Phase 1: isolation (1 week). One week was sufficient to remove memory of previous social experiences (Guiasu & Dunham 1999; Zulantz Schneider et al. 2001). Each crayfish, numbered on the cephalothorax using a white typing correction fluid, was kept isolated in an opaque PVC aquarium (30 × 16 cm) and fed every day as in the maintenance phase.

Phase 2: familiarization (1 h). We formed a total of 90 size-matched pairs (maximum difference, cephalothorax length: 5–6%; width of both chelae: 2%; length of both chelae: 6%; weight: 4%). The two opponents were kept in an experimental container (a circular opaque PVC container, diameter: 30 cm) separated by an opaque PVC divider for 10-min acclimatization. Experiments started with the removal of the divider and consisted of video-recording (Sony DCR-TRV33E) crayfish behaviour for subsequent analyses. Simultaneously, an experienced observer (ET) recorded the winner of each fight so that at the end of this phase, we could determine the dominant crayfish, alpha (and consequently the subordinate crayfish, beta) for each pair, that is, the winner (and the loser) of more than 50% of the total fights. The 'winner' was defined as the crayfish that did not retreat or that retreated after the opponent showed a motionless posture, which is typical for a subordinate (Bruski & Dunham 1987). Before this phase started, we measured the chelar force (see below) exercised by the members of 30 pairs, designated at random.

Phase 3: the two sets of experiments diverged: the 30 pairs, where chelar force of their members had been measured, were assigned to experiment 1 and the remaining 60 to experiment 2.

### Experiment 1: Duration and Effects of Bioamines on Posture and Force

After the familiarization phase, the members of each of the 30 pairs designated for this experiment were randomly assigned to one of the three following treatments. Ten alphas and 10 betas were subject to the injection of an octopamine solution (0.03 ml/g) to obtain 'octopaminized' alphas and betas, respectively; 10 alphas and 10 betas received an injection of a serotonin solution (0.03 ml/g) to obtain 'serotonized' alphas and betas, respectively; and 10 alphas and 10 betas were subject to the injection of physiological solutions (0.03 ml/g). During this experiment, crayfish were maintained isolated in the individual aquaria of Phase 1.

Solutions were prepared following McRae (1996) and Huber et al. (1997b), that is, we used physiological solutions of 0.125 M NaCl (sodium chloride, Sigma 71378) and solutions of 5-HT (5-HT creatinine sulphate complex, Sigma H-7752) and OA (DL-octopamine hydrochloride, Sigma 74980) at concentrations of 0.0123 M diluted in the physiological solution of 0.125 M NaCl. Before being used, the solutions were stored in the dark at 4°C for a maximum of 5 weeks. The volume of the solutions injected to the animals was set from the results of a preliminary experiment that, based on the studies of Livingstone et al. (1980), McRae (1996), and Huber et al. (1997b), explored the dose that induced the maximum duration of the bioamines combined with the minimum postural effect (see below).

All the injections were made through the dorsal abdo- cephalothoracic membrane into the abdominal extensor muscle using a 25 gauge  $\times$  5/8" needle fitted to a 1-ml syringe (McRae 1996). As shown by Livingstone et al.

(1980), the injections of the bioamines immediately caused rigid postures in the crayfish (the serotonin caused flexion and the octopamine caused hyperextension of the legs and abdomen) and sometimes vomiting, especially with serotonin. So, treated crayfish were left undisturbed for 30 min to acquire a normal posture.

For each crayfish, we took a total of 17 consecutive records (16 taken every 30 min for 8 h followed by one taken after 24 h) of whether (1) its body was raised or lowered, (2) its abdomen was extended or flexed, (3) its chelipeds were raised or lowered, and (4) its chelae were open or close. According to Bruski & Dunham (1987), the body of dominant individuals is usually raised with their abdomen extended and their chelipeds are most often raised with the chelae open to easily grasp any offered object. The opposite was found for subordinate crayfish. To each postural category, we assigned a decreasing score from 2 to 1 with 1.5 for an intermediate posture. So, for each animal and for each record we calculated a general postural score, computed as the sum of the scores of the four postural categories as above. The maximum score, 8, denoted the typical behaviour of a dominant individual, while the minimum, 4, that of a subordinate.

