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BEHAVIORAL RESPONSES TO 'ALARM ODORS' IN POTENTIALLY INVASIVE AND NON-INVASIVE CRAYFISH SPECIES FROM AQUACULTURE PONDS

by

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Summary

Two North American crayfish species, the Eastern white river crayfish, *Procambarus acutus acutus*, and the red swamp crayfish, *P. clarkii*, were studied in the laboratory for their responses to food odors and to cues released by injured conspecifics and heterospecifics. The two species differ in that only *P. clarkii* is known to behave as an invasive species. All the test individuals were collected from aquaculture research ponds, in which they had had no prior contact with the other species and predation risks, excluding cannibalism, were reduced. The experimental design consisted in subjecting 20 crayfish per species to (1) a 3-min control phase after the injection of 20 ml of water and (2) a 3-min test phase after the injection of 20 ml of one of three test solutions (food odor, conspecific odor plus food odor, heterospecific odor plus food odor). We found that the two species differ on one hand for their background behavior and on the other for the intensity and quality of their responses to the three types of cues. Firstly, *P. clarkii* appeared more active than *P. acutus acutus* during the control phase and responded in a stronger fashion to the injection of the solutions. Secondly, we recorded an increased locomotion in *P. acutus acutus* with food and heterospecific cues (by moving crayfish maximize the chance of finding food), but not with conspecific odors (by not moving, crayfish reduce their exposure to visual predators). To the contrary, at the injection of the

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three test solutions *P. clarkii* displayed clear feeding-related activities (although less intense with conspecific odors) as opposed to the danger reactions shown in a previous study on individuals from a naturalized population of the same species. This result suggests that crayfish reared in an environment where predation risks are reduced (*e.g.* in aquaculture ponds) may respond differently to cues that in other, more risky habitats inform of a danger.

Keywords: chemical communication, alarm signals, crayfish, invasive species, aquaculture.

Introduction

The ability to gather information from the chemical medium is of great selective advantage to many aquatic organisms from different taxa and functional groups (reviewed in Mackie & Grant, 1974; Daloze *et al.*, 1980; Atema, 1985), especially when their habits are nocturnal and the environment they occupy is highly structured and/or characterized by a poor light transmission (Brönmark & Hansson, 2000).

Several crayfish species have been found to detect chemical stimuli released by both food (Ameyaw-Akumfi, 1977; Tierney & Atema, 1988; Hazlett, 1994a; Willman *et al.*, 1994; Giri & Dunham, 1999; Moore & Grills, 1999; Steele *et al.*, 1999) and predators (Blake & Hart, 1993; Shave *et al.*, 1994; Willman *et al.*, 1994; Hazlett & Schoolmaster, 1998). Chemicals are also used for status recognition in dominance hierarchies (Zulandt Schneider *et al.*, 1999), as well as for mate and parent recognition (*e.g.* Little, 1975, 1976; Bechler, 1995).

An abundant literature shows that, together with a variety of other aquatic invertebrates (*e.g.* Snyder & Snyder, 1970; Stenzler & Atema, 1976; McKillup & McKillup, 1992; Rittschof *et al.*, 1992; Hazlett, 1994a), several crayfish species are capable of detecting chemical stimuli released by disturbed (Hazlett, 1985, 1989, 1990; Zulandt Schneider & Moore, 2000) or injured conspecifics (Hazlett, 1994b; Mitchell & Hazlett, 1996). These substances function as indicators of predation risk and may cause appropriate changes of behavior in the alerted individuals by inducing, *e.g.* avoidance of areas of potential danger, freezing or reduced activity, and increased use of cover and watchful posture (Hazlett, 1994b). It is expected, and proved in other taxa (Hews, 1988; Mathis & Smith, 1993; Wisenden *et al.*, 1999), that such a behavior confers obvious survival benefits to the reactive animals by decreasing their vulnerability to predation.

Since predators of crayfish often have generalist and opportunistic feeding habits (Hobbs, 1993), it seems advantageous for a species to use a broad range of information about predation risks, for instance by associating to the enemy the alarm substances emitted by heterospecifics that are members of the same 'prey guild' (Hazlett, 1994b, 2000). This allows individuals of that species to cope with new types of predators and is particularly advantageous when they occupy a novel environment. The breadth of information gathering about predators (Hazlett, 2000) is in fact viewed as a remarkable feature of the behavioral flexibility that characterizes the biology of invasive species (Schweitzer & Larson, 1999).

