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Individual aggressiveness in the crab *Chasmagnathus*: Influence in fight outcome and modulation by serotonin and octopamine

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ABSTRACT

In a previous work we found that size-matched *Chasmagnathus* crabs establish winner–loser relationships that were stable over successive encounters but no evidence of escalation was revealed through fights. Here, we evaluated the hypothesis that size-matched fights between these crabs would be resolved according to the contestants' level of aggressiveness. Moreover, we aim at analysing the proximate roots of aggression, addressing the influence of the biogenic amines serotonin (5HT) and octopamine (OA) in crab's agonistic behaviour. To achieve these purposes, the following experiments were carried out. First, we performed successive fight encounters between the same opponents, varying the number of encounters and the interval between them, to assess the stability and progression of the winner–loser relationship. Then, we analysed dominance relationships in groups of three crabs, evaluating the emergence of linearity. Thirdly, we examined the effects of 5HT and OA injections over the fight dynamics and its result. Our findings show that contest outcome is persistent even through four encounters separated by 24 h, but a comparison between encounters does not reveal any saving in fight time or increase in the opponent disparity. Within a group of crabs, a rank-order of dominance is revealed which is reflected in their fight dynamics. Interestingly, these results would not be due to winner or loser effects, suggesting that fight outcome could be mainly explained as resulting from differences in the level of aggressiveness of each opponent. Moreover, this individual aggressiveness can be modulated in opposite directions by the biogenic amines 5HT and OA, being increased by 5HT and decreased by OA.

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1. Introduction

Aggressive interactions between conspecifics are found in a wide range of animal taxa, serving in the acquisition or defense of vital resources such as food, shelter, or access to mates [1–3]. A fight can lead to a dominance relationship which, in some animal species, is stable and becomes more pronounced through successive encounters, up to the point of forming part of a social hierarchy [4]. Dominance relationships are based on differences between the contenders either in intrinsic factors, i.e., individual attributes that modify fighting ability; or in extrinsic factors, such as previous agonistic experience that include winner and loser effects [5–9]. Moreover, within a group, dominance relationships can lead to a linear structure where one individual dominates all the others; a second dominates all but the first; and so on. It is suggested that such linearity would be accounted either by differences in prior attributes or instead by the dynamics of social interaction among group members [4]. Individual attributes that influence fight result are of varied sorts, depending on study and species, but body size, age, sex, reproductive state, and the level of aggressiveness are among the most recurrent [10–12]. While most of

these factors can be assessed by the experimenter, the level of aggressiveness is usually obscured by other traits, making it difficult to determine its relative contribution to the fight outcome.

Crustacean species represent excellent study systems to explore the structure of aggression and its causation [13–18]. In a typical fight between a size-matched pair of animals, they exchange stereotypical behaviours that escalate until one of the contestants withdraws, resulting in dominant and subordinate individuals. Subsequent fights between the same contenders become shorter, until fighting is avoided altogether by the retreat of the subordinate in the face of the dominant's advance [19]. That is, fight generates an agonistic status, which is kept by individual or status recognition [16,20–22].

We started to explore the agonistic behaviour of the crab *Chasmagnathus granulatus*, a species that has been extensively studied in our laboratory with respect to learning and memory processes [23–25]. In a previous work, we found that size-matched male crabs display a conspicuous agonistic behaviour and establish winner–loser relationships that were stable over successive encounters [26]. However, no ritualized aggression was shown throughout the fights, and our prior results did not suggest any changes in the contest intensity from a first to a second encounter, which contrasts with a typical case of dominance status. These findings led us to propose the hypothesis that size-matched fights in *Chasmagnathus* would be resolved according to the contestants' level of

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aggressiveness. Moreover, this would offer unique vistas for a search for the proximate roots of aggression.

Serotonin (5HT) and octopamine (OA) are considered key physiological regulators of agonistic behaviour in crustaceans, where 5HT is generally associated with a dominant role and OA with a subordinate one [27]. When injected into the hemolymph of lobsters, crayfishes and squat lobsters, 5HT produces a characteristic posture resembling that of dominants, whereas OA causes a posture typical of subordinate individuals [28–30]. Besides, an infusion of 5HT was proposed to affect the aggressive motivation in crayfish, with subordinates becoming more likely to initiate encounters and less likely to withdraw [31–33].

Here, three lines of experiments with the crab *Chasmagnathus* are shown. Firstly, we performed successive fight encounters between the same opponents, varying the number of encounters and the interval between them, to assess the stability and progression of the dominance relationship. Secondly, we analysed dominance relationships in groups of three crabs staging dyadic encounters between all members of the group. By performing fight encounters between unfamiliar opponents we could evaluate the influence of winner and loser effects over the outcome of a fight. Besides, the analysis of all the fights of each triad allowed us to evaluate the emergence of linearity and its expression in the agonistic performance of each member of the triad. Finally, we analysed the influence of the biogenic amines 5HT and OA over the fight outcome and its dynamics. Our results suggest that fight outcome in *Chasmagnathus* could be mainly explained as resulting from differences in the level of aggressiveness of each opponent in a size-matched contest. Furthermore, we show that this individual aggressiveness could be modulated in opposite directions by the biogenic amines 5HT and OA, being increased by 5HT and decreased by OA.

