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Assessing mate size in the red swamp crayfish *Procambarus clarkii*: effects of visual versus chemical stimuli

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SUMMARY

1. Mate assessment requires an efficient system of information exchange between the sexes and often relies on several forms of communication. Both sexes of the crayfish, *Procambarus clarkii*, select a mate on the basis of its body size, independently of chelar size/symmetry and dominance status, large size being a proxy for a high quality partner. Here, we explored the effects that visual and chemical stimuli, emitted alone or in combination, exert on the assessment of size of a potential mate.

2. We followed a binary choice test paradigm, in which two male or female 'targets', of either large or small size, were simultaneously presented to a female or a male 'chooser'. We recorded the first target visited, the total duration of the visits per target, and the behaviour of the chooser when approaching the target area.

3. Our results show that females require a combination of visual and chemical stimuli to select the larger male. A more complex pattern was found when males were the choosers. While visual and chemical cues together rendered them willing to mate with the larger female, their initial choice and the total time spent near her depended only on smell. In mate assessment by *P. clarkii*, visual and chemical information seem to act as 'non-redundant signals'.

Keywords: body size, crayfish, invasive species, mate assessment, multimodality, *Procambarus clarkii*

Introduction

Mate assessment requires an efficient system of information exchange between the sexes and often relies on composite stimuli of more than one sense (Guilford & Dawkins, 1991; Rowe & Guilford, 1999; Sneddon *et al.*, 2003; Partan & Marler, 2005). Indeed, the synchronous use of different senses provides the chooser with more reliable information compared to a single sense (Partan & Marler, 1999; Rowe, 1999). Such multimodality improves the ability of the assessor to detect and localize target individuals by both modulating its responses and reducing the ambiguity of the signals (Partan & Marler, 2005).

Multimodal communication is adopted by several invertebrate species in a wide range of contexts, including defensive warning in many insect species (e.g. Marples, van Veelen & Brakefield, 1994; Rowe & Guilford, 1999), recruitment of nest mates in eusocial insects (e.g. Tautz & Rohrseitz, 1998; Hölldobler, 1999), and courtship displays in crickets (e.g. Balakrishnan & Pollack, 1997), fruitflies (e.g. Rybak, Sureau & Aubin, 2002), moths (e.g. Takács, Mistal & Gries, 2003) and spiders (reviewed in Uetz & Roberts, 2002). Despite this long list of studies, however, there has been relatively little interest in multimodal communication during mate assessment.

With a few exceptions (Hughes, 1996; Shuster & Wade, 2003), the literature underlines the importance of chemical communication in the sexual behaviour of crustaceans (Atema, 1995), although a glaring gap in knowledge is still the lack of information about the

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chemical identity of the putative sexual pheromones (Atema & Steinbach, 2007). In several species, sex identification and mating behaviour have been found to be affected by ablation of the antennules (Christofferson, 1970; Cowan, 1991; Bamber & Naylor, 1996) but not by blindness (Snyder, Ameyaw-Akumfi & Chang, 1993; Bushmann, 1999). There are few examples in which the use, during mate assessment by crustaceans, of either visual stimuli alone (Dunham & Oh, 1996; Corotto *et al.*, 1999) or chemical and visual stimuli in combination has been investigated. Females of the blue crab, *Callinectes sapidus* Rathbun, recognize mates by smell, but their execution of sexual displays is stimulated by the co-occurrence of smell and sight (Teytaud, 1971). The combined effects of sight and smell have been described in the snapping shrimp *Alpheus heterochaelis* Say (Hughes, 1996) and in stomatopods (Christy & Salmon, 1991; Marshall *et al.*, 1999). Similarly, male *Austropotamobius pallipes* Lereboullet require both visual and chemical stimuli to respond to a female (Acquistapace *et al.*, 2002).

