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Original Citation:

Extended mother-offspring relationships in invasive crayfish / L. AQUILONI; F. GHERARDI. - In: ETHOLOGY.
- ISSN 0179-1613. - STAMPA. - 114:(2008), pp. 946-954. [10.1111/j.1439-0310.2008.01547.x]

Availability:

This version is available at: 2158/329870 since:

Published version:

DOI: 10.1111/j.1439-0310.2008.01547.x

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RESEARCH PAPERS

Extended Mother–Offspring Relationships in Crayfish: The Return Behaviour of Juvenile *Procambarus Clarkii*

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Received: March 14, 2008

Initial acceptance: April 25, 2008

Final acceptance: May 1, 2008

(J. Schneider)

doi: 10.1111/j.1439-0310.2008.01547.x

Abstract

Crayfish shows a relatively complex parental behaviour compared with other invertebrates, but the literature provides only anecdotal accounts of this phenomenon. In *Procambarus clarkii*, we described the ‘return’ behaviour of third-stage juveniles when offered four types of adults: biological mothers, foster mothers, non-brooding females and males. Then, we analysed the posture and behaviour of the adults to understand the role played by the putative mother in attracting the juveniles. Contrary to non-brooding individuals, both biological and foster mothers displayed a relatively rare locomotion, executed few cleaning and feeding acts, and never attempted to prey on juveniles. They often assumed a ‘spoon-like telson posture’ that seemed to facilitate offspring’s approaches. Juveniles increased the frequency of tail-flips away in the presence of non-brooding adults; conversely, they accepted foster mothers, along with biological mothers, but not as fast as the latter. Taken together, these results suggest that mother–offspring relationships in *P. clarkii* are more refined than previously thought, being possibly a key factor enabling this species to thrive in harsh environmental conditions.

Introduction

Parental investment in animals spans from allocating metabolic energy in eggs or protecting zygotes from environmental stresses to more advanced forms, usually gathered under the category of ‘extended parental care’ (XPC; Thiel 2003). The species engaged in XPC display a wide array of behaviours of increased complexity, from providing food and/or protection to the juveniles to aiding them in learning processes. Although it is often difficult to distinguish when the ‘usual’ parental care ends and when XPC starts, we classify here the instances of parental care involving potentially self-sufficient juveniles as XPC (Thiel 1999).

Crustacean decapods show forms of active parental care, such as egg grooming (Pandian 1994), increased pleopod beating in response to changed microclimatic conditions (Ameyaw-Akumfi 1976),

and selective removal of non-viable eggs (Tack 1941), along with burrowing and migrating to protect the brood (Hazlett 1983); in 60 species (on a total of 130 crustaceans showing parental care; Thiel 2003), a form of XPC has been described often in association with the stressful conditions encountered in terrestrial and freshwater environments (Hazlett 1983). The mothers engaged in XPC (see Thiel 1999 and Tallamy 2000 for the few cases of paternal care in arthropods) exhibit relatively complex behaviours: they protect offspring against adverse abiotic conditions and predators (e.g. in potamonid crabs; Cumberlidge & Sachs 1991) and even provision food to them (in the tree crab *Metapaulius depressus*; Diesel 1989, 1992; Diesel & Schubart 2000).

Parental behaviour in crayfish is relatively complex if compared with other invertebrates (Bechler 1981; Hazlett 1983; Figler et al. 1995, 1997). Young stages

are not planktonic and do not undergo significant metamorphoses but resemble the adults in their morphology right after hatching. They remain attached to their mother's abdomen by transient structures (the telson thread and pereopodal hooks; Vogt & Tolley 2004) and then freely crawl onto her body for a period ranging between few weeks in most species and 3–4 mo or more in *Procambarus clarkii* (Huner & Barr 1991; Huner 1994) and *Paranephrops zealandicus* (Whitmore & Huryn 1999). Weaning consists in the young definitively leaving their mother and digging their first burrows at some distance from her (e.g. in *Procambarus layi*; Payne 1972). Exceptions are species of the genus *Engaeus*, in which family groups (Healy & Yaldwyn 1970), similar to those described in desert isopods (Linsenmair & Linsenmair 1971), were found to inhabit communal burrows (Clark 1936; Suter & Richardson 1977).