Here we examined the behavioral responses to con- and heterospecific odors produced by injured individuals in two cambarid species, the Eastern white river crayfish (*Procambarus acutus acutus*) and the red swamp crayfish (*Procambarus clarkii*). *Procambarus acutus acutus* is a relatively common species, occurring in moderately flowing streams or rivers and lentic waters (swamps, ditches, sloughs, and ponds) within an area extending between Maine and Georgia, Florida and Texas, and Minnesota and Ohio (Williams & Bivens, 1996). *Procambarus clarkii* is one of the most extensively studied crayfish due to its invasive properties (Lindqvist & Huner, 1999). From its natural range (north-eastern Mexico and the south-central USA westward to Texas and eastward to Alabama and northward to Tennessee and Illinois; Hobbs, 1972), it has been translocated by humans for aquaculture purposes in different states of North America and in all the other continents, except Australia and Antarctica (Huner, 1977; Huner & Avault, 1979). In many regions, specimens escaped from farm ponds and invaded natural water bodies, where they exert a strong negative impact to the indigenous community and crayfish species as well (Holdich, 1991; Gherardi *et al.*, 1999). This phenomenon is underway in Delaware and may pose a threat to the native *P. acutus acutus*.

Our aim here was to determine whether *P. acutus acutus* and the invasive *P. clarkii* show alarm responses to cues from injured con- and heterospecifics. Since our results from *P. clarkii* reared in aquaculture ponds were not completely in accordance with the conclusion of previous studies on a naturalized population of the same species (Hazlett, 2000; Hazlett *et al.*, 2003), we will finally discuss the potential that the rearing condition in aquaculture ponds might have altered crayfish responses to cues that in other, more risky habitats inform of predation dangers.

Methods

Experimental design

Individuals of *P. acutus acutus* and *P. clarkii* were made available from aquaculture research ponds located in Delaware and Louisiana (USA). During January 2002, in the laboratory at the Delaware State University (Dover, Delaware), 20 adult Form I males of *P. acutus acutus* (cephalothorax length: 35-44.1 mm) and 20 adult Form I males of *P. clarkii* (cephalothorax length: 47.8-56.5 mm) were kept in individual aquaria (50 × 25 cm bottom), visually isolated from one another and from possible sources of disturbance, for at least one week before testing. The aquaria were constantly aerated and contained 12.5 liters of dechlorinated tap water at a temperature of 16.9-19.6°C and a 15 cm-long piece of a straight PVC pipe (5 cm internal diameter) as a shelter. During their maintenance in the laboratory, animals were fed *ad libitum* with commercial crayfish pellets once every second day and water was changed an hour later. Both in the acclimation day and during the experiment crayfish were not fed.

Experiments were conducted between 0900 and 1400 hours. The experimental protocol followed the design of previous studies (e.g. Hazlett, 2000). After 24 h of acclimation in individual aquaria, records were taken once every 15 s for 3 min on 20 individuals per species during two consecutive time phases: (1) a 3-min control phase following the injection of 20 ml of control water, and immediately after (2) a 3-min test phase following the injection of 20 ml of test solution.

Test solutions were composed of: (1) 10 ml food odor plus 10 ml water (FOOD), (2) 10 ml food odor plus 10 ml conspecific odor (CONS), or (3) 10 ml food odor plus 10 ml heterospecific odor (HETE). Solutions were prepared by macerating commercial crayfish pellets (5 g) for producing food odor and two 20-25 g male con- or heterospecific crayfish for producing con- and heterospecific odors, respectively, in 400 ml of tap water and by filtering the product with coarse filter paper. Solutions were used immediately after their preparation. We tested con- and heterospecific odors in conjunction with food odor, and not alone, because previous studies conducted in other species (Hazlett, 1994b; Hazlett & McLay, 2000; Bouwma & Hazlett, 2001) and pilot observations conducted in the here analyzed species had revealed that alarm odors may act as modulatory cues altering the probability of reactions to other stimuli. Therefore, the detection by crayfish of alarm cues is usually made more evident when feeding-related activities, executed by crayfish in the presence of food odor alone, are depressed.

Control water and test solutions were injected with a syringe to one of the two corners of the aquarium (the farthest from the tested individual). Crayfish of the two species were presented with all test solutions, a single trial every day. The sequence of solutions tested was varied systematically. After each trial, water was changed and the aquaria were thoroughly cleaned to eliminate any odor that could influence the response by crayfish to the solutions tested the subsequent day.