Procambarus clarkii (Girard) is a well-known invasive crayfish in Europe (Gherardi, 2006; Gherardi & Acquistapace, 2007). A previous study (Acquistapace, 2003) had shown that males of this species are able to distinguish the sex of a conspecific using chemical cues alone, whereas females need both visual and chemical stimuli to do so. In addition, a conspecific of the opposite sex with a large body size is usually selected by either a male or a female as a high quality mate (Aquiloni & Gherardi, 2007). This choice is independent of the chelar size/symmetry and hierarchical status of the target individual. The reasons for this form of sexual selection are several. On the one hand, females may benefit by selecting large males because they produce more sperm and are more likely win fights for vital resources, such as burrows (Gherardi, 2002). Large size might be also an expression of high quality genes, and females mating with a large male can transmit this quality to their offspring (Hunt, Brooks & Jennions, 2005). Alternatively, a slight ancestral preference for large males might have led both to an increased frequency of 'large-body genes' among males and to an increased female preference for large males (Weatherhead & Robertson, 1979). On the other hand, *P. clarkii* males, who are known to invest much energy and time in the production of spermatophores and often have a limited number of gametes (Gherardi, 2002), may

seek for a highly fecund female as is honestly expressed by her large body size (Nobblitt, Payne & Delong, 1995).

In this paper, we aimed to investigate the sensory channel/s (sight, smell or the two combined) used by a chooser to get information about the quality (i.e. the size) of a potential mate.

Methods

Collection and holding conditions

About 200 individuals (females and Form I – reproductive-males) were collected using baited traps from the Massaciuccoli Lake (Tuscany, Italy) in June, before the onset of the reproduction. Once in the laboratory, the cephalothorax length (from the tip of the rostrum to the posterior edge of the carapace) of each individual was measured using a vernier caliper to the nearest 0.1 mm. Sexes were kept apart in tanks ($80 \times 60 \times 60$ cm) containing clay pots as refuges at a density of 15 animals m^{-2}. They were maintained in a natural light-dark cycle at room temperature (20 °C) and fed *ad libitum* with live *Calliphora* sp. larvae. Water was changed weekly.

Experimental design

We followed a binary choice test paradigm (modified from Sato & Goshima, 2007), in which pairs of live crayfish, the 'targets', of different body size ('large' and 'small'), were simultaneously offered to a test animal of the opposite sex, the 'chooser'. The apparatus was designed so that the chooser was able to perceive either visual or chemical stimuli from both targets or both stimuli. In particular, we assessed the ability of the chooser to discriminate between a large and a small target subject to three treatments: (1) sight only (Vi = visual), (2) smell only (Ch = chemical) and (3) sight and smell (Vi + Ch = visual plus chemical). A control (C) was also run to analyse the chooser's movement in the experimental apparatus in the absence of any stimulus emitted by targets.

The experimental apparatus (Aquiloni & Gherardi, 2007) consisted of an elliptical plastic aquarium (65×40 cm, water level: 10 cm) containing 26 L of still tap water. Two circles (diameter: 20 cm), 20 cm apart, were drawn on the bottom. The release point of the chooser was at the centre of the ellipse,

equidistant from the centre of the two circles. Twenty minutes before the start of any trial, each of the two targets was inserted randomly into one of two 22 cm-high transparent plastic containers (10 × 4 cm) each put on the centre of a circle, hereafter called 'target area'. The size of the containers was small enough to impede any movement of the individual held within. According to treatment, the plastic containers were: transparent (in Vi); opaque and finely drilled with holes (3 mm of diameter) at a density of 4 cm^{-2} (in Ch); transparent and similarly drilled (in Vi + Ch); or empty (in C).

Each chooser was acclimatized to the experimental condition by keeping it for 5 min at the release point inside a cylinder of wire netting. The wire netting was then removed and the experiment started. Between trials, the experimental apparatus was thoroughly washed with clean tap water.

Choosers were used only once; each pair of targets was used in a maximum of three trials on different and non-consecutive days. Before and after each trial, both chooser and targets were tested for their responsiveness to a sexual partner. Individuals being defined as responsive when, once a couple was placed in a circular tank, the male tried to turn the female over for copulation. If a crayfish, after being used, was found to be non-responsive, the corresponding trial was excluded from the analysis. Each treatment was replicated 20 times for each sex, following a random sequence.