This study stems from previous observations on the behaviour of third-stage juveniles in some crayfish species. Once sense organs have been fully developed (Vogt & Tolley 2004), juveniles occasionally leave their mothers to shortly explore the environment and feed. At the end of their excursions or if disturbed, they return under their mother's abdomen or on other parts of her body (Ameyaw-Akumfi 1976; Figler et al. 1997) using a brood pheromone (Little 1975, 1976), possibly species-specific, released by the maternal female. A similar 'return' behaviour has also been described in a number of peracarids (*Neohaustorius schmitzi*, Croker 1968; *Gammarus palustris*, Borowsky 1980; *Parallorchestes ochotensis*, Kobayashi et al. 2002), but in these species the mothers simply allow juveniles to associate with them (Dick et al. 1998).

Here, we analyzed mother–offspring relationships in *Procambarus clarkii*, an invasive crayfish traditionally defined as an *r*-selected species (Gherardi 2006). The first aim of the study was to describe the return behaviour of third-stage juveniles when presented with adults of one of four types: (1) biological mothers, (2) non-biological (foster) mothers, (3) non-brooding females and (4) males. Secondly, we analyzed the posture and behaviour exhibited by the adults with the aim of understanding the role played by the putative mother in attracting the juveniles back to her.

Materials and Methods

Collection of Animals and Maintenance

A total of 200 crayfish of both sexes was collected with baited traps from the Massaciuccoli Lake

(Tuscany, Italy) in July 2005 at the start of the reproductive season. Once in the laboratory, they were kept, sexes apart, at a density of 15 animals per m² in plastic tanks (80 × 60 × 60 cm) containing 48 l of still tap water and clay pots as refuges. For the entire period of the experiment, they were maintained in a natural light:dark cycle at 20°C and fed *ad libitum* with live *Calliphora* sp. larvae. Water was changed weekly.

The cephalothorax length (from the tip of the rostrum to the posterior edge of the carapace) of each crayfish was measured with a vernier caliper and an individual mark was applied on them using a water-proof paint. Only hard-shelled crayfish with all appendages intact were used.

After a week of acclimation, crayfish were checked for their sexual responsiveness by observing their behaviour towards a potential mate. If the male tried to turn the female over for copulation, the two individuals were defined as sexually responsive. Responsive individuals were removed before the copulation took place and placed into individual aquaria (25 × 20 × 20 cm) with a clay pot as refuge and still tap water (5 l). In this way, we selected 60 males and 60 females with an average cephalothorax length of 42.8 mm (±0.3) and 44 mm (±0.9), respectively. From these crayfish, we randomly formed 40 mating pairs of appropriate relative size (difference in cephalothorax length: ±2 mm) and placed each of them in a 25 × 20 × 20 cm aquarium as above. Copulation took place in all pairs and all the females successfully spawned. Immediately after spawning, females were separated from their mates to avoid the possibility that males might cannibalize the eggs, as often recorded in captivity, and they were placed with the developing eggs in individual aquaria.

The juveniles from 26 females hatched in October, broods being composed of 153–314 hatchlings each. Brooding females were used in two treatments, 13 as 'biological mothers' (Bm) and 13 as 'foster mothers' (Fm). In the other two treatments, we used non-brooding individuals of our original sample, i.e. 13 females ('non-brooding females', Fe) and 13 males ('males', Ma). From each Bm's brood, we randomly formed four groups of 30 juveniles to be tested each separately with one of the four types of adults (see below).

Experimental Design and Apparatus

The experiment was run in November 2005, when the juveniles had reached their third inter-moult stage (approx. 30 d old). At this age, juveniles start

swimming or walking short distance away from the female on feeding forays before returning to the mother freely or upon any disturbance (Gherardi 2002). The experimental apparatus consisted of one circular plastic tank (diameter 30 cm, water level 10 cm) per treatment.