Chelar force was measured before Phase 2 (familiarization) and 30, 90 and 150 min after the injections. By chelar force we mean the force exerted by the two dactyli of the crayfish major chela on a rubber tube. Following in part Gabbanini et al. (1995), chelar force was measured using an electronic device (FCE, Florence), consisting of a gauge and a sensor connected to a voltmeter. The sensor was a rubber tube crossed by a beam of infra-red light; the force exerted on the tube reduces the cross-sectional area of the tube and, therefore, decreases the intensity of the light arriving at the detector. The detector transforms light intensity into electric signals measured by the voltmeter. To translate the recorded voltage to the force exerted, we applied the equation:  $F = av + bv^2$  where  $v$  is the voltage recorded on the voltmeter (mV),  $F$  (in N) is the force exerted, and  $a$  and  $b$  are constants ( $a = 0.221$ ;  $b = 431.4 \times 10^{-6}$ ). Each test animal was induced to grasp the rubber tube with its major chela in three successive trials for every measure. The highest value was used for the analysis.

### Experiment 2: Effects of Bioamines on Ranks

After the familiarization phase, the members of 60 pairs were placed back into the same individual aquaria as used in Phase 1 and randomly assigned to one of the following treatments.

(1) 'control pairs' (CP,  $N = 20$ ). Physiological solutions (0.03 ml/g), prepared as above, were injected into the haemolymph of both alphas and betas.

(2) 'reinforced pairs' (RP,  $N = 20$ ). A serotonin solution (0.03 ml/g) and an octopamine solution (0.03 ml/g), prepared as above, were injected into the haemolymph of alphas and betas, respectively, to obtain 'serotonized' alphas and 'octopaminized' betas.

(3) 'inverted pairs' (IP,  $N = 20$ ). An octopamine solution (0.03 ml/g) and a serotonin solution (0.03 ml/g) as



prepared above were injected into the haemolymph of alphas and betas, respectively, to obtain 'octopaminized' alphas and 'serotonized' betas.

The injected crayfish were left undisturbed for 30 min to acquire a normal posture. Meanwhile, the experimental aquaria were washed and the water was changed. The original pairs were reconstituted after no more than 35 min from the injection. After 10-min acclimatization as above, crayfish behaviour was video-recorded for 2 h.

## Data Recorded

During Phases 2 and 3 of experiment 2, we recorded the following parameters.

(1) Number of fights. A fight began when one crayfish approached the rival and ended when one of the opponents retreated to a distance of 10 cm for at least 10 s (Gherardi & Daniels 2003).

(2) Total duration of fights (s).

(3) Percentage of dominance for each individual (the number of fights won as a percentage over the total fights fought).

(4) Behavioural patterns (as described by Bruski & Dunham 1987) performed by alphas and betas: in particular, we recorded the strong contacts (chela strikes and interlocked) that allowed us to estimate the aggressiveness of each animal.

## Statistical Analyses

We followed Zar (1984) and Siegel & Castellan (1988). Data from experiment 2 were analysed among three time periods, each lasting 1 h, that is, the familiarization phase and the first and second hour of Phase 3. The data were checked for homogeneity of variance using the Levene test. For those measures that met the assumptions for parametric analyses (i.e. the percentage of dominance), the data were first normalized using the arcsine ( $\sqrt{x}/100$ ) transformation and then, to correct temporal autocorrelations arising from measurements repeated in time and to prevent temporal pseudoreplication, a three-way, two-way or one-way repeated measures ANOVA (statistic:  $F$ ) was used. If the difference among samples was significant after ANOVA, we applied the Student Newman Keuls' multiple comparisons test (SNK) to determine which pairs differed significantly, while the comparison between alphas and betas was made by paired samples Student's  $t$  test (statistic:  $t$ ).

When the assumptions for parametric analyses were not met (in the cases of the postural scores, the duration and number of fights, and the number of strong contacts), independent samples (i.e. alphas and betas) were compared using Mann–Whitney  $U$  tests (statistic:  $U$ ), while the difference among treatments (i.e. CP, RP, IP) were analysed using Kruskal–Wallis analyses of variance (statistic:  $H$ ). For dependent samples (e.g. the frequencies of the strong contacts or the number and duration of fights through time) we used Friedman two-way analyses of variance (statistic:  $Fr$ ). If the difference among samples was significant after Kruskal–Wallis and Friedman analyses,

we applied multiple comparisons tests to determine which pairs differed significantly. In the case of strong contacts, the interaction between the rank and the treatments was analysed using the nonparametric two-factor analysis of variance Scheirer–Ray–Hare (statistic:  $H$ , Zar 1984).