2. Material and methods

2.1. Animal housing conditions

Animals were adult male crabs *C. granulatus* (Dana, 1851), of 2.7–3.0 cm across the carapace, weighing around 17.0 g, collected from water less than 1 m deep in narrow coastal inlets of San Clemente del Tuyú, Argentina, and transported to the laboratory. They were lodged initially in plastic tanks (35×48×27 cm) filled to 2-cm depth with diluted marine water, to a density of 20 crabs per tank. When isolated, animals were kept in individual opaque containers (12 cm diameter, 15 cm height). Water used in tanks and containers during experiments was prepared using hw-Marinex (Winex-Germany), salinity 10–14‰, pH 7.4–7.6, and maintained within a range of 22–24 °C. The holding and experimental rooms were maintained on a 12 h light–dark cycle (light on 07:00–19:00 h). Animals received no food until experiments were concluded; afterwards they were fed rabbit food pellets *ad libitum*. None of the crabs was harmed during the course of this investigation, and at the conclusion of the study they were returned to their original place of capture. Principles of laboratory animal care were followed in all cases (NIH publication no. 85-23, revised in 1985).

2.2. Animal selection

We selected only mature intermoult males in pristine conditions, i.e., hard exoskeleton, no epifaunal growth, no missing or recently regenerated limbs, no obvious shell disease or carapace punctures, and no apparent asymmetry of chelipeds [34]. Thus, animals were uniform in these characteristics that could otherwise influence their fight performance [35].

2.3. General experimental procedure

Animals were isolated in individual opaque containers for 6 days (unless otherwise noted) before the experiment. Previous works have shown that this isolation period can increase aggressiveness [36–38]

and also remove any prior social effects [16,39]. We staged dyadic encounters between male crabs matched to within 1 mm for both carapace width and claw length. One member of the dyad was marked with a small dot of White Out (BIC) on its carapace. Each animal of a pair was moved from its respective individual container to the opposite sides of an experimental arena, where the encounter took place. The arena consists of an opaque plastic box (12.5×25×15 cm), illuminated from above. Previous results [26] demonstrated that *Chasmagnathus* crabs fight readily in these laboratory conditions, with an agonistic repertoire comparable to the one observed in the field (Fathala M.V., personal communication). The encounter duration was 10 min, since limited interactions were shown after such time period. When the encounter finished, crabs were moved from the arena back to their respective individual opaque containers. We recorded the encounters by means of a Sony digital camcorder DCR-TRV22. When watching the films, the observer was blind to the experimental conditions of the contestants. A computer was used as an event recorder and custom designed software allowed us to record the time each animal spent in each category of agonistic behaviour.

2.4. Categories of agonistic behaviour

We defined the following categories of agonistic behaviour to evaluate the animal's performance during the encounters. See Supplementary material (Movie 1 SM) for a brief extract of a fight encounter, indicating all these behavioural categories.

Approach stands for a walk of the animal towards the opponent, either facing it or not, either leading or not to contact it.

Attack stands for sudden movements of one animal towards another that lead to physical contact. It includes diverse kinds of physical contact between the chela (e) and the opponent body, as jabbing (vigorous and rapid touching), pushing or enveloping (a movement of one or both chelae to partially or totally embrace a conspecific).

Retreat stands for the movement of one animal away from the other in response to an attack or approach; including escapes, i.e., quick retreats.

Approaches and attacks are considered as “dominant acts”, while retreats as “submissive acts”.

Apart from these three items of behaviour, we distinguished a fourth category of acts, the “non-agonistic behaviours”, that includes wandering (i.e., movements not oriented towards its opponent) and resting.

Preliminary observations revealed that fight initiation did not predict fight outcome in *Chasmagnathus* size-matched fights. Thus, we did not include this variable in the current study.

2.5. Evaluation of agonistic performance

We coined diverse indexes for assessing the agonistic performance of each opponent during the encounter, to establish which is the winner and which is the loser and to compare fight intensity and duration between encounters.

The individual dominance level (IDL) for each opponent is the difference between the total time of dominant acts (approaches plus attacks) and the total time of submissive acts (retreats), during the whole encounter period. The contender with the higher IDL is termed the winner and the other the loser.

In contrast with the IDL, all the following estimations involve measurements corresponding to both contenders.

The total interaction time (TIT) adds up the total time both contenders spend in performing agonist acts, either dominant or submissive acts, during the encounter.