Before the start of any trial (at approx. 1200 h), an adult (Bm, Fm, Fe or Ma) and a group of 30 similarly sized juveniles of the same brood, each gently detached from the mother with tweezers, were kept for 20 min at the opposite sides of the same experimental tank inside a cylindrical wire netting to acclimatize. The experiment started with the lifting of the wire nettings and lasted 22 h. Interactions among adult crayfish and juveniles were video-taped *in continuum* for 30 min from the start of the trial using a Samsung digital camera (VP-L800) (Samsung Electronics Inc., New Jersey, USA) activated at distance to avoid disturbance to the experimental animals. After recording, each group was kept in the same aquarium for the remaining 1290 min before being separated and used for other laboratory experiments. Four trials, one for each treatment, were run daily following random temporal sequences. Each treatment (Bm, Fm, Fe and Ma) was replicated 13 times, using adults and juveniles only once to avoid pseudo-replication. Between trials, the tank was thoroughly washed with clean tap water.

Collection of Data

Video-tapes were analyzed to record:

- the distance covered (in cm) by each adult and by three randomly selected juveniles per group and the speed (in cm/s) of their movement; the latter was computed from the distance covered on the overall time spent in locomotion;
- the total time (in seconds) spent by each adult (1) displaying one of three postures: with the abdomen bent (i.e. the telson touched the ventral surface of the abdomen), with the telson in a spoon-like posture (i.e. the abdomen was extended and the caudal fan opened and slightly folded), and raised (i.e. the crayfish body was elevated off the substratum, the chelipeds held off the substratum and parallel to it, or higher, and the abdomen and tail fan were extended); (2) executing cleaning acts (i.e. the crayfish rubbing of body parts and appendages with either the tiny chelae of the first and second walking legs or the sharp spines of the propodus of the third and the fourth walking legs; cleaning includes grabbing at the juveniles and shaking them off from the adult's body); and (3) executing feeding acts (i.e. the

crayfish scraping the substratum using chelipeds and/or pereopods or moving its maxillipeds);

- the number of attempts by each adult to grab the juveniles with its chelae (predation attempts);
- the relative frequency of the juveniles (1) approaching the adult (computed from the ratio between the number of the total approaches to less than 10 ± 2 mm from the adult in 1 min and the number of the juveniles free on the bottom of the tank during the record; the record was repeated every 5 min obtaining six measures per trial); and (2) tail-flipping away from the adult (computed from the ratio between the total number of tail-flips away executed in each trial and the total number of approaches to less than 10 ± 2 mm from the adult; tail-flips away were the backward swimming responses of crayfish, involving the sudden forward contraction of the abdominal section).

Additionally, we counted:

- the total number of the juveniles eaten by the adults in 1320 min (i.e. the number of missing juveniles at the end of each trial);
- the ratio between the number of the juveniles that were found to crawl on parts of the adult's body (e.g. chelipeds, antennae, carapace) ('attached juveniles') and the number of them still alive after 30 and 1320 min from the start of each trial.

The records obtained in the first 5 min were discarded from the analysis to reduce the disturbance consequent to the release.

Data analyses

Data were tested for normality using the Kolmogorov–Smirnov test and for homogeneity of variance using the Levene test. One-way ANOVAS (statistic: *F*) were used to analyze the variables that were shown to be normally distributed and homogeneous in their variance (i.e. distance covered and speed of movement) followed by a *post hoc* Tukey test. For the other variables (abdomen-bent posture, spoon-like telson posture, raised posture, cleaning acts and feeding acts), we used non-parametric Kruskal–Wallis tests (statistic: *H*), followed by multiple comparisons tests, and for frequencies (number of predation attempts, number of juveniles eaten) *G* tests after William's correction (statistic: *G*). The relative frequency of the attached juveniles with time was compared among treatments fitting a generalized linear model (GLM, statistic: *F*) for repeated measures, in which the treatments were entered as between-subject factors and the relative frequency of attached juveniles after 30 and 1320 min as within-subject factor. We

investigated the significance of between-subject factors (treatments) using a *post hoc* Tukey test and the significance of within-subject factor (time) using a Wilcoxon test (statistic: Z).