Once every 15 s for each 3-min phase, an experienced observer (always F.G.) recorded whether crayfish: (a) executed feeding acts (*i.e.* scraped the substratum using chelipeds and/or pereopods or moved its maxillipeds) (FE), and (b) displayed locomotory movements (LO). Previous studies (e.g. Hazlett, 1994b, 1999) had shown that, when presented with food cues only, crayfish increase the frequency of FE and LO, and reduce the time spent inside the shelter. On the contrary, when alarm signals (and predator odors as well) are combined with food cues, FE and LO become less (Blake & Hart, 1993; Hazlett & Schoolmaster, 1998; Hazlett, 1999) and shelter occupancy more (Hazlett, 1994b) frequent, respectively. In the present study, the decrease of LO seemed to be more indicative of the detection of alarm

substances than the reduction of FE (P. Acquistapace, pers. obs.), since the latter often persist as displacement activities. Besides, crayfish of both species were rarely observed hiding in the shelter, notwithstanding previous observations (F. Gherardi, pers. obs.) had shown that the type of shelters we offered was normally occupied by the same species and test individuals were previously accustomed to the PVC pipe. Therefore, shelter occupancy was excluded from our analysis.

For both the control and the test phases, we then computed the percentage of the time spent in each of the two behaviors recorded.

Statistical analyses

Following Siegel & Castellan (1988), we used nonparametric tests since the assumption of normality of data was not always met and variances were unequal.

To evaluate background differences between species, the Wilcoxon-Mann-Whitney test (z for large samples) was applied to make between-species comparisons of the percentages of time spent in the two behaviors during the three control phases. Comparisons between control and test phases were made by the Wilcoxon signed ranks test for the percentages of time spent in the two behavior patterns.

Because of the diverse background behavior, comparisons among test solutions and species were made by analyzing the magnitude of change in crayfish behaviors (in %) between control and test phases and its direction, that is, if test solutions induced significant increases (+) or decreases (-) in responses with respect to the control. A Friedman two-way analysis of variance by ranks (F_r) was used to compare responses by each species to the three test solutions followed by a Multiple Comparisons test to show hierarchies among test solutions when a difference was found after the Friedman test. Species were compared with a Wilcoxon-Mann-Whitney test (z for large samples).

The level of significance under which the null hypothesis was rejected is $\alpha = 0.05$. Text, tables and the figure provide medians and 95% confidence intervals.

Results

Interspecific differences to control water

During the control, the two species did not differ significantly ($p > 0.05$) in executing FE (*P. acutus acutus*: 0% and 0-5.64%, $N = 60$; *P. clarkii*: 0% and 0-3.46%, $N = 60$). However, background LO was more frequent in *P. clarkii* (0% and 0-14.66%, $N = 60$) than in *P. acutus acutus* (0% and 0-6.31%, $N = 60$; $z = 2.028$, $p < 0.05$).

Control water vs test solutions

The injection of test solutions induced evident changes in the two behaviors analyzed in 60-85% *P. clarkii*, while the responses by *P. acutus acutus* were weaker and occurred in a fewer number of individuals, *i.e.* 25-70% (Table 1).

TABLE 1. Comparisons between control and test phases (3 min) for the time (%) spent in executing feeding acts and locomotory movements

		Control		Test		T ⁺	N
		median	95% c.i.	median	95% c.i.		
<i>Procambarus acutus acutus</i>							
Feeding acts	FOOD	0	0-11.40	20.83	18.10-46.07	97.5**	14 (70)
	CONS	0	0-4.42	0	0-14.38	36**	8 (40)
	HETE	0	0-5.41	0	0-19.56	46.5	10 (50)
Locomotion	FOOD	0	0-14.41	4.17	4.16-30.17	40.5*	9 (45)
	CONS	0	0-4.70	0	0-25.31	18.5	5 (25)
	HETE	0	0-3.58	0	0-10.97	40.5*	9 (45)
<i>Procambarus clarkii</i>							
Feeding acts	FOOD	0	0-3.04	33.33	28.09-56.08	151.5**	17 (85)
	CONS	0	0.10-4.90	8.33	5.24-19.76	67*	12 (60)
	HETE	0	0-5.43	25	23.17-47.67	136.5**	16 (80)
Locomotion	FOOD	0	0-12.94	16.67	16.47-41.03	102**	14 (70)
	CONS	0	0-15.54	16.67	14.44-42.22	93**	14 (70)
	HETE	0	0-25.44	33.33	17.37-38.47	73.5*	13 (65)

Control phases followed the injection of water and test phases followed the injection of three test solutions (FOOD = food odor, CONS = food odor + cues from injured conspecifics, and HETE = food odor + cues from injured heterospecifics). Medians (and 95% confidence intervals, c.i.) were compared using the Wilcoxon signed ranks test (T⁺). * $p < 0.05$, ** $p < 0.01$. N is the number of individuals (and their percentages on the total of 20, in brackets) that showed a change in behavior from control to test phases. p values less than 0.05 in bold.