The disparity index (DI) stands for the asymmetry in the behaviour of the contenders during the time they interact. We distinguished between “positive” or “negative” agonistic acts, according to their consistence with the agonistic condition of each animal of the dyad (loser or winner). The DI is the difference between the total time of the

205 “positive” acts (dominant acts of the winner plus submissive acts of the
206 loser) and the “negative” acts (dominant acts of the loser plus
207 submissive acts of the winner), over $TIT \times 100$. This value is a
208 dimensionless measure that ranges from 0 to 100. A 100% would
209 represent a case where the winner only performed dominant acts, and
210 the loser only submissive acts, that is, only “positive” acts that reinforce
211 the dyadic asymmetry. We only included in our analysis the encounters
212 where the winner and loser roles could be clearly differentiated
213 (a DI higher than 20%).

214 2.6. Experimental protocols

215 2.6.1. Successive fight encounters between the same opponents

216 In this series of experiments we analysed the course of a dyadic
217 relationship through successive fight encounters between the same
218 opponents, assessing its stability and progression. Animals remained
219 isolated for 6 days (except in experiment D), after which they were
220 staged in a fight arena with a sized-matched contestant. Then, crabs
221 were re-isolated and after an interval of at least 24 h they were staged
222 again with the same contestant. The following experimental variables
223 were modified in each case (Table 1, Experimental procedure): 1 –
224 between-encounters interval: 24, 48 or 72 h was used between two
225 fight encounters (exp. A, B, C, respectively); 2 – isolation time: animals
226 were isolated for 14 days before the first fight took place (exp. D); 3 –
227 number of fight encounters: animals underwent four encounters, each
228 separated by 24 h (exp. E).

229 2.6.2. Dominance relationships in groups of three crabs

230 In this experiment we used 37 groups of three individuals each
231 (triads), staging dyadic encounters between all members of the group
232 (round-robin competition [4]) separated by a 24 h interval. Unlike the
233 previous series of experiments, in this case animals confront a new
234 opponent in each encounter. The encounters were preceded by 6 days of
235 isolation and were separated by a 24 h interval from each other. The first
236 fight was staged between a randomly selected pair; the second one
237 included one of the former opponents (named focal crab, randomly
238 chosen) and the other crab of the triad; finally a third fight was staged, in
239 such a way that all the crabs have met one another. The rationale for
240 using separate dyadic encounters is that it allows individual qualities
241 (such as the level of aggressiveness) to control the outcome of the
242 contests, and prohibits all the other interactions that might occur
243 normally in a group context and affect the results of the fights [4].

244 2.6.3. Influence of 5HT and OA on crab's agonistic behaviour

245 We examined the effect of OA and 5HT over the dynamics and the
246 outcome of the fights. Two experiments were performed. In the first

247 one, one of the animals of the dyad received an injection of 5HT 1 mM
248 (5HT group, N=66), or OA 4 mM (OA group, N=51), whereas its
249 opponent received a saline injection. Besides, we included a control
250 group (SAL, N=60) where both animals received a vehicle admin-
251 istration. In the second experiment, both opponents received the
252 same treatment: OA 4 mM (OA group, N=36), or saline (SAL group,
253 N=32). In all cases, the injections were applied 15 min before the
254 fights took place. The fighting period in these experiments was
255 extended to 15 min.

256 2.7. Drugs and injection procedure

257 Crustacean saline solution [40] was used as a vehicle. Fifty microliters
258 of saline or drug solution was given through the right side of the dorsal
259 cephalothoracic-abdominal membrane, by means of a syringe fitted with
260 a sleeve to control the depth of penetration to 4 mm, thus ensuring that
261 the injected solution was released in the pericardial sac. The lack of an
262 endothelial blood–brain barrier in crabs [41], together with the fact that
263 blood is distributed throughout an extensive capillary system [42] makes
264 it possible for the injected drugs to reach the various neuropil areas of the
265 brain. Drug solutions were 4 mM for OA (2.24 $\mu\text{g/g}$) and 1 mM for 5HT
266 (0.6 $\mu\text{g/g}$). However, the final hemolymph drug concentrations were
267 1:100 fold diluted, considering that the hemolymph volume is approx-
268 imately 5 ml [43]. Ascorbic acid 0.1 mM was included in all solutions to
269 prevent oxidation of the amines. Solutions were freshly prepared each
270 time. The amine concentrations used in the present work did not produce
271 any noticeable changes in the animal's posture, exploratory activity or
272 escape response, as demonstrated in a large series of previous experi-
273 ments [44, Pedetta and Maldonado, in preparation]. Octopamine and
274 serotonin were purchased from Sigma (USA).

275 2.8. Data analysis

276 In the first series of experiments, persistence in fight outcome was
277 estimated using a chi-square test, contrasting the obtained values with
278 the 50% expected by chance. The comparison of fighting parameters
279 between encounters was performed by a one-tailed paired t-test or a
280 Wilcoxon matched-pairs test when normality was not met. Only dyads
281 with a persistent outcome were included in this analysis.