Text and figures give means (and SE) for percentages of dominance and medians (and interquartile ranges, first–third quartiles) for the other measures. The level of significance at which the null hypothesis was rejected is  $\alpha = 0.05$ .

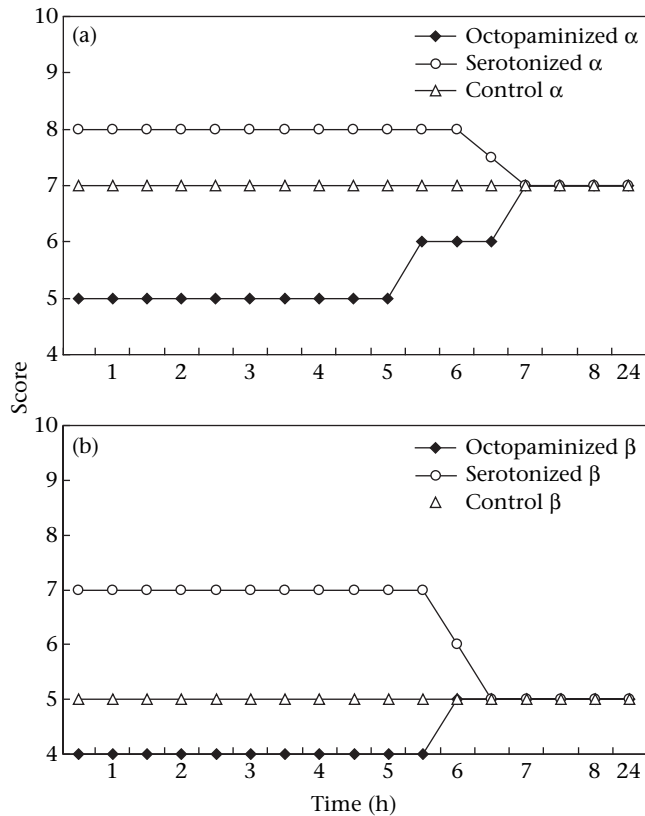
## RESULTS

### Experiment 1: Duration and Effects of Bioamines on Posture and Force

Bioamines made the posture of a given hierarchical rank significantly more conspicuous, serotonized alphas obtaining higher scores than serotonized betas and control alphas ( $H_2 = 31.66$ ,  $P < 0.0001$ ; after multiple comparisons test: serotonized alphas > control alphas > serotonized betas) and octopaminized betas lower scores than octopaminized alphas and control betas ( $H_2 = 27.06$ ,  $P < 0.0001$ ; after multiple comparisons test: octopaminized alphas > control betas > octopaminized betas). However, alphas and betas (serotonized, octopaminized, or control) did not differ in terms of their chelar force (alphas: 0.003–0.012 N; betas: 0.002–0.010 N;  $U$  ranged between 424 and 411,  $N = 40$  for each comparison;  $P > 0.05$ ), either before or after the treatments ( $Fr_4 = 7.20$ ,  $P = 0.13$ ). The effects of the bioamines on crayfish posture remained unchanged for about 6 h and then significantly decreased (octopaminized alphas:  $Fr_{16} = 154.34$ , octopaminized betas:  $Fr_{16} = 155.30$ ; serotonized alphas:  $Fr_{16} = 143.38$ ; serotonized betas:  $Fr_{16} = 137.81$ ;  $P < 0.001$ ; after multiple comparisons test, the hierarchy for serotonized alphas and betas was: 0–6 h 30 min > 7 h > 8 h = 24 h; for octopaminized alphas was: 24 h = 8 h > 7 h > 6 h 30 min > 0–5 h; for octopaminized betas was: 24 h = 8 h = 7 h > 6 h 30 min > 0–5 h), while the control individuals maintained the same posture all the time (control alphas:  $Fr_{16} = 0.53$ ,  $P = 0.47$ ; control betas:  $Fr_{16} = 2.88$ ,  $P = 0.09$ ) (Fig. 1a, b).