Correlation-based principal component analyses were used for potential-independent variables on transformed data, with a Varimax rotation. All factors with eigenvalues greater than 1.0 were retained and variables with absolute loadings of at least 0.5 were taken as important indicators of factors. To determine the association between the behaviour of the adults and the behaviour of the juveniles, enter linear regression analyses were performed (statistic: r^2), using orthogonal behaviours and postures factors (VAR 1–3) as independent variables and the relative frequencies of attached juveniles or of tail-flips away as a dependent variable. All tests were carried out with a significance level of $\alpha = 0.05$.

Results

An overall significant difference among the four treatments was found for the distance covered by adults ($F = 3.194$, $df = 3.48$, $p = 0.032$; Fig. 1a) and for their speed of movement ($F = 4.821$, $df = 3.48$, $p = 0.005$; Fig. 1b), as the result of the more intense locomotion shown by Fe and Ma than Bm and Fm. The spoon-like telson posture was maintained for a longer time by Bm and Fm than by non-brooding adults (Table 1). The latter, on the contrary, spent more time executing cleaning and feeding acts (Table 1) and performed significantly more frequent predation attempts ($G = 121.085$, $df = 3$, $p < 0.001$).

The relative frequency of juveniles found attached to their putative mother's body was significantly higher for brooding, rather than non-brooding, adults (GLM treatments: $F = 33.668$, $df = 3.48$, $p < 0.001$), although the latter always hosted about 20% of them (Fig. 2). This frequency increased with time (GLM time: $F = 13.825$, $df = 1,48$, $p = 0.001$)

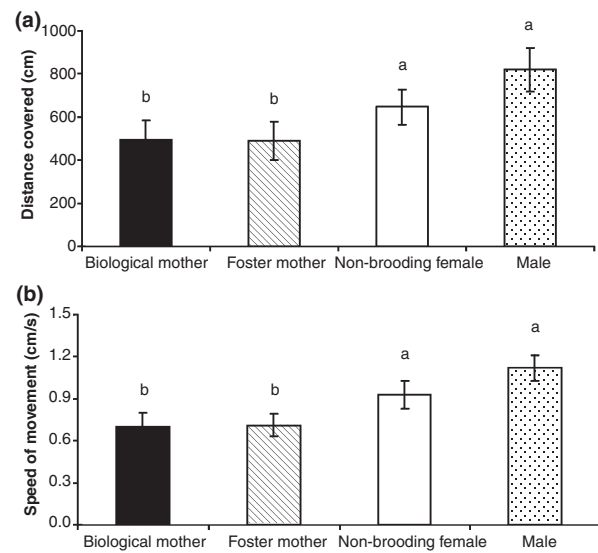


Fig. 1: Mean (\pm SE) distance covered (cm) by (a) and mean (\pm SE) speed of movement (cm/s) of (b) *Procambarus clarkii* adults of four types in the presence of 30 third-stage juveniles of the same brood after 30 min from the start of the experiment (the first 5 min have been discarded from the analysis). $N = 13$. Letters denote the hierarchy after a *post hoc* Tukey test.

only in Bm ($Z = -2.764$, $N = 13$, $p = 0.003$) and in Fm ($Z = -3.181$, $N = 13$, $p < 0.001$; conversely, Fe: $Z = -1.015$, $N = 13$, $p = 0.332$ and Ma: $Z = -0.280$, $N = 13$, $p = 0.800$) and, after 30 min from the start of the experiment, it was higher when the adult was a biological mother. At the end of the experiment, however, no difference was found between Bm and Fm (GLM treatments \times time: $F = 7.668$, $df = 3.48$, $p < 0.001$, followed by a *post hoc* Tukey test).