282 In the experiment using groups of three crabs, winner and loser
283 effects were evaluated with a chi-square test, contrasting the
284 persistence in fight outcome of the focal crab with the 50% expected
285 by chance. Fight dynamics of the top animal's two encounters (top-
286 second vs. top-last) was compared with a one-tailed paired t-test.

287 In the pharmacological experiments, the amines' effect on fight
288 outcome was evaluated by a chi-square test. Agonistic performances

t1.1 **Table 1**

t1.2 Experimental procedure and between-encounters comparison for the first series of experiments. *Experimental procedure*: isolation period, number of fight encounters, inter-fight
t1.3 interval and number of pairs for each experiment are indicated. *Between-encounters comparison*: persistence in fight result, expressed as the % of persistent dyads, is compared to that
t1.4 expected by chance (50%) by a chi-square test; progression of the dominance relationship is evaluated by comparing total interaction time and disparity index between E1 and E2
t1.5 (one-tailed paired t-test). Data are expressed as mean \pm SE. Significant differences are shown in bold. #: In this case comparisons are performed between E1 and E4.

t1.6	t1.2 Experimental procedure				t1.3 Between-encounters comparison								
	Exp.	Isolation	# of fight encounters	Inter-fight interval	N (pairs)	t1.4 Fight outcome		t1.5 Total interaction time (s)		t1.6 Disparity index (%)			
t1.7						Persistence	Statistics (chi-square)	TIT 1 (mean \pm SE)	TIT 2 (mean \pm SE)	Statistics (t-test)	DI 1 (mean \pm SE)	DI 2 (mean \pm SE)	Statistics (t-test)
t1.8	A	6 days	2	24 h	41	83%	$\chi^2 = 17.68$ p < 0.0001	229.2 \pm 28.22	257.7 \pm 27.71	t = -0.73 p = 0.5	55.1 \pm 5.11	51.3 \pm 5.43	t = 0.53 p = 0.6
t1.9	B	6 days	2	48 h	80	72%	$\chi^2 = 16.2$ p < 0.0001	324.3 \pm 17.78	333.5 \pm 14.03	t = -0.44 p = 0.6	56.7 \pm 3.73	60.6 \pm 3.2	t = -1.06 p = 0.3
t1.10	C	6 days	2	72 h	32	75%	$\chi^2 = 8.00$ p < 0.005	257.0 \pm 36.67	287.3 \pm 27.93	t = -0.80 p = 0.4	54.5 \pm 5.99	44.2 \pm 6.97	t = 1.19 p = 0.2
t1.11	D	14 days	2	48 h	85	80%	$\chi^2 = 30.60$ p < 0.0001	312.0 \pm 37.83	285.1 \pm 34.57	t = 1.36 p = 0.18	58.8 \pm 3.56	66.3 \pm 3.84	t = -1.65 p = 0.1
t1.12	E	6 days	4 (#)	24 h	37	70%	$\chi^2 = 6.08$ p < 0.05	212.7 \pm 28.72	256.2 \pm 33.35	t = -0.96 p = 0.3	47.4 \pm 5.72	53.1 \pm 6.2	t = -0.66 p = 0.5

within each pair were compared by a one-tailed paired t-test. Fighting parameters of 5HT, OA and SAL groups were compared using a one-tailed t-test for independent samples. We normalized all data with respect to the total interaction time of the SAL group, in order to combine experiments from different capture efforts.

In all cases, normality of the data was determined by the Kolmogorov–Smirnov test, and equality of variance by the F-test.

3. Results

3.1. Dominance relationships persist through successive encounters without saving in fight time or increasing dominance disparity

We analysed fight dynamics in a first encounter between size-matched crabs and compared it with a successive one. We modified three different experimental conditions in order to see whether we could disclose an improvement in the dominance relationship between encounters. All procedures, results and statistics of this section are displayed in Table 1 and Table 1 SM (Supplementary material).

Initially, we performed three experiments using 24, 48 or 72 h between encounters. Results show, in all cases, a significantly greater number of persistent dyads than expected by chance. In neither case were there any significant differences between encounters for the total interaction time (TIT) or the disparity index (DI). Secondly, we performed an experiment with 14 days of isolation in the individual containers. The rationale for this procedure is that isolation time is considered to increase the level of aggression [36]; then, if this period were extended, a more intense fight at the first encounter (E1) would be obtained and a progression in the W–L disparity could be found at the second encounter (E2). Results contradict this production, showing values of DI and TIT for the first encounter similar to those shown in the previous experiment with 6 days of isolation. Thus, a longer isolation period previous to E1 induces no higher level of fight during such encounter. Besides, no significant differences were found for DI or for TIT between the two encounters, consistently with findings of the previous experiments. Thirdly, animals underwent four fight encounters separated by 24 h, based on the proposal that a larger number of confrontations between the same animals could result in a change in aggressive behaviour during the last fight [45]. The comparison between the first and the fourth encounters revealed a significant persistence in the fight outcome. However, no significant difference was shown in the fight dynamics between encounters.