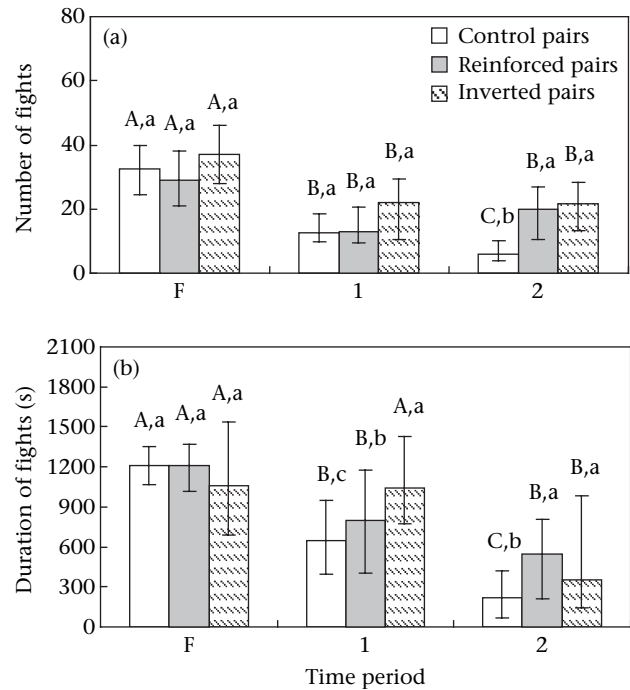
### Experiment 2: Effects of Bioamines on Ranks

The number and duration of the fights significantly changed with time (Fig. 2a,b, Table 1). While both measures constantly decreased in CP from the familiarization to the end of the experiment, fight number, in RP and IP, and duration, in RP, underwent a significant reduction with the injection of the bioamines but remained constant throughout the whole of Phase 3. Only in IP did fights decrease their duration in the second hour of Phase 3. If compared to the control, the number of fights fought by RP and IP were relatively more numerous in the second hour of Phase 3 and longer throughout the whole of Phase 3. Fights were even longer in IP than in RP, at least during the first hour after the treatment.



**Figure 1.** Effects of bioamines over time on the general posture of alpha (a) and beta (b) individuals that have been subject to injections of serotonin (serotonized alphas:  $N = 10$  and betas:  $N = 10$ ), octopamine (octopaminized alphas:  $N = 10$  and betas:  $N = 10$ ), or physiological solutions (control alphas:  $N = 10$  and betas:  $N = 10$ ). General posture was assessed by computing a score from four categories that took into account body height, abdomen extension, cheliped height and chelar openness.

A three-way ANOVA showed a significant interaction between treatments, ranks, and time for the percentage of dominance ( $F_{4,341} = 13.92$ ,  $P < 0.0001$ ). Specifically, in CP and RP dominance was not affected by the injected



**Figure 2.** Medians (and interquartile ranges) of (a) the number and (b) the duration of fights in the three treatments (control pairs:  $N = 20$ ; reinforced pairs:  $N = 20$ ; and inverted pairs:  $N = 20$ ), during three time periods (i.e. familiarization phase, F; first, 1, and second, 2, hour of the experimental phase). Capital letters over bars denote the hierarchy among time periods within each pair, small letters, the hierarchy among the three treatments within each time period, both obtained by multiple comparisons tests.

solutions, but it slightly changed with the time of cohabitation (Table 2); however, it was always significantly higher in alphas than in betas (Fig. 3a, b, Table 2). On the contrary, after the injection of the bioamines in IP, betas increased (and alphas decreased) their dominance during the first hour of Phase 3 (Fig. 3c, Table 2), reaching

**Table 1.** Number and duration of fights: comparisons among the three treatments (CP, RP, IP;  $N$  for each treatment = 20) within each time period and comparisons among time periods (F, 1, 2) within each treatment

	Number			Duration		
	H/Fr	P	Hierarchy	H/Fr	P	Hierarchy
Comparisons among treatments						
F	5.08	0.08	CP=RP=IP	0.03	0.10	CP=RP=IP
1	4.90	0.09	CP=RP=IP	8.19	<b>0.02</b>	IP>RP>CP
2	17.98	<b>&lt;0.001</b>	RP=IP>CP	9.26	<b>0.01</b>	CP<RP=IP
Comparisons among time periods						
CP	22.51	<b>&lt;0.001</b>	F>1>2	29.20	<b>&lt;0.001</b>	F>1>2
RP	9.80	<b>0.008</b>	F>1=2	10.00	<b>0.007</b>	F>1=2
IP	13.85	<b>0.001</b>	F>1=2	9.10	<b>0.01</b>	F>1=2

The three treatments: CP = control pairs; RP = reinforced pairs; IP = inverted pairs.

Time periods: F = familiarization; 1 = first hour of Phase 3; 2 = second hour of Phase 3.

