



Female mate choice by chemical signals in a semi-terrestrial crab

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ABSTRACT

Information about the roles of both sexes in pair formation is required to better understand the mechanisms involved in sexual selection. Mate choice could depend on the courtship behavior, involving chemical, tactile and visual signals. We determined if *Neohelice granulata* mate choice is based on female or male choice, considering visual and chemical with contact and without contact signals between partners and different categories of individuals: receptive and unreceptive females; and large, small, mated or unmated males. Experiments showed that mate selection was based on receptive female's choice using chemical signals, but not visual ones. Since copulation occurs during high and low tides, water-borne chemical signals would be preferentially used during high tide, while contact ones during low tide. Females preferred large and unmated males, while males did not seem to recognize receptive females using chemical neither visual signals. Females were capable of detecting the presence of the chemical signals released by large and unmated males, but not its amount. It is proposed that small and mated males are probably releasing different types of chemical signals, not attractive to females, or that they are not emitting any signal.

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1. Introduction

Many studies have reported non-random mating due to female or male choice of sexual partners (Harvey and Bradbury, 1991). Females are assumed to increase their reproductive success when choosing a single “high quality” male (Hunt et al., 2004), or by mating with several males, thus increasing the genetic diversity of their offspring (Andersson, 1994; Jennions and Petrie, 2000). In turn, males are assumed to increase their fitness by reproducing with as many females as possible (Clutton-Brock and Parker, 1992; Trivers, 1972). Specifically in Brachyura crabs, females are benefited by having their seminal receptacles filled with as much sperm as possible, while males are favored by occupying with their sperm as much female seminal receptacle space as possible (McLay and López Greco, 2011).

The mechanisms used in mate choice are the result of the sexual selection process. Sexual selection has been traditionally considered as the result of direct competition among males and female choice (Bateman, 1948; Darwin, 1871; Trivers, 1972), although male choice was also recorded in some species (Berglund et al., 1996; Reading and Backwell, 2007). Mate choice would depend on signals that involved intrinsic and extrinsic factors (Thiel and Duffy, 2007). In the former, morphological and physiological traits of the individuals are considered, while in the latter, different types of signals are

included such as visual, acoustic, tactile and water-borne chemical signals used to attract mates.

Considering intrinsic factors, the mating systems of different Brachyura species in which the larger dominant males acquire the greatest reproductive success are commonly described (Aquiloni and Gherardi, 2008; Gosselin et al., 2003; Sal Moyano et al., 2012a). Thus, choice by females is expected in those mating systems. Theory predicts that intra-sexual selection favors traits such as large body size that improves the potential of males to monopolize females. Moreover, when there is strong intra-specific competition and males defend resources used for mating purposes, such as self-constructed burrows, female mate choice is also predicted (Backwell and Passmore, 1996; Popper et al., 2001; Reading and Backwell, 2007). There is evidence that multiple criteria are often employed during mate assessment (Balmford et al., 1992; Burley, 1981; Pomiankowski and Iwasa, 1993; Thornhill, 1983), and females could select mates according to both male phenotype and the quality of defended resources (Balmford et al., 1992; Hebets and Rundus, 2011; Thompson, 1986; Thornhill, 1983).

Females of some species have the capacity to mate repeatedly with different males throughout a single reproductive period (Hartnoll, 2000). Thus, females would be more selective than males since copulation with alternative males promotes sperm competition inside their seminal receptacle (Thiel and Duffy, 2007). The mating frequency of males is another factor that may influence female choice because the amount of sperm reserve decreases with increased mating frequency, promoting sperm limitation in mated males (Sato and Goshima, 2006). Evidence indicates that females do not always prefer dominant

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males since sperm limitation may be a cost to females that mate with dominant males (Qvamström and Forsgren, 1998).