Additionally, we performed a detailed analysis of all individuals' performances from this series of experiments (Table 1 SM), comparing approaches, attacks and retreats of winners and losers, in their first and second encounters. No significant differences were shown in any case. Besides, no recurrent trends in dominance disparity through encounters were revealed, supporting our previous findings using a dyadic analysis.

Results of this first series of experiments show, firstly, that the dominance disparity is kept through encounters, even when separated by 72 h. Secondly, that the dyadic dominance is rebuilt during a successive encounter, in similar qualitative and quantitative terms.

3.2. Linearity in the rank-order would be supported by differences in prior attributes between group members

In this experiment we used groups of three individuals, staging dyadic encounters between all members of the group, in a way that animals confront a new opponent in each encounter.

Firstly, we asked whether there is an influence of the previous agonistic experience in the outcome of a new contest. In order to standardize the animals' previous agonistic experience, we restricted our analysis to the first two encounters of one animal (i.e., the focal crab) of each triad. The procedure was as follows: on the first encounter both contestants were naïve, while in the second one, 24 h later, the focal crab (randomly chosen, either a previous winner or loser) was faced with a

naïf opponent. Four possible arrangements can be obtained. In two of these cases, the focal crab wins (or loses) both fights with a different opponent in each combat (role persistence); while in the other two, the focal crab wins (or loses) the first encounter and loses (or wins) the second one (role change). If past experience influences the outcome of a second fight, we should expect the former two arrangements to outnumber the two latter combinations. Results contradict such prediction. Nineteen focal crabs exhibited role persistence (51.30%) vs. 18 showing role change (48.60%), which does not differ significantly from a chance distribution ($\chi^2 = 0.027$; $p = 0.87$). This result shows that the dyadic dominance in a fight would not be biased by the outcome of a previous one.

Secondly, to determine whether linearity can be disclosed in a group of three animals, we analysed the entire sequence of combats of each triad. That is, we determined the emergent group structure from the analysis of the three dyadic relationships. Results show 30 out of 37 triads with a linear rank-order where the top animal won both encounters, the second-rank crab won just one and the last one none. Fig. 1A displays all IDL values according to each animal's position in the ranking. Results reveal a wide variety in animals' performances, both within and between rank-positions, which would correlate with the existing differences in individuals' aggressiveness that account for the emergent triadic rank-order.

Finally, to analyse whether this linearity is reflected on the fight dynamics we compared the two encounters of the top animal ($E_{\text{top-second}}$ vs. $E_{\text{top-last}}$). The DI of the encounter between the top animal and the last one, $E_{\text{top-last}}$, was significantly higher than that between the top animal and the second one, $E_{\text{top-second}}$ (Fig. 1B, $t = -2.11$, $p < 0.05$, $N = 30$). A closer analysis disclosed the factors that are mainly weighing on the dominance disparity. The total interaction time (TIT) was significantly lower for $E_{\text{top-last}}$ than for $E_{\text{top-second}}$ (Fig. 1C, $t = 4.32$, $p < 0.0005$), as well as the “negative” acts (i.e., the time allocated to dominant acts by the loser, plus that allocated to retreats by the winner, $t = 2.93$, $p < 0.01$, $N = 30$). That is, the higher the distance in the rank-order of the triad, the higher the dominance disparity, the shorter the time dedicated to the agonist interaction and the smaller the weight of the “negative” acts.

3.3. Serotonin extends the agonistic interaction, while OA decreases it

Initially, we analysed whether 5HT or OA could modify the outcome of a fight and its dynamics. Thus, we performed an experiment where one of the animals of the dyad received an injection of 5HT, OA, or vehicle, while its respective opponent received vehicle in all cases. Results showed that neither amine has a noticeable effect over the outcome of the fights [5HT: $\chi^2 = 0.25$, $p = 0.62$; OA: $\chi^2 = 0.02$; $p = 0.88$]. That is, neither OA nor 5HT increases the tendency to be loser or winner, respectively. Similarly, no differences were found in the time of approaches, attacks and retreats performed by the amine-injected animals with respect to their vehicle-injected opponents (data not shown). However, when combining the performance of both contenders (Fig. 2), results demonstrate that the fight episodes were longer in the 5HT-group with respect from both the SAL group [TIT, t-test: $t = 1.68$; $p < 0.05$] and the OA group [$t = 2.11$; $p < 0.05$] (Fig. 2A). A more detailed analysis of these results revealed that the increase in the fight duration of the 5HT group was due to an intensification of the time dedicated by both opponents to dominant acts [$t = 1.82$; $p < 0.05$ with respect to SAL, and $t = 1.97$; $p < 0.05$ with respect to OA] (Fig. 2B). Thus, our findings suggest that 5-HT administration would enhance an animal's aggressive state, which in turn would evoke more aggression in its vehicle-injected opponent, leading to prolonged agonistic interactions.