For comparisons among the three treatments, we used Kruskal–Wallis analyses of variance (statistic:  $H$ ;  $df = 2$ ), while for comparisons among time periods we used Friedman two-way analyses of variance (statistic:  $F$ ;  $df = 2$ ). Both analyses were followed by multiple comparisons tests. Significant differences are shown in bold.

**Table 2.** Percentage of dominance and number of strong contacts: comparisons among time periods (F, 1, 2) within each of the three treatments (CP, RP, IP;  $N$  for each treatment = 20) per rank (alpha and beta); comparisons between ranks (alpha,  $N = 20$ , and beta,  $N = 20$ ) among the three treatments (CP, RP, IP) per time period (F, 1, 2)

		Dominance			Strong contacts		
	Rank	$F/t$	$P$	Hierarchy	$Fr/U$	$P$	Hierarchy
Comparisons among time periods within treatments							
CP	$\alpha$	1.64	0.20	$F=1=2$	33.31	<b>&lt;0.001</b>	$F>1>2$
	$\beta$	7.58	<b>0.001</b>	$F>1>2$	31.50	<b>&lt;0.001</b>	$F>1>2$
RP	$\alpha$	2.23	0.12	$F=1=2$	12.33	<b>0.002</b>	$F=1>2$
	$\beta$	7.28	<b>0.002</b>	$F>1>2$	33.42	<b>&lt;0.001</b>	$F>1>2$
IP	$\alpha$	5.66	<b>0.006</b>	$F=2>1$	29.50	<b>&lt;0.001</b>	$F>1>2$
	$\beta$	5.70	<b>0.006</b>	$1>F=2$	17.41	<b>&lt;0.001</b>	$1>F=2$
Comparisons between ranks among treatments							
	Time period						
CP	F	10.98	<b>&lt;0.001</b>		16.50	<b>&lt;0.001</b>	
	1	14.63	<b>&lt;0.001</b>		60.00	<b>&lt;0.001</b>	
	2	11.15	<b>&lt;0.001</b>		102.50	<b>0.003</b>	
RP	F	17.12	<b>&lt;0.001</b>		121.50	<b>0.03</b>	
	1	26.16	<b>&lt;0.001</b>		52.50	<b>&lt;0.001</b>	
	2	15.70	<b>&lt;0.001</b>		75.00	<b>&lt;0.001</b>	
IP	F	20.59	<b>&lt;0.001</b>		99.00	<b>0.006</b>	
	1	0.28	0.78		92.00	<b>0.003</b>	
	2	2.12	<b>0.04</b>		152.50	0.20	

The three treatments: CP = control pairs; RP = reinforced pairs; IP = inverted pairs.

Time periods: F = familiarization; 1 = first hour of Phase 3; 2 = second hour of Phase 3.

For the percentage of dominance we used two-way repeated measures ANOVAs (statistic:  $F$ ;  $df = 2$ ), followed by Student Newman Keuls' multiple comparisons for the comparisons among time periods, and paired samples Student's  $t$  tests (statistic:  $t$ ) for the comparisons between ranks. For the number of strong contacts we used Friedman two-way analyses of variance (statistic:  $F$ ;  $df = 2$ ), followed by multiple comparisons tests for the comparisons among time periods, and Mann-Whitney  $U$  tests (statistic:  $U$ ) for the comparisons between ranks. Significant differences are shown in bold.

the same level as the opponents (Table 2). However, except for five pairs (out of 20), betas were not able to reverse their status. In the second hour after the treatment, the original alphas reached the same dominance level as in the familiarization phase (Fig. 3c, Table 2).