Criteria for choosing crayfish for experiments

The experiment was carried out at the beginning of July 2006. Only hard-shelled crayfish with all the appendages and the rostrum intact were used. The mean (±SE) cephalothorax length of large and small male targets was 53.8 ± 5.71 and 36.4 ± 2.67, respectively, and for female targets was 56.36 ± 3.89 and 37.55 ± 2.55 respectively. The size of choosers was 43.7 ± 0.72 for males and 42.0 ± 0.5 for females. Information about the appropriate size of the individuals used to form pairs was taken from the literature on other crayfish species (Woodlock & Reynolds, 1988) and from personal observations on the study species. Due to the allometric growth of chelae (Gherardi, Barbaresi & Raddi, 1999), a difference in the cephalothorax length was accompanied by a difference in the size of the chelae, which seemed

not to influence mate choice in the study species (Aquiloni & Gherardi, 2007).

Crayfish were kept in isolation for at least a week before the experiment. In no case did the chooser meet the targets prior to the experiment, at least in the 4 weeks of their maintenance in the laboratory, so that we can exclude any effect of previous social experience (Bergman & Moore, 2005).

Collection of data

The data were collected between 08:00 and 14:00 hours. Crayfish behaviour was video-taped for 20 min using a digital camera activated at a distance to avoid disturbance to the experimental animals. Video-tapes were subsequently analysed to acquire the following data:

1. The first target visited, i.e. the first target area entered by the chooser. We excluded instances in which the chooser entered the area by swimming or walking backwards.

2. The total duration of visits in seconds, as an estimate of the chooser's persistence in his/her choice. The duration of each single visit was computed as the time between the rostrum of the chooser entering a target area and its telson leaving it.

3. The behaviour of the chooser approaching a target area; this allowed us to infer whether the chooser was willing to interact with the target individual and if it was motivated either to mate or to fight. A score from 0 to 2 was arbitrarily assigned to each visit, and the most frequent score was assigned to each chooser per target. Scores were 0 (when the chooser approached the target area following a meander trajectory while exploring the apparatus), 1 (when the chooser approached the target with its chelipeds lowered) and 2 (when it did so with its chelipeds raised). As previously shown (e.g. Ameyaw-Akumfi & Hazlett, 1975; Acquistapace, 2003), in this species posture is an expression of either a 'peaceful' (chelipeds lowered) or an 'aggressive' (chelipeds raised) attitude towards a conspecific.

Statistical analyses

Data were first checked for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene test, respectively, which allowed us to use parametric tests when appropriate. The total duration

of visits was compared within each treatment using Student's *t*-tests for paired data (statistic: *t*) and among treatments using two-way ANOVAS (statistic: *F*), in which the target animal's size and treatments were fixed factors and the duration of visits was the variable. Frequencies were analysed by *G*-tests after Williams correction (H_0 = uniform distribution of frequencies) (statistic: *G*). The level of significance under which the null hypothesis was rejected is $\alpha = 0.05$. Text and figures report mean ± SE.

Results

The first target visited

The large individual was usually the first target to be visited in Ch when the chooser was a male (16 out of 20; $G_1 = 7.522$, $P < 0.01$) and in Vi + Ch when the chooser was a female (18 out of 20; $G_1 = 14.363$, $P < 0.001$) (Fig. 1). The other comparisons ranged between $G_1 = 0.195$ and $G_1 = 1.783$ ($P > 0.05$). No difference was found among treatments in either sex (males: $G_3 = 2.319$, $P > 0.05$; females: $G_3 = 4.125$, $P > 0.05$). No significant difference was found in the control ($P > 0.05$).
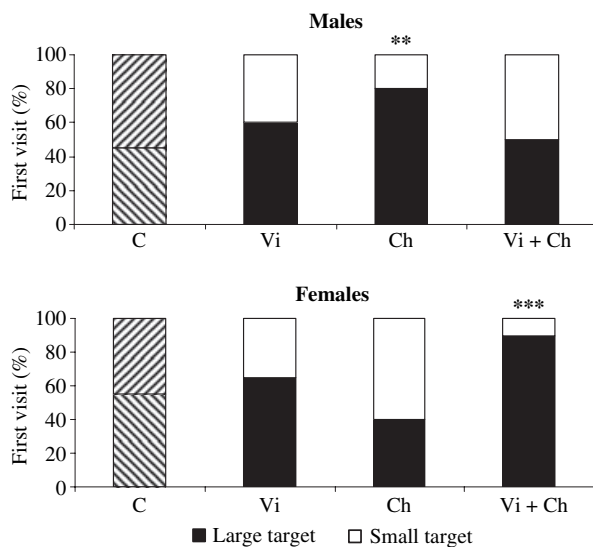
Total duration of visits