As the previous experimental design did not allow us to reveal a net effect of OA over fight dynamics, we performed another experiment to analyse if OA could have a modulatory role on aggressiveness. In this case both animals received an OA injection and were compared with a SAL group. Results (Fig. 3A) showed that the total interaction time of the

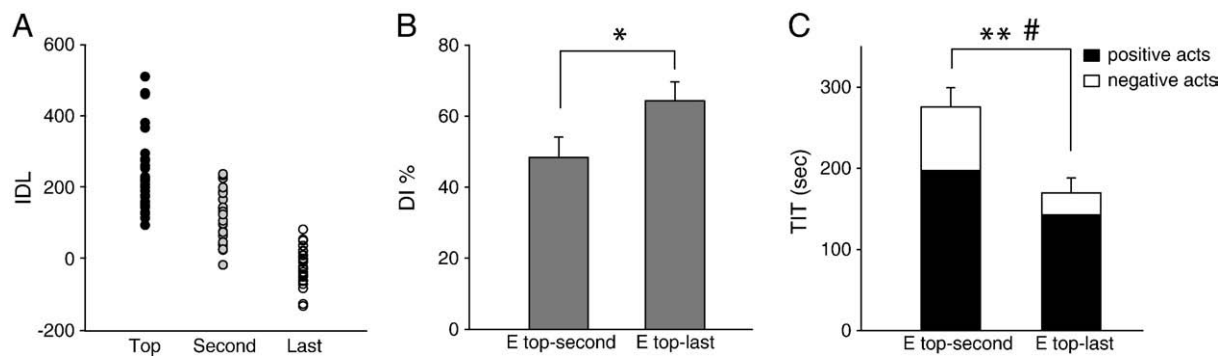


Fig. 1. Rank-order differences are expressed in fight dynamics. A. Individual dominance level (IDL) is displayed according to the animal's position in the rank-order (top, second or last). The values are obtained by adding the IDLs of each animal's two encounters. B. Indexes of agonistic performance are estimated for the encounter between top- and second-ranked individuals ($E_{\text{top-second}}$) and for that between top- and last-ranked individuals ($E_{\text{top-last}}$). Disparity index % differs between both encounters of top individual (one-tailed paired t-test; *: $p < 0.05$, $N = 30$). C. Total interaction time. Time allocated to positive acts (black) and negative acts (white) are differentiated in the figure. We found significant differences for TIT (one-tailed paired t-test; #: $p < 0.01$ $N = 30$) and for negative acts (**: $p < 0.01$).

414 OA group was significantly lower than the SAL group [TIT: $t = 2.09$,
 415 $p < 0.05$]. This difference in the fight duration was due to a decrease in
 416 the time allocated to dominant acts of the OA group (Fig. 3B), in
 417 particular the attacks [$t = 2.39$; $p < 0.01$].

418 Thus, although neither amine could modify the outcome of the
 419 fights, they were able to modulate the fight dynamics: while 5HT
 420 increased the time allocated to dominant acts, OA decreased it.

421 4. Discussion

422 4.1. Aggressiveness as a determinant of fight outcome

423 When two animals engage in a fight, an observer could usually
 424 predict which is going to win based on their differences in attributes
 425 such as body size, sex, or weapon size [34,46]. However, if these
 426 individuals are paired in a large series of measurable attributes, as was
 427 the case in our study, which factor would be determining fight
 428 outcome? Some authors argue that extrinsic factors, such as winner
 429 and loser effects, have a main influence in the fight success [47,48].
 430 We propose here that a contest between *Chasmagnathus* crabs could be
 431 defined by unmeasured dominance-related asymmetries between
 432 members [6,35,49]. In particular, our suggestion is that the intrinsic
 433 level of aggressiveness could be a key factor determining fight outcome.
 434 Our conclusion stemmed from a series of results.

435 Firstly, we showed that fight outcome was persistent through
 436 encounters, even when separated by 72 h (Table 1). In spite of this
 437 constancy in the W-L relationship, a comparison between encounters

438 did not reveal any differences in the fight dynamics. This result would
 439 imply that every time the same contestants meet, they fight in a similar
 440 way, reconstructing the W-L roles according to their individual
 441 aggressiveness. However, it could be argued that it is the past agonistic
 442 experience that determines the result of a subsequent fight encounter.
 443 We showed that this would not be the case in our study. Our results did
 444 not reveal any winner or loser effects when animals met a new
 445 opponent in each encounter, indicating that previous agonistic
 446 experience has no evident influence in the outcome of a successive
 447 one. Finally, we found a rank-order of dominance (i.e., linearity) in
 448 groups of three crabs that is reflected in its fight dynamics. That is, when
 449 the top animal faces the last-ranked crab, the disparity between them is
 450 more pronounced, and the interaction time is reduced, with respect to a
 451 fight against the second-ranked animal (Fig. 1). Therefore, there would
 452 be a pre-existent ranking between these individuals, based on the level
 453 of aggressiveness of each opponent, ready to be revealed when they
 454 meet. Our results open the possibility of disclosing the level of
 455 aggressiveness of each individual within a group of size-matched
 456 crabs, through the systematic assessment of dominance in a sequence of
 457 paired contests. This would allow us, in turn, to study correlates
 458 between this intrinsic factor and other attributes of the same animal, as
 459 its memory ability [26].