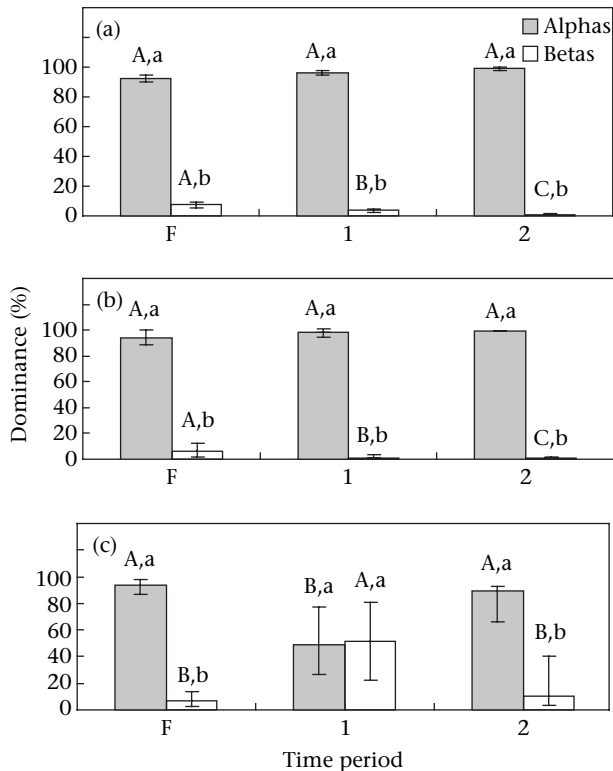
The number of the strong contacts executed by crayfish while fighting significantly differed between ranks during the familiarization phase, but not among treatments ( $H_{\text{rank } 1} = 35.71$ ,  $P < 0.001$ ;  $H_{\text{treatment } 2} = 5.10$ ,  $0.05 < P < 0.1$ ;  $H_{\text{interaction } 2,116} = 2.08$ ,  $P > 0.1$ ). However, a significant interaction between rank and treatment was found in the Phase 3 (first hour:  $H_{\text{rank } 1} = 6.96$ ,  $P < 0.01$ ;  $H_{\text{treatment } 2} = 20.03$ ,  $P < 0.001$ ;  $H_{\text{interaction } 2,116} = 27.44$ ,  $P < 0.001$ ; second hour:  $H_{\text{rank } 1} = 7.38$ ,  $P < 0.01$ ;  $H_{\text{treatment } 2} = 15.53$ ,  $P < 0.001$ ;  $H_{\text{interaction } 2,116} = 11.20$ ,  $P < 0.01$ ). In CP and RP, the number of strong contacts made was always higher in alphas than betas and constantly decreased with time (Fig. 4a, b, Table 2), except for the alphas in RP (Fig. 4b).

Interestingly, the serotoninized betas executed more numerous strong contacts during the first hour after the treatment than in the familiarization phase, and more often than both the octopaminized alphas and the serotoninized alphas analysed in the same time period ( $U = 122.5$ ,  $N = 40$ ,  $P = 0.04$ ; Fig. 4c, Table 2). However, in the second hour of Phase 3, the frequencies of strong contacts significantly decreased, reaching the same value in the two ranks ( $U = 166.5$ ,  $N = 40$ ,  $P = 0.4$ ), whereas octopaminized alphas appeared more aggressive than octopaminized betas in the whole of Phase 3 ( $U = 64$ ,  $U = 77$ ,  $N = 40$ ,  $P < 0.001$ ).

## DISCUSSION

Previous research on the behavioural changes induced in crayfish by exogenous bioamines (e.g. Livingstone et al. 1980; Huber et al. 1997a, b; Huber & Delago 1998) offered different results and divergent interpretations (Kravitz & Huber 2003). Our study analysed for the first time the effects of both serotonin and octopamine on the agonistic behaviour of crayfish that have had prior social experience. Consistent with the findings obtained for *Homarus americanus* (Livingstone et al. 1980), *Astacus astacus* (Huber et al. 1997a, b; Huber & Delago 1998), and *Munida quadrispina* (Antonsen & Paul 1997), we showed that the injection of bioamines modified the posture and the aggressive level of the treated *P. clarkii*, but also found that these effects did not lead to a permanent inversion of the dominance hierarchy as we might expect. On the contrary, when octopaminized alphas were confronted with serotoninized betas, the original hierarchy was reconstituted only 1 h after the treatment.

Contrary to McRae (1996), who showed that females of *Cherax destructor* display submissive postures if injected with serotonin and dominant postures if injected with octopamine, in *P. clarkii* exogenous serotonin and octopamine induced the expression of dominant and subordinate postures, respectively, independent of the hierarchical rank of the treated crayfish. However, the acquired rank of an individual enhanced the effects of the bioamines, making its postural responses more conspicuous, as evident from the comparison between treated and