The time that most *P. clarkii* individuals spent in both FE and LO was longer during the test than during the control phases. This was found also in *P. acutus acutus* for FE in the presence of FOOD and CONS and for LO in the presence of FOOD and HETE.

Comparison among test solutions

A comparison among test solutions for the magnitude of changes in the two analyzed behaviors (Fig. 1) showed that FE were more frequent with FOOD and HETE than with CONS in *P. clarkii* ($F_r = 25.057$, $p < 0.01$). No significant differences were found in the other comparisons (in *P. acutus acutus*, FE: $F_r = 3.354$, $p > 0.05$, LO: $F_r = 2.542$, $p > 0.05$; in *P. clarkii*, LO: $F_r = 3.176$, $p > 0.05$).

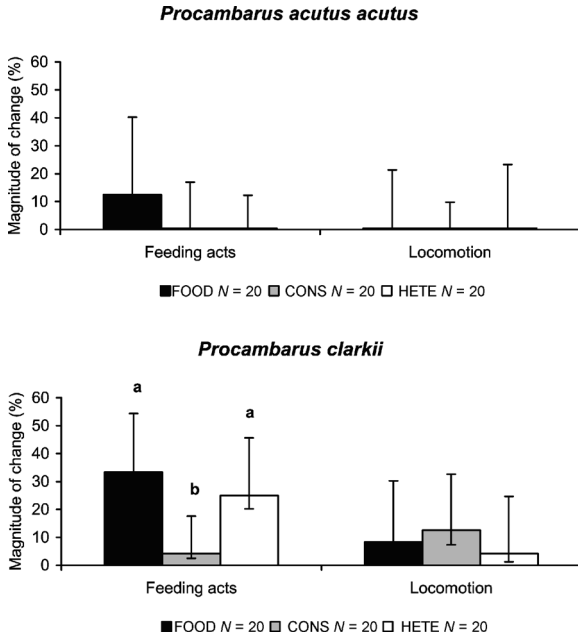


Fig. 1. *Procambarus acutus acutus* and *P. clarkii*. Magnitude of changes (median and 95% confidence intervals) between test phases and controls (3 min) in the time (%) spent in executing feeding acts and locomotory movements. Control phases followed the injection of water and test phases followed the injection of three test solutions, *i.e.* food (FOOD), food + conspecific alarm cues (CONS), and food + heterospecific alarm cues (HETE). Comparisons among test solutions were done using the Friedman two-way analysis of variance by ranks (F_r , $df = 2$) followed by a Multiple Comparisons test. Different letters indicate significant differences (at $p < 0.05$) between magnitude of changes.

Comparison between species

Interspecific differences in the magnitude of changes were found with CONS and HETE only (Table 2). *Procambarus acutus acutus* spent less time than *P. clarkii* in FE after the injection of HETE and in LO after the injection of CONS.

Discussion

Results from this study demonstrate that *P. acutus acutus* and the potentially invasive species *P. clarkii* differ firstly in their background behavior, and secondly in their responses to the odors released by food on the one hand and by injured con- and heterospecific individuals on the other.

TABLE 2. *Magnitude of changes between test solutions*

	FOOD		CONS		HETE	
	z	Direction of change	z	Direction of change	z	Direction of change
Feeding acts	1.547		0.232		3.366**	Pc > Pa +
Locomotion	1.410		2.314*	Pc > Pa +	0.128	

Magnitude of changes between test solutions (FOOD = food odor, CONS = food odor + cues from injured conspecifics, and HETE = food odor + cues from injured heterospecifics) and control water for the time (%) spent in executing feeding acts and locomotory movements. Comparisons between species (Pa = *P. acutus acutus*, $N = 20$; Pc = *P. clarkii*, $N = 20$) were made using the Wilcoxon-Mann-Whitney test (z for large samples). Species ranked in decreasing order of magnitude of change after a Multiple Comparisons test. Significant differences at $p < 0.05$ in bold. * $p < 0.05$, ** $p < 0.01$.