These results are independent of the speed of movement of the juveniles ($F = 1.157$, $df = 3.48$, $p = 0.036$), the distance covered by them ($F = 0.283$, $p = 0.837$), or the relative frequency of their approaches ($F = 0.126$, $df = 3.48$, $p = 0.944$). At the end of the experiment, the total number of eaten

Table 1: Difference in the time spent by adults in different postures and in the behaviours executed

Time spent in postures and executing behaviours (s)	Bm	Fm	Fe	Ma	H	df	p	Hierarchy
Abdomen bent posture	37 (0–124.5)	0 (0–379)	85 (2–287)	15 (0–61.5)	2.176	3	0.537	Bm = Fm = Fe = Ma
Spoon-like telson posture	1017 (165–1324)	822 (334–1285)	28 (0–88)	0 (0–14)	27.526	3	0.000	Bm = Fm > Fe = Ma
Raised posture	120 (0–242)	90 (0–253)	127 (39–362)	240 (75–462)	3.632	3	0.304	Bm = Fm = Fe = Ma
Cleaning acts	0 (0–6.5)	0 (0–19)	121 (8–378)	53 (7–166)	19.106	3	0.000	Bm = Fm < Fe = Ma
Feeding acts	0 (0–0)	0 (0–0)	66 (23–311)	46 (14–117)	38.829	3	0.000	Bm = Fm < Fe = Ma

Comparisons among treatments (Bm, biological mother; Fm, foster mother; Fe, non-brooding female; Ma, male) for median (and interquartile range) of the time spent by *Procambarus clarkii* adults in three postures and executing cleaning and feeding acts after Kruskal–Wallis tests (statistic: H). $N = 13$. Significant differences are in bold.

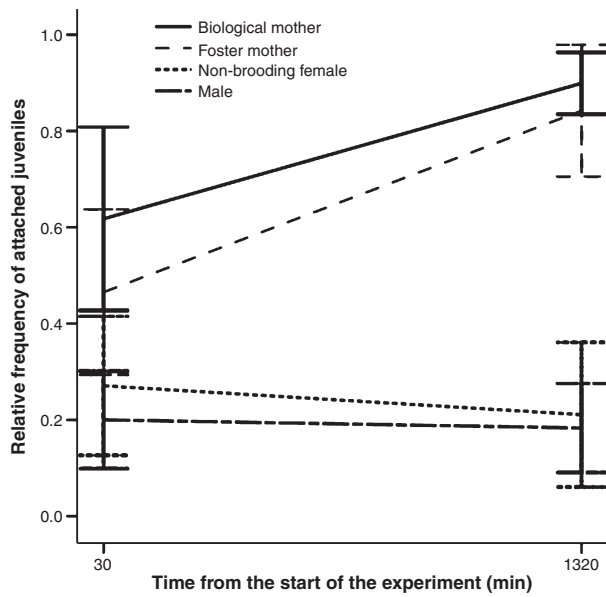


Fig. 2: Relative mean frequency (\pm SE) of the juveniles that were counted on their putative mother's body 30 and 1320 min after the start of the experiment compared among treatments. $N = 13$.

Table 2: Factor loadings of postural/behavioural variables (VAR 1–3) produced by principal component analyses with Varimax rotation

Source	VAR		
	1	2	3
Variance explained (%)	36.778	24.530	12.923
Eigenvalues	2.942	1.962	1.034
Behavioural/postural variables			
Distance covered (cm)	0.915	0.155	-0.056
Speed of movement (cm/s)	0.864	0.147	0.259
Abdomen-bent posture (time, s)	-0.491	0.039	0.598
Spoon-like telson posture (time, s)	-0.090	-0.593	-0.369
Raised posture (time, s)	0.794	0.085	-0.198
Cleaning acts (time, s)	0.087	0.150	0.864
Feeding acts (time, s)	0.039	0.879	0.132
Number of predation attempts	0.213	0.918	-0.092

Absolute loadings of 0.5 and greater are in bold.

juveniles significantly differed among treatments ($G = 410.450$, $df = 3$, $p < 0.001$), reaching 53.1% in Fe and 44.6% in Ma and only 1.5% and 3.1% in Bm and Fm, respectively.