460 An intriguing finding of the present work is that we found no
 461 evidence of an increase in the dominance disparity through successive
 462 encounters. That is, the winner-loser relationship would be fully
 463 rebuilt each time, without saving fight time or reducing the number of
 464 negative acts. Such scenario is at variance with that described for

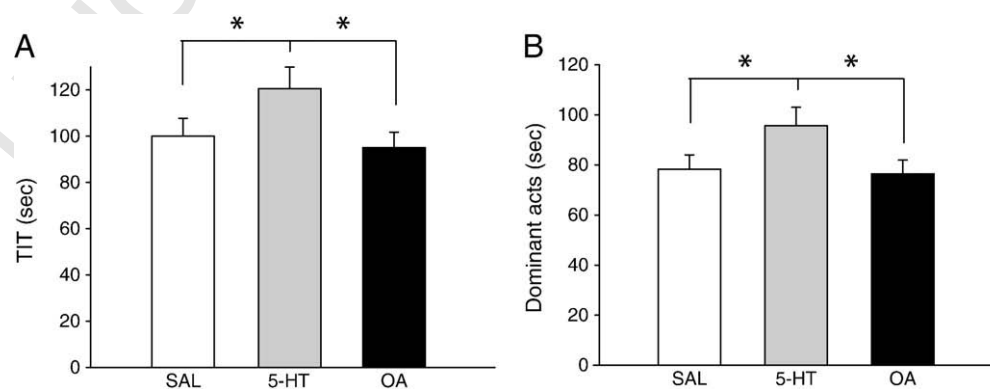


Fig. 2. Serotonin enhances aggression when administrated to one of the contestants. Indexes of agonistic performance for the three experimental groups: SAL (white bar, $N = 60$), 5HT (grey bar, $N = 66$) and OA (black bar, $N = 51$). The combined performance of both contestants is displayed. Data are expressed as mean \pm SE. A. Total interaction time. Agonistic episodes from the 5HT group are longer than those of the SAL and OA groups (one-tailed t-test; *: $p < 0.05$). B. Dominant acts. The intensification of the agonistic interactions is due to an increase in dominant acts of the 5HT group (one-tailed t-test; *: $p < 0.05$).

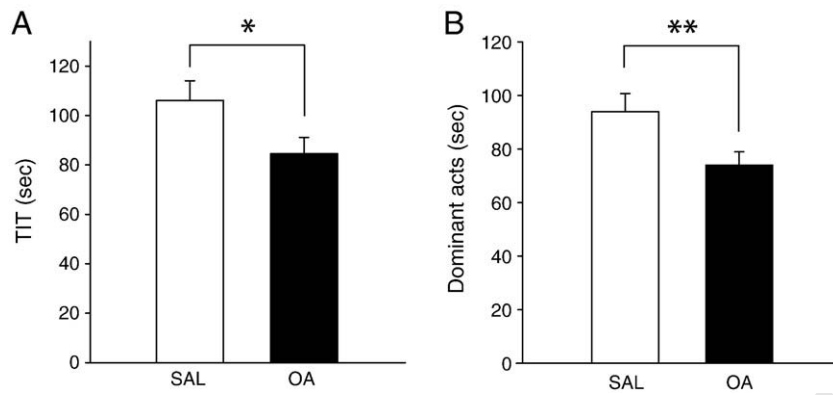


Fig. 3. Octopamine reduces aggression when administered to both contenders. Indexes of agonistic performance for the two experimental groups: SAL (white bar, N = 32) and OA (black bar, N = 36). The combined performance of both contestants is displayed. Data are expressed as mean \pm SE. A. TIT. Agonistic episodes from the OA group are shorter than those of the SAL group (one-tailed t-test; *: $p < 0.05$). B. Dominant acts. The reduction of the agonistic interaction is due to a decrease in the dominant acts of the OA group (one-tailed t-test; **: $p < 0.01$).

several other animal species, where fights on subsequent combats appear to be resolved more rapidly (time saving), at lower intensity, and with increased polarity [13,15,16,47,50–52]. It is worthwhile to consider these results in relation to the crab's life in its natural habitat. *Chasmagnathus* build their burrows in the mudflat and live in highly dense populations, with up to 60 burrows per m^2 [53,54], exposed to daily tidal fluctuations that may imply rearrangements in burrow ownership. Field observations reveal that these crabs are solitary animals and their limited interactions with conspecifics are of aggressive nature, mainly occurring in the context of burrow disputes between a resident and a wondering crab [55]. In such scenario, the selective pressure towards a recognition mechanism is probably weak, because animals have a very low chance to be engaged in a fight repeatedly with the same opponent. Therefore, our results showing that the fight generates a W–L relationship and not a long lasting agonistic status seem to be consistent with their natural conditions.