Considering extrinsic factors mate choice depends on the different kinds of signals involved in the attraction and assessment of the mate. A recent definition of signal indicates that “it is a feature of an individual's phenotype that evolves because it elicits a response from other organisms that increase the signaler's fitness” (Christy and Rittschof, 2011; after Hasson, 1994). However, little is known about the way in which individuals obtain information related to the quality of the potential mates and how this information is used in mating. Some crustacean studies recognized the importance of chemical signals during reproductive behavior interactions (Bushman and Atema, 2000; Dunham, 1988). Chemical signals are commonly named pheromones, emitted and received by individuals of the same species (Wyatt, 2010). Mate choice using chemical signals by one or the other sex, or both of them, would seem to vary among species and may depend on their social context (Borowsky, 1985; Kamio et al., 2000, 2002; Raethke et al., 2004). Alternative mechanisms involved in mate recognition include: visual signals (Baldwin and Johnsen, 2012; Christy and Rittschof, 2011; Christy and Salmon, 1991; Salmon, 1983), chemical signals detected through the contact between partners, since the existence of “contact” sexual pheromones characterized as cuticle exudates was described (Bauer, 2011; Borowsky, 1991; Caskey and Bauer, 2005; Ekerholm and Hallberg, 2005; Herborg et al., 2006; Kamio et al., 2002), and acoustic signals (Popper et al., 2001; Salmon and Horch, 1972). Visual and acoustic signals were commonly described for semi-terrestrial decapod species, principally ocapodids, while “contact” chemical signals were registered for aquatic and semi-terrestrial species.

The burrowing and semi-terrestrial crab *Neohelice granulata* (Dana, 1851) is considered a keystone species in South American salt marshes, mud flats and estuaries. This crab ranges from northern Patagonia, Argentina (42°25'S; 64°36'W), through Uruguay, to Rio de Janeiro, Brazil (22°57'S; 42°50'W) (Spivak, 2010). It is distributed in high densities in habitats of varying salinity, temperature and tidal patterns, inhabiting both *Spartina* salt marsh and intertidal mud flats, generating extensive beds of burrows (Luppi et al., 2013; Spivak et al., 1994). Burrows reach high densities, up to 60 burrows m⁻² (Iribarne et al., 1997). The mating system of the population of *N. granulata* inhabiting the Mar Chiquita Coastal Lagoon (37°45'S; 57°19'W, Buenos Aires, Argentina) was characterized recently (Sal Moyano et al., 2012a), as belonging to the “resource center competition” type, defined by Emlen and Oring (1977) and Christy (1987). Large males construct burrows with chambers where a successful copulatory and post-copulatory guarding behavior occurs, while small males construct burrows without chambers, and their mating commonly occurred on the surface or less frequently inside empty burrows with chambers constructed by large males (Sal Moyano et al., 2012a). Thus, male size may be influencing female mate choice since burrow chambers constructed by large males are safer places used for mating purposes. Additionally, this difference in the mating success among large and small males related to the burrow form and the capacity of females to copulate with several males throughout its receptive period (Sal Moyano et al., 2012b) could influence male mating frequency, another important factor affecting mate choice.

Field observations showed that individuals are exposed to a semi-diurnal tidal pattern; females sample male burrows by getting inside and outside them initiating a courtship behavior and copulate inside or outside burrows during low and high tides. During low tide water recedes and burrows become totally exposed, thus an important effect of visual and “contact” chemical signals used in mate recognition and copulation is predicted. During high tide, burrows are totally covered by water and the use of water-borne chemical signals is expected to be involved in mating. This would indicate that mate-attraction is mediated by different kinds of signals at the interface of two different environmental conditions. The objective of the present study was to determine if mate choice is based on female or male choice, considering (1) visual and chemical signals and (2)

different categories of individuals: receptive versus unreceptive females, large versus small and mated versus unmated males.

2. Material and methods

N. granulata crabs were collected by hand from the field one month before the beginning of the reproductive season (August) of 2010/2011 and 2011/2012 in Mar Chiquita Coastal Lagoon and transported to laboratory. In the laboratory individuals were maintained in seawater aerated aquaria (0.3 × 0.35 × 0.25 m, containing 3 cm of seawater), and sexes were kept separate. Individuals were fed three times a week with pet-food pellets and water was changed weekly. Crabs were maintained under natural daylight conditions, and temperatures that ranged between 22 and 28 °C (mean 24 °C). Individuals were measured with a caliper accurate to 0.1 mm using the carapace width (CW) as the reference variable. Only morphologically and physiologically mature crabs of both sexes were used during experiments (>19 mm CW; López Greco and Rodríguez, 1998). Three categories of individuals were employed for the experiments: unreceptive (UF) and receptive (RF) non-ovigerous females ranging from 24.2 to 30.1 mm CW and from 23.3 to 29.5 mm CW, respectively; large males (LM) of 30–34 mm CW, and small males (SM) of 25–28 mm CW; and recently unmated males (UM) of 26.9–31.8 mm CW, and recently mated males (MM) of 26.4–32.1 mm CW. The experiments begun on the same day that a receptive female was recognized. Receptive females were recognized by the presence of mobile vulvae opercula since it could be pushed inwards like a trapdoor when checked under a binocular microscope: the pleon was lifted and the two opercula were gently probed daily using fine forceps (see Sal Moyano et al., 2012b). Recently mated males were obtained after they were placed in an aquarium with a receptive female and successful copulation was observed. Copulation was considered to have occurred when both the male and female pleon were observed open and the male gonopods were inserted into the female vulvae. Immediately after copulation ended and the pair separated, the male was used for the experiments. In all the experiments, in order to avoid a possible effect of the size difference between sexes in mate choice and, according to the size of the mating partners observed copulating in the field, the size difference between males and females never exceeded 4 mm CW.