Principal component analyses were used to categorize three orthogonal VAR factors that accounted for 74.23% of the observed variance. Factors loadings of VAR 1–3 are given in Table 2. Regression analyses show that the relative frequency of attached juveniles was associated with VAR 2. This indicates that return behaviour of *P. clarkii* juveniles is related in a

Table 3: Results of enter linear regression analyses using the relative number of the attached juveniles as the dependent variable and postural/behavioural factors (VAR 1–3) as independent variables

Variable	r^2	t	p
VAR 1	0.001	0.177	0.860
Distance covered (cm) 0.915			
Speed of movement (cm/s) 0.864			
Raised posture (time, s) 0.794			
VAR 2	0.138	-2.832	0.007
Spoon-like telson posture (time, s) -0.593			
Feeding acts (time, s) 0.879			
Number of predation attempts 0.918			
VAR 3	0.060	-1.786	0.080
Abdomen-bent posture (time, s) 0.598			
Cleaning acts (time, s) 0.864			

Important variables for each VAR (absolute loadings of 0.5 and greater) are shown with the respective factor scores.

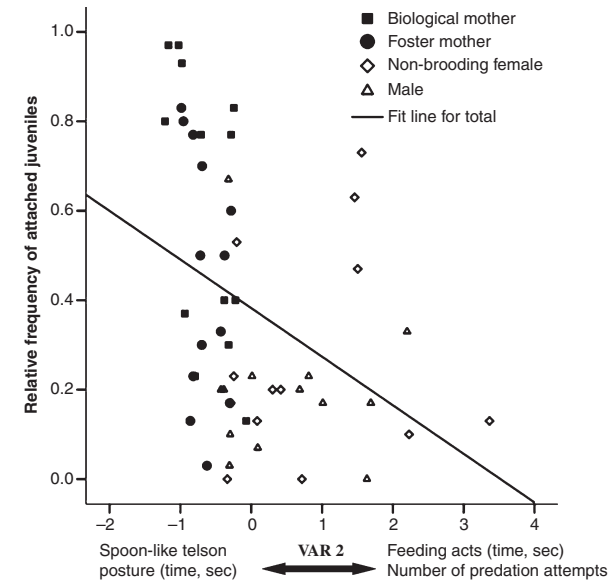


Fig. 3: Relationship between the postural/behavioural factor 2 (VAR 2) and the relative number of attached juveniles. Important variables (absolute loadings of 0.5 and greater) are shown.

positive fashion to the time spent by the adult in the spoon-like telson posture and in a negative fashion both to the time spent by its executing feeding acts and to the number of its predation attempts (Table 3, Fig. 3). Similarly, regression analyses show that the frequency of tail-flips away was associated with both VAR 1 and VAR 2. This typical escape behaviour seems to be directly linked with the increased distance covered by the adult, its speed of movement, the time spent by it in a raised posture

Table 4: Results of enter linear regression analyses using the relative frequency of tail-flips away executed by 30 juveniles per replicate as the dependent variable and postural/behavioural factors (VAR 1–3) as independent variables

Variable	r^2	t	P
VAR 1	0.110	2.487	0.016
Distance covered (cm) 0.915			
Speed of movement (cm/s) 0.864			
Raised posture (time, s) 0.794			
VAR 2	0.363	5.335	0.000
Spoon-like telson posture (time, s) -0.593			
Feeding acts (time, s) 0.879			
Number of predation attempts 0.918			
VAR 3	0.010	0.716	0.477
Abdomen-bent posture (time, s) 0.598			
Cleaning acts (time, s) 0.864			

Important variables for each VAR (absolute loadings of 0.5 and greater) are shown with the respective factor scores.

(VAR 1) and executing feeding acts, and the number of its predation attempts (VAR 2). Conversely, the relative frequency of tail-flips away decreased significantly with the time an adult spent in the spoon-like telson posture (Table 4, Fig. 4).

Discussion

Our study has pinpointed the complexity of mother–offspring relationships in *P. clarkii* and has described

a relatively high degree of maternal responsiveness towards potentially self-sufficient juveniles. Taken together, these results suggest that crayfish mothers are involved in a form of XPC that may be more refined than previously thought.