Male choosers spent more time near large, rather than the small, targets in both Ch ($t_{19} = 2.642$, $P = 0.016$) and Vi + Ch ($t_{19} = 3.239$, $P = 0.004$), while this was true of female choosers in Vi + Ch only ($t_{19} = 2.441$, $P = 0.025$) (Fig. 2). The other comparisons ranged between $t_{19} = -0.219$ and $t_{19} = 1.474$ ($P > 0.05$). The total duration of visits did not significantly differ among treatments when the chooser was either a male ($F_{2,120} = 0.747$, $P = 0.476$) or a female ($F_{2,120} = 2.085$, $P = 0.129$). No significant difference was found in the control ($P > 0.05$).

Behaviour of the chooser approaching the target area

As expected, the score obtained by the choosers in C was nearly always 0 (males: $G_2 = 65.487$, $P < 0.001$; females: $G_2 = 70.827$, $P < 0.001$) and did not differ between target areas ($P > 0.05$) (Fig. 3). In both sexes, the chooser showed an aggressive behaviour (i.e. chelipeds raised) when it could only see a conspecific (males: $G_2 = 19.625$, $P < 0.001$; females: $G_2 = 6.432$,
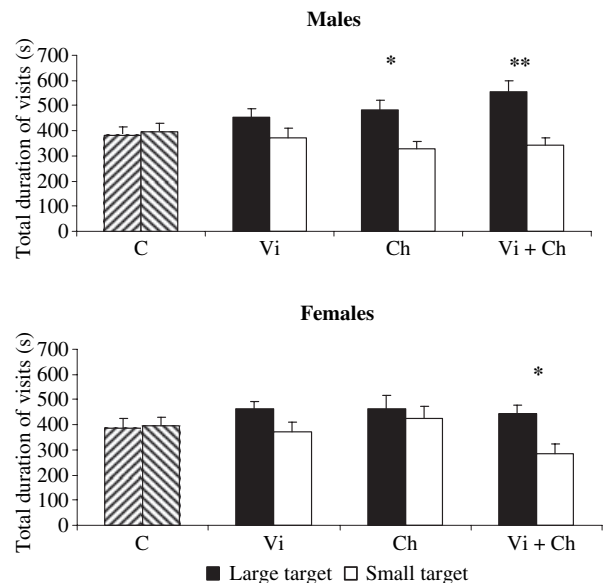

Fig. 1 Frequency (%) of the first visit to a target by a chooser of the opposite sex in the three treatments (Visual, Vi; Chemical, Ch; and Visual plus chemical, Vi + Ch) and in the control (C, striped bar). The target was of either large (black bar) or small (white bar) size. Two and three asterisks denote significant differences at $P < 0.01$ and $P < 0.001$, respectively, after a *G*-test. Each treatment and the control were replicated 20 times.