As expected from previous ecological studies (Gherardi *et al.*, 2000; Gherardi & Barbaresi, 2000), *P. clarkii* appeared more active than *P. acutus acutus* and responded to a stronger extent to the injection of the three test solutions. A large proportion of *P. clarkii* individuals (always >60%) changed their behavior from the control to the test phases by increasing the time they executed feeding acts or displayed locomotory movements. The different responses of the two species were made more evident from their direct comparison. In fact, feeding acts and locomotion had a longer duration in *P. clarkii* than in *P. acutus acutus* in the presence of, respectively, hetero- and conspecific cues.

This interspecific difference was not only a matter of intensity, but also of the quality of responses. In *P. acutus acutus*, notwithstanding that differences among solutions did not reach significance, we found that the injection of food and heterospecific solutions was followed by an increased locomotion when compared to the control phase. And locomotory movements are reliable indicators of the detection by crayfish of food odors in that they maximize animal chance of finding food (P. Acquistapace, pers. obs.). To the contrary, in the presence of cues from conspecifics, individuals of this species executed feeding acts (possibly to be interpreted as displacement activities) without however intensifying their locomotion. By not moving, the exposure to visual predators is obviously reduced (see, *e.g.* Werner & Anholt, 1993; Hazlett, 1994b). Taken together, these data would suggest that, while substances from heterospecifics elicit in *P. acutus acutus* the same responses as food odors, this species associates cues from injured conspecifics with a

danger situation, in analogy with what was found in several other crayfish species (see, e.g. Hazlett, 1994b; Mitchell & Hazlett, 1996).

In *P. clarkii*, the injection of the three test solutions was followed by a significant increase of feeding-related activities that were however less intense in the presence of conspecific cues. In other words, while this crayfish behaved in the same fashion as *P. acutus acutus* with heterospecific odors, the eventual alarm substances from conspecifics were not sufficient to annul (but slightly inhibited) its responses to food. To the contrary, in a previous work on a naturalized population of the same species, Hazlett *et al.* (2003) demonstrated that *P. clarkii* individuals do recognize as alarm signals the odors emitted by injured crayfish. However, both in Hazlett *et al.* (2003) and in the present study, responses to con- and heterospecific cues differed for feeding acts only.

Obviously, much work has to be done to explain such a variability among species (between *P. acutus acutus* and *P. clarkii*) and among populations (in *P. clarkii*). In particular, further studies are required to explore the innate or learned basis in the recognition of alarm signals in crayfish, as already done in fish (e.g. Pfeiffer, 1963; Waldman, 1982) and in amphibians (e.g. Wildy & Blaustein, 2001). Our results can only provide suggestions to interpret differences of crayfish responses with the environmental context.

Firstly, the alarmed responses to conspecific cues shown by *P. acutus acutus* reared for generations in the absence of predators may be exclusively innate. Alternatively, they may be a consequence of the cannibalistic nature of crayfish (Nyström, 2002). In fact, individuals that experience odors released by injured conspecifics in combination with visual, chemical or tactile cues of other conspecifics acting as predators can learn to associate alarm odors and the danger of being cannibalized also in aquaculture ponds. On the other hand, the same *P. acutus acutus* individuals appeared incapable to extend the significance of alarm signals to substances (presumably of a similar chemical nature) emitted by the congeneric *P. clarkii*. This would be due to test individuals having never experienced *P. clarkii* in their life, but alternatively may be inherent to the non-invasive nature of *P. acutus acutus*. In fact, all the other non-invasive crayfish studied to date showed the same incapability to respond to alarm signals of other crayfish species (Hazlett, 1994b, 2000; Hazlett *et al.*, 2003), although living in syntopy.

However, when both predation and cannibalism are rare or absent (only juveniles and molting individuals are subject to be cannibalized; Abrahamsson,

1966), adult crayfish can lose or forget the either innate or learned association between cues from injured individuals and danger; or, in the presence of conflicting information, food searching behavior will dominate over defensive responses to predator-associated cues. Alternatively, adults may assign to the cues emitted by wounded conspecifics the meaning of food odors, as a consequence of their prevalent cannibalistic habit. One of these three events would have induced *P. clarkii* individuals to respond to cues from injured crayfish with feeding-related activities.

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