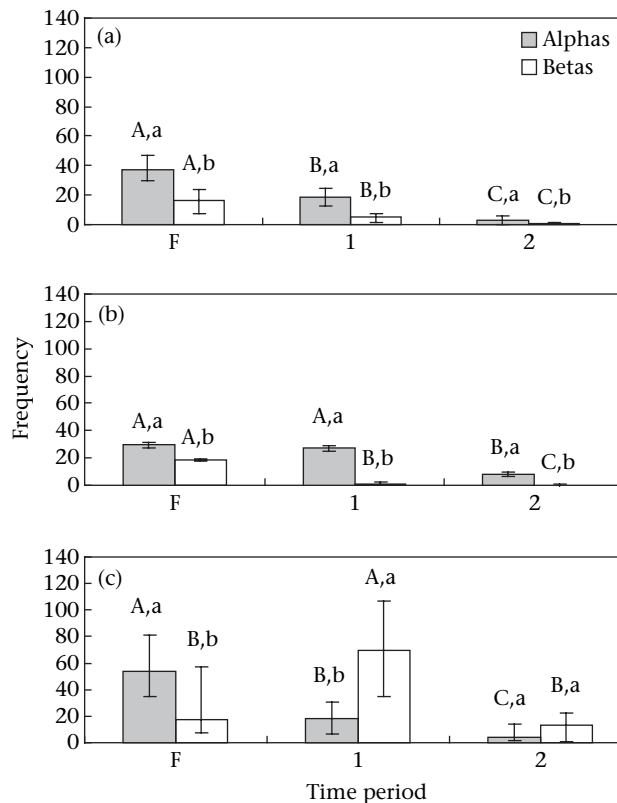


**Figure 3.** Means (and SE) of percentage of dominance recorded in (a) control pairs ( $N = 20$ ), (b) reinforced pairs ( $N = 20$ ) and (c) inverted pairs ( $N = 20$ ) during the familiarization phase (F) and in the first (1) and in the second (2) hour of the experimental phase. Capital letters over bars denote the hierarchy among time periods within each rank after the Student Newman Keuls' multiple comparisons, small letters, the hierarchy between alphas and betas within each time period after a paired samples Student's  $t$  test.

control crayfish. Nevertheless, the bioamines did not affect its chelar force.

As expected, in the control and in the reinforced pairs, the number and duration of fights decreased with time and alphas slightly increased their percentage of dominance. On the contrary, in the inverted pairs the number of fights decreased but their duration increased. Certainly, the higher motivation to fight shown by the serotoninized betas, combined with the not completely inhibited aggressiveness of the octopaminized alphas, increased the duration of fights in the inverted pairs, especially within the first hour after the treatment, as observed also in *Astacus astacus* (Huber et al. 1997b; Huber & Delago 1998).

A higher (or lower) dominance level was recorded in serotoninized betas (or octopaminized alphas) but this effect lasted for only an hour after the treatment. During the second hour of the experimental phase, the original alphas reached the same dominance level as in the familiarization phase, a phenomenon that was unlikely to have been caused by a reduction in the effects of the bioamines that, on the contrary, lasted about six hours (i.e. three times longer than the duration of our observations). Notwithstanding that serotoninized betas showed a higher aggressiveness, the large majority of the analysed pairs did not reverse their hierarchical rank. This was also



**Figure 4.** Medians (and interquartile ranges) of the number of strong contacts recorded in (a) control pairs ( $N = 20$ ), (b) reinforced pairs ( $N = 20$ ), and (c) inverted pairs ( $N = 20$ ) during the familiarization phase (F) and in the first (1) and in the second (2) hour of the experimental phase. Capital letters over bars denote the hierarchy among time periods within each rank, small letters, the hierarchy between ranks within each time period, both obtained by multiple comparisons tests.

observed in subordinate *Astacus astacus* individuals, which, once infused with serotonin, advanced their rank; however, this effect was transient and completely reversible in the majority of the cases, with the former dominant re-establishing its position (Huber et al. 1997b; Huber & Delago 1998).

Serotonin increased crayfish aggressiveness, in terms of the number of strong contacts executed, as previously observed in *Astacus astacus* (Huber et al. 1997a, b; Huber & Delago 1998). In fact, immediately after the injection of serotonin, strong contacts were executed more often in the serotoninized individuals than in the control ones. Octopamine had the effect of decreasing aggressiveness, even if octopaminized alphas still executed strong contacts more often than octopaminized betas. On the contrary, due to the phenomenon of 'social inertia' (Guhl 1968), their occurrence decreased in both members of the control pairs.

Other studies obtained different results than ours. For example, Tierney & Mangiamale (2001) found that the level of aggression in *P. clarkii* was reduced by serotonin, being, however, enhanced by a serotonin analogue, 5-carboxamidotryptamine maleate. In *Homarus americanus* juveniles, low doses of 5-HT (<3 mg/kg) did not lead to any effect on dominance and competition, while higher



doses (>3 mg/kg) produced subordinate animals (Peeke et al. 2000). Finally, specimens of *Orconectes rusticus* implanted with 5-HT were indistinguishable from the controls in terms of fighting behaviour when the rate of the substance release was slow, but were more aggressive when it was fast (Panksepp & Huber 2002).