4.2. Proximate roots of aggression: modulation by biogenic amines

A comprehensive understanding of the causation of aggressive behaviour requires behavioural variability to be mapped onto its underlying proximate mechanisms. Monoamine systems are attractive candidates as they modify neural function at multiple levels and may thereby bring a coherent behavioural response when the animal interacts with conspecifics or intruders [56–58]. In particular, serotonin (5HT) is strongly implicated in the control of agonistic behaviour and social dominance across a wide variety of invertebrate and vertebrate taxa, including humans [59–62]. Increased 5HT function is often considered to lower aggression in vertebrates, while the opposite scenario seems to hold true for invertebrate taxa [63,64]. From this divergence, it is possible to consider the hypothesis that the role of 5HT systems underlying aggression could have undergone a sign change during early vertebrate evolution [65]. Alternatively, this discrepancy could be attributed to the differences in behavioural paradigms used, manipulation of serotonergic levels, as well as to the diverse connotations of the terms “aggression” and “dominance”. Regarding octopamine (OA), which is considered the invertebrate's counterpart of noradrenaline [66], it was repeatedly associated with the regulation of aggressive behaviour in arthropods [57]. In insects like crickets and fruit flies, OA was found to increase the motivation to fight [67–70]. Conversely, in decapod crustaceans an injection of OA was shown to decrease aggression and lead to a posture typical of subordinate individuals [28–30]. All in all, beyond the existing differences across taxa, 5HT and OA are recognized as key physiological regulators of agonistic behaviour, and particularly in decapod crustaceans these amines appear

to play opposite roles in the control of aggressiveness and the establishment of dominant and subordinate roles [57,58,63,71].

By using a pharmacological approach we aimed to determine the role of these amines in *Chasmagnathus* agonistic behaviour. Our results show that 5HT and OA modulate crabs' aggressiveness in opposite directions: while 5HT tends to increase it, OA would lead to a reduction in the aggressive drive. This assertion is based on the following set of results. On the one hand, 5HT treatment produces an increase in the time allocated to dominant acts, leading to extended agonistic episodes and to a longer interaction time with respect to the saline group (Fig. 2). In contrast, OA decreases the time devoted to attacks and subsequently, the total interaction time, compared to the saline group (Fig. 3). In spite of these changes in fight dynamics, neither amine altered the probability of becoming winner or loser. At first, this may seem inconsistent with our previous assertion that these amines modulate crabs' aggressiveness. However, it should be noticed that injections of 5HT and OA are applied blindly, without knowing the intrinsic aggressiveness of each opponent. Thus, the amine administration may alter the animal's internal state, and thus modify its behavioural output, but we should not expect it to convert forthcoming winners into losers, or vice versa. Therefore, it seems more appropriate to look for differences in the fight dynamics regardless of the outcome of the confrontation. In this sense, we found that both amines modified the time dedicated to dominant acts, leading to a change in the intensity of the agonistic interactions.

Our results are in accordance with previous studies in crustaceans. Subordinate lobsters injected with 5HT exhibited an enhanced aggressive motivation [31] and in crayfish, OA and 5HT were proposed to modulate aggressiveness without affecting the hierarchical rank of the individuals [30]. There are, nevertheless, alternative visions regarding the effect of 5HT on aggression. Some authors have found a decrease in aggression in crayfish injected with 5HT [72] while others proposed that the prolonged fights of the subordinates injected with 5HT could be attributed to a motor inhibition that makes an animal less able to retreat, instead of an enhanced aggression [73]. Such argument does not apply to our study, since the doses used in this paper do not produce any postural changes, and do not have any effects on the animal's exploratory activity or escape response [44, Pedetta and Maldonado in preparation].

In the present study it is proposed that an intrinsic level of aggressiveness would determine fight outcome in symmetrical contests. We hypothesize that during a fight encounter this individual's aggressiveness would be translated from an internal state into a proper behavioural output, via the action of a number of mediator signals, including serotonin and octopamine. These substances are thought to alter the activity of specific neural decision-making centres [74]. In this way, they could modulate the decision of whether to continue fighting

554 or to retreat in front of the contestant's advance, contributing greatly to
555 an individual's fitness.

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561 Appendix A. Supplementary data

562 Supplementary data associated with this article can be found, in the
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