Fig. 2 The mean total duration of visits (±SE) to a target by a chooser of the opposite sex in the three treatments (Visual, Vi; Chemical, Ch; and Visual plus chemical, Vi + Ch) and in the control (C, striped bar). The target was of either large (black bar) or small (white bar) size. One and two asterisks denote significant differences at $P < 0.05$ and $P < 0.01$, respectively, after a Student's *t*-test. Each treatment and the control were replicated 20 times.
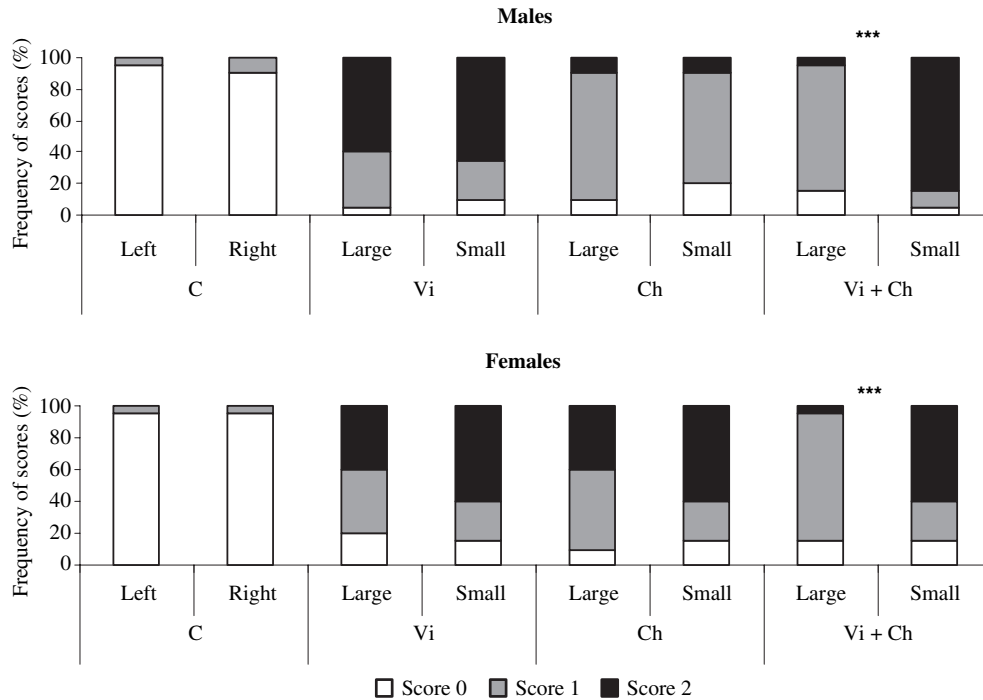
Fig. 3 Frequency (%) of Score 0 (white bar), of Score 1 (grey bar) and Score 2 (black bar) obtained by a chooser approaching the target area in the three treatments (Visual, Vi; Chemical, Ch; and Visual plus chemical, Vi + Ch) and in the control (C). Scores are indices of the chooser's behaviour, 0, 1 and 2 indicating willingness to explore, to mate and to fight, respectively. The two equal sides in the control are indicated with Left and Right. The three asterisks denote significant differences at $P < 0.001$ after a G-test. Each treatment and the control were replicated 20 times.

$P < 0.05$), independent of the target animal's size ($P > 0.05$), whereas in the presence of both visual and chemical stimuli it approached the larger target most often with the chelipeds lowered (male choosers: $G_2 = 28.294$, $P < 0.001$; female choosers: $G_2 = 15.968$, $P < 0.001$). Conversely, independently of the target animal's size ($P > 0.05$), the two sexes differed when they were presented with odour only: males appeared to be willing to mate ($G_2 = 28.959$, $P < 0.001$), whereas females behaved aggressively ($G_2 = 9.781$, $P < 0.01$).

Discussion

Contrary to expectation, the results of this study suggest that male and female *P. clarkii* use composite stimuli to assess the size of a potential mate, although in different ways. Females always used visual and chemical stimuli in combination to choose large (i.e. high quality) mate, whereas the sight or odour only of an individual of the opposite sex, independently of size, elicited aggression. A more complex pattern was found when the chooser was a male. On the one hand,

males used smell to make their initial choice of the larger female and to persist in this choice, staying for a longer time in her proximity. On the other hand, their willingness to mate with the larger target depended on a combination of information from sight and smell, as shown by their approaching the target animal with the chelipeds lowered. No synergistic effect was ever found when visual and chemical stimuli co-occurred.