Vertebrates have been the widespread subject of experiment manipulations of rank. When lizards were treated with serotonin or sertraline (a selective 5-HT reuptake inhibitor), mixed results were obtained (Larson & Summers 2001) and only occasional were inversions of dominance found. When dyads of the dark-eyed junco, *Junco hyemalis*, were subject to colour changes in their plumage, the subordinates initially won most fights, but in the end the original dominant individuals started to win again (Grasso et al. 1996). A likely explanation was that the colour of plumage alone does not suffice to determine dominance, appropriate behaviour being possibly needed, as shown in *Zonotrichia querula* (Rohwer & Rohwer 1978), where subordinates, both dyed and injected with testosterone, became successful cheaters. However, although an individual can fake a badge of dominance, it may be incapable of equating its fighting ability to its faked status and therefore it is disadvantaged by the disproportionate costs it encounters (Grasso et al. 1996). For instance, the house sparrow, *Passer domesticus*, once experimentally implanted with testosterone, showed reduced survival, possibly because of its increased energy consumption due to an excessive breeding effort it was constitutionally unable to sustain (Veiga 1995).

From the results of our study we can hypothesize that a similar phenomenon may occur in crayfish. The subordinate crayfish treated with serotonin display the typical behaviour and posture of dominant individuals, but they have neither the physical capability nor the experience to sustain the role of an alpha. The prolonged fighting, particularly expensive when conducted against similarly strong opponents, could result in losing time and energy, and contemporaneously increases the risks of injury that eventually may lead to death. In the end, the former dominance relationship is the best compromise with minimal costs and it is most often re-established, except in five cases in which betas were, however, particularly aggressive during the familiarization phase.

It is likely that intrinsic characteristics of crayfish other than body size, weight and chelar dimensions determine the structure of dominance hierarchies in decapods. For instance, in the American lobster, *Homarus americanus*, the outcome of contests between size-matched individuals was predicted from subtle cues such as plasma protein level and exoskeleton calcium concentration (Vye et al. 1997). These variables are not clearly visible to the rivals, but fighting lobsters may indirectly assess them by the effect of the above variables on claw contraction forces, the resistance of the exoskeleton to pressure, and general fighting vigour (Vye et al. 1997). We did not measure these variables, so we cannot exclude their relevance in establishing dominance order and in maintaining it after the injection of bioamines.

Our study suggests that also prior experience of wins/losses might exert a higher effect than bioamines on the

long-lasting maintenance of dominance in crayfish. This hypothesis is in part confirmed by previous physiological studies (Yeh et al. 1996, 1997). Different populations of serotonin receptors, responsible for escape responses, were found to occur in the lateral giant neurons of crayfish (Yeh et al. 1996, 1997; Edwards & Kravitz 1997; Kravitz 2000) and seemed to be either transformed or replaced following a change in social status (Yeh et al. 1997). Huber (2005) hypothesized that compensatory mechanisms might counteract the altered functions of serotonergic neurons as induced by chronic infusion of serotonin in social crayfish (e.g. Panksepp & Huber 2002). We may expect that a similar mechanism, although still unexplored, functions also for octopamine.

An alternative hypothesis is that bioamines are not the only, and possibly not the most important, hormonal substance influencing aggression in crustaceans (Kravitz 2000; Peeke et al. 2000; Panksepp & Huber 2002). Recent studies have demonstrated close interactions between amines, neurons and circulating steroids (e.g. Bonson et al. 1994; Edwards & Kravitz 1997; Ferris et al. 1997). Some authors supported the involvement of dopamine in agonistic behaviour (Barthe et al. 1989) and L-dopa, a precursor for dopamine, has been shown to affect the social status of fish (Winberg & Nilsson 1992). Also 5-carboxamidotryptamine maleate has been reported to enhance the performance of several agonistic behaviours (Tierney & Mangiamele 2001). The moulting hormone ecdysone is another candidate, since lobster fighting and escape behaviours change dramatically over the moult cycle (Kravitz 2000).

In synthesis, the issue of agonistic behaviour in crayfish is still full of questions and doubts. Further studies are clearly needed to investigate all the alternative hypotheses and to clarify the factors that are responsible for the initial establishment of dominance, its maintenance, and its restoration, and the nature of the chemicals involved in crayfish aggression.

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