Procambarus clarkii mothers show a relatively scarce locomotion and a low speed of movement; this is a reflection of the reduced activity displayed by brooding females in natural conditions, where they extrude their eggs while confined in individual burrows and remain secluded there until the young have reached independence (Thiel 2007). Contrary to non-maternal adults, mothers execute few cleaning and feeding acts, do not show any attempts to grab the approaching juveniles, and were never observed cannibalizing them (see also Scudamore 1948; Levi et al. 1999). However, a relatively small fraction of juveniles (1.5% and 3.5% in Bm and Fm, respectively) was eaten by brooding females. This might be explained by *P. clarkii* mothers being inhibited from cannibalizing alive but not dead juveniles as has also been reported by Ameyaw-Akumfi (1976) and Hazlett (1983). As *P. clarkii* mothers remain in their burrow for many weeks, feeding on dead juveniles probably serves as a source of nutrient for them and contemporaneously avoids excess contamination of the water in the burrow (Little 1976); it obviously requires a mechanism – still unexplored – that the mothers should

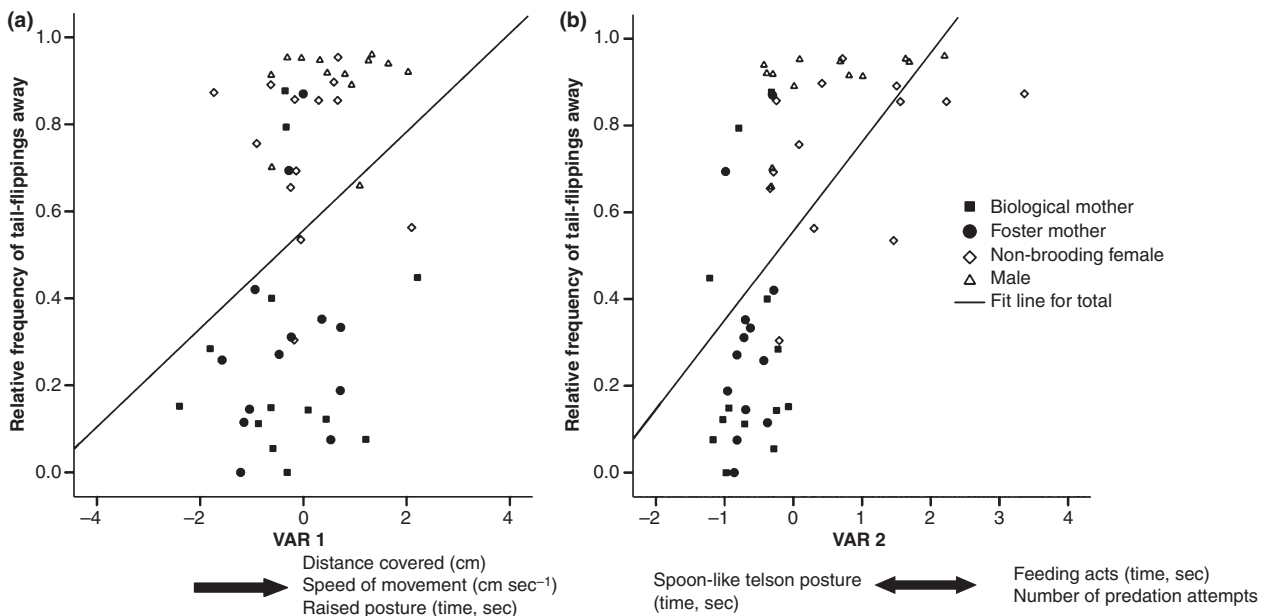


Fig. 4: Relationship between the postural/behavioural factor 1 (VAR 1) (a) and factor 2 (VAR 2) (b) and the relative frequency of tail-flips away executed by 30 juveniles per replicate. Important behavioural variables (absolute loadings of 0.5 and greater) are shown.

have to discriminate between alive and dead offspring.