Traditionally, olfactory stimuli have been classified as long-distance signals (Dusenbery, 1992). Their use may be advantageous in the turbid water where *P. clarkii* usually lives (Smith, 1992). Generally, the sex actively involved in mate searching detects a potential mate at a relatively long distance using smell. In the crabs *Carcinus maenas* Linnaeus (Bamber & Naylor, 1996), *Telmessus cheiragonus* Tilesius (Kamio, Matsunaga & Fusetani, 2002) and *Chionoectes opilio* Fabricius (Bouchard, Sainte-Marie & McNeil, 1996), males make use of chemical stimuli, being the sex involved in mate searching. On the contrary, female *Homarus americanus* Edwards (Bushmann & Atema, 1997) are able to chemically detect males at a distance of about

5 m, while males discriminate between the sexes by smell and between the receptivity state of the approaching female at close range (Bushmann & Atema, 2000). During visits to shelters, both sexes greatly increase their urine release (Bushmann & Atema, 2000) revealing the possible exchange of chemical signals between potential mates.

Since *P. clarkii* males seem to make a greater use of odours, it is probably the sex involved in mate search. In this study, due to the experimental setup we used, we were unable to identify the distance at which males could smell their potential (and large) mate. A novel result is that the putative chemical stimuli emitted by *P. clarkii* females can convey information not only about the species and the sex they belong to (Ameyaw-Akumfi & Hazlett, 1975). Possibly complemented by visual information at a shorter distance, odours also apparently inform the chooser about the body size of the mate, although we cannot yet explain how this is done. An alternative (and more parsimonious) explanation might be that, in our experimental apparatus, large individuals produce currents that favour the more effective transport of their odour to the chooser. However, this seems unlikely because of the slight movements that target individuals executed in the holding container.

Laboratory experiments on the chemosensory systems used in aquatic predator-prey relations support the notion that chemicals may yield information about the age/size of other individuals. In salmonid fishes, the char *Salvelinus fontinalis* Mitchill responds more intensively to the chemical alarm odours emitted by individuals of their own age/size (Mirza & Chivers, 2002), and in fathead minnows *Pimephales promelas* Rafinesque the chemical cues from large and more dangerous pike are more effective than those from small ones (Kush, Mirza & Chivers, 2004). Similarly, the crucian carp *Carassius carassius* Linnaeus discriminates by smell between large and small pike (Pettersson, Nilsson & Brönmark, 2000).

Visual stimuli are used in short-distance communication systems once the individuals are in close proximity to each other. Their role in mating has been investigated in several terrestrial decapods (Salmon, 1983; Burggren & MacMahon, 1988; Backwell *et al.*, 1998; Pope, 2000) and in species from shallow waters, such as several stomatopods (Hatziolos & Caldwell, 1983; Christy & Salmon, 1991; Marshall *et al.*, 1999). In the aquatic *medium*, visual stimuli are most often accompanied by the emission of chemical cues (reviewed in Salmon, 1983), as found in the snapping shrimp *A. heterochelis* (Hughes), in the rock shrimp *Rhynchocinetes typus* Edwards (Diaz & Thiel, 2003) and in the crayfish *A. pallipes* (Acquistapace *et al.*, 2002). This is confirmed in the present study, which shows that the co-occurrence of chemical and visual information makes both sexes of *P. clarkii* willing to mate.

Several female decapods select high quality males on the basis of the visual stimuli produced, for instance, by a sand pyramid (Linsenmair, 1967), a shelter (Emlen & Oring, 1977; Christy, 1983; Rabeni, 1985), or a display (Murai & Backwell, 2006), which may all function as reliable indicators of male fitness (reviewed in Atema & Cobb, 1980; Christy, 1987). Similarly, body size seems to be the best honest expression of male mate quality (Hyatt, 1977; reviewed in Atema & Cobb, 1980; Greenspan, 1980). Surprisingly, in *P. clarkii* the sight of a mate of a larger size is not used *per se* as an index of the 'best' partner but this must be confirmed by chemical stimuli that, in their turn, might inform about the species, the sex of the individual, his/her moulting state, and/or his/her reproductive condition (for instance, whether the male is sperm depleted; Markow, Quaid & Kerr, 1978; Nakatsuru & Kramer, 1982; Sato & Goshima, 2007).

In conclusion, our results suggest that sight and smell work differently in male and female *P. clarkii*, possibly due to the diverse role played by the two sexes during mating. However, their responsiveness towards a high quality (i.e. large-sized) mate relies on the co-occurrence of visual and chemical stimuli that seem to provide different kinds of information and thus are not redundant (Partan & Marler, 2005).

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