In order to test for mate choice and to determine the type of signals involved, two sets of experiments were employed using the different categories of individuals described above. The experiments were designed following Sato and Goshima (2007). Two kinds of signals were studied: visual and chemical. For chemical signals, two different criteria were considered: “contact” chemical signals (the release of chemical signals occurs through cuticle pores and communication is mediated through physical contact between partners), and waterborne chemical signals (release of chemical signals to water and communication occurs without contact between partners).

2.1. Experimental design 1

An experimental plastic aquarium (1 × 0.4 × 0.15 m) contained two equal plastic cylinders (12 cm diameter, 9 cm high) which were surrounded at its circular base by a circle drawn in the experimental plastic aquarium, at a distance of 6 cm from the two experimental plastic aquarium walls (Fig. 1A). Experimental crabs were located in each cylinder. Test crabs were located in another single cylinder in the center of the experimental plastic aquarium, at a similar distance from the two other cylinders (Fig. 1A). Test crabs were acclimated during 15 min until the experiment began. Once the experiment started, the cylinder containing the test crab was removed allowing the crab to move around the aquarium (Fig. 1B). Each time the test crab crossed the drawn lines surrounding each cylinder with some part of its body, the time spent on the drawn line or inside the circle was measured (seconds) and considered as the dependent

variable. Whether the test crab spent more time in a circle than in the other was considered indicative of its preference since it was expected that the test crab was attracted towards the putative pheromones presumed to be released by the crabs inside the cylinders. The duration of the experiments was 30 min. To test both chemical signals, with and without contact between partners, individuals were left in the cylinders a minimum of 2 h, allowing for the buildup of putative pheromones presumed to be released, as it was employed for spider crabs (Jones and Hartnoll, 1997).

To test visual or chemical with and without-contact signals during mate choice, plastic cylinders were used in three arrangements. Arrangement A: to test the existence of the visual signal transparent cylinders without perforations were used. Individuals could be seen at one and the other side of the cylinder through its transparent wall although no emission neither contact of putative chemical signals occur because of the absence of cylinder perforations (Fig. 1C). A seawater level of 3 cm was used in the cylinder and in the experimental plastic aquaria which allowed individuals to see each other under or above water, reproducing high and low tide conditions observed in field: individuals were observed in an erect, vertical position, and thus could see out of water, or stayed in a horizontal position and see inside water. In each experiment during the entire experimental time individuals alternate both vertical and horizontal positions.

Arrangement B: to test the existence of the contact chemical signal opaque cylinders with perforations of 1 cm² were used to allow the individuals inside or outside of the cylinders to protrude/insert the chela or legs (Fig. 1D). Opaque cylinders prevented individuals to be seen at one and the other side. A low seawater level of 0.2 cm in the cylinder and in the experimental plastic aquaria was used in order to maintain the individuals wet, but to avoid communication through waterborne chemical signals and to favor communication only using contact chemical signals. In all the experiments the individuals inside and outside the cylinder contacted each other during the trials, although we did not quantify the number of contacts.

Arrangement C: to test the existence of chemical without contact signals opaque cylinders with perforations of 2 mm² were used to prevent the crab inside or outside of the cylinder to protrude parts of its body and to allow communication only through the release of putative waterborne chemical signals (Fig. 1E). In this case, a seawater level of 3 cm in the cylinder and in the experimental plastic

aquaria was used in order to favor the communication through the release of chemical signals to water.

According to the different categories of individuals described, four experiments were conducted.