Procambarus clarkii mothers assume a characteristic posture – here indicated as spoon-like telson posture – that seems to facilitate the juveniles' return to the female's abdomen. The persistence in this posture, in fact, increase the number of the juveniles clinging on their mothers' body and reduce the frequency of tail-flips away by them, whereas the adults' feeding acts and predation attempts had opposite effects. Juveniles also seemed to escape in response to the intensity of locomotion and to the time the adult spent in a raised posture, both symptoms of aggressiveness.

As a confirmation of previous observations, *P. clarkii* females, separated for a relatively short time from their brood, behave as mothers in the presence of juveniles other than their own. Fostering, which occurs when an alloparent adopts the offspring of another individual providing it with exclusive care (Riedman 1982), has been described in a number of invertebrates (e.g. in the burying beetle, *Necrophorus vespilloides*; Müller & Eggert 1990), but it is obviously most prevalent in vertebrates, particularly in birds and mammals (Packer et al. 1992). Differently from the desert isopod *Hemilepistus reaumuri* (Linsenmair 1985) and from the burrow-dwelling amphipod *Casco bigelowi* (Thiel 1999), crayfish females seem to be unable to discriminate between their own and a foreign brood (Figler et al. 1997) as a consequence of their being confined for a long time in the same shelter. In reproductive burrows, in fact, the likelihood of a mother to encounter foreign offspring would be extremely low. As a consequence, a refined mechanism to recognize the own brood might not have an adaptive value in this taxon.

The maintenance of the maternal behaviour may be the result of a complex and still unexplored interaction between the hormonal *milieu* of the brooding female and the feedback she receives from her brood (Levi et al. 1999). Studies by Little (1976) suggested that, without any physical contact with the juveniles for more than 12 d, the female starts to cannibalize them. Cannibalism also appears when the number of young attached to the female body becomes less than 10 (Little 1976), as an indication that mechanoreceptors on the female pleopods are involved (Figler et al. 1997, 2004).

The intensity of movement of the juveniles, their average distance from the putative mother and their approaches to her seemed not to depend on the maternal state of the offered adult. Conversely, the frequency of tail-flips away and the number of

the attached juveniles were significantly affected. This in part confirms findings by Little (1975, 1976) and Ameyaw-Akumfi (1976), who showed that third-stage juveniles of at least five crayfish species orient towards the water in which a brooding female (either the biological or the non-biological mother) has been held, but not towards the water conditioned by the odour of a male or of a non-brooding female, thus suggesting the release by the former of a maternal pheromone. However, our results show that this issue has not been completely solved. In fact, the behaviour of juveniles changes at a short distance (1 cm on average) from the putative mother, when they possibly start to smell the adult. However, their decision of whether to escape from or to attach to the adult may be more likely made by combining chemical information with visual stimuli associated with maternal behaviour (Hazlett 1983). Besides, the ability of juveniles to recognize mothers from other non-maternal conspecifics seems to depend on the offspring's individual experience. We found that the number of attached juveniles increased with time in the presence of a maternal female but remained constant when offered a non-brooding adult. Juveniles accepted foster mothers but not as fast as biological mothers. In fact, the number of attached juveniles was lower with foster mothers than biological mothers after 30 min from the start of the experiment, but not after 1320 min. These results might suggest that juveniles can discriminate their biological mother from other brooding females but are ready to accept also the latter after having experienced their maternal behaviour.

Extended parental care is a property of K-selected species (Thiel 2003); it typically involves substantial expenditure of energy and of other resources by the parents (Clutton-Brock 1991) but also yields elevated costs in terms of losing time for growth and for further reproduction (Thiel 1999). While carrying developing offspring, *P. clarkii* females are in fact inhibited in moulting and in producing a second brood for at least 3 mo (Huner & Barr 1991; Huner 1994). However, the mother-offspring relationships shown by this species, possibly more refined than those described for other crayfish, might enable it to thrive in harsh environmental conditions.

Acknowledgements

The experiments comply with the current laws of Italy, the country in which they were done.

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