2.1.1. Experiment 1

A small male and a large male located in each cylinder + a receptive test female were used in order to test whether male size affects mate choice. The three signals (visual, contact chemical signal and without contact chemical signal) were tested.

2.1.2. Experiment 2

A receptive and an unreceptive female of similar size located in each cylinder + an unmated test male were used in order to test whether the female receptivity condition affects mate choice. The three signals (visual, contact chemical signal and without contact chemical signal) were tested.

2.1.3. Experiment 3

An unmated and a mated male of similar size + a receptive test female were used in order to test whether the male mated condition affects mate choice. In this experiment, according to the results obtained in Experiments 1 and 2 (see Results section), only both chemical signals were tested.

2.1.4. Experiment 4

A receptive and an unreceptive female of similar size + a mated test male were used in order to test whether female receptivity condition affects mate choice conducted by mated males. In this experiment, according to the results obtained in Experiments 1 and 2 (see Results section), only both chemical signals were tested.

To determine which signal was involved in mate choice and which sex was choosing, for each treatment, paired *t*-tests were performed in order to test for differences in the time spent by the test crab within the two circles. In order to test whether differences in the intensity of both chemical signals exist (with and without contact), *t*-tests were performed in the experiments where differences in the response of the test crab to spend more time in one or the other cylinder were found. Ten replicates were obtained for each experiment.

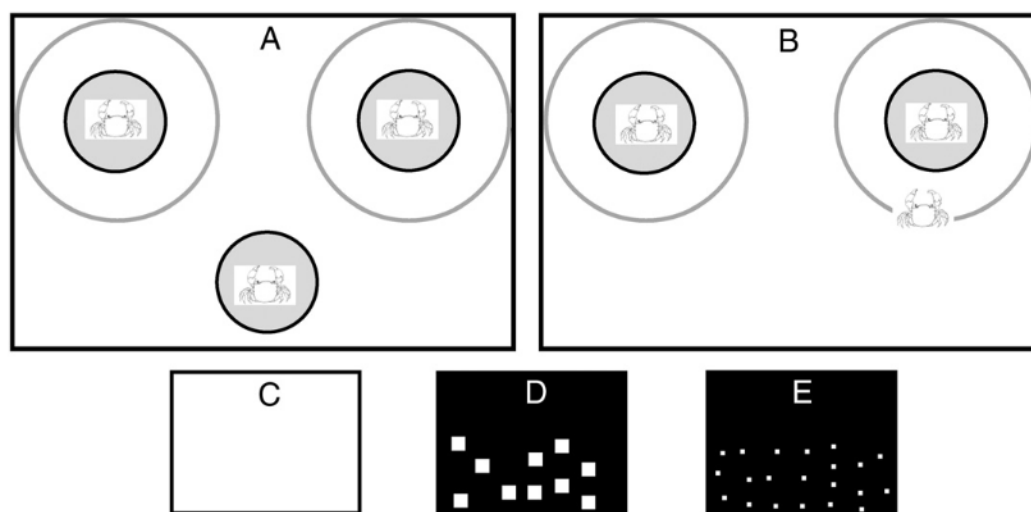


Fig. 1. Schematic representation of Experimental design 1. (A) The experimental plastic aquarium containing the two equal sized cylinders used in the treatments (gray color). Surrounding each cylinder the circle drawn could be visualized at a distance of 6 cm. In the middle of both cylinders, a third cylinder where the test crab was acclimated before the beginning of the experiment is observed. (B) Upon release the test crab could move towards one or the other cylinders. The total amount of time the crab spent within each of the surrounding circles was recorded. Cylinders used in the three different treatments: (C) translucent without perforations, (D) opaque with perforations of 1 cm², (E) opaque with perforations of 2 mm².

2.2. Experimental design 2

The same experimental design described above was used, although, in this case, two additional plastic containers ($0.15 \times 0.10 \times 0.15$ m) were located above each cylinder, at a distance of 1 m, and connected to them through a plastic hose (4 mm diameter) with its own flow (Fig. 2). A continuous flow of seawater (0.16 ml/s) was controlled. Inside each additional plastic contained, a level of 5 cm of seawater was reached. According to one of the results obtained in [Experimental design 1](#) (females chose unmated males using chemical signals, see [Results](#) section) and in order to test whether crabs could detect a quantity of the putative waterborne chemical signals involved in mate choice, two experiments were conducted.

2.2.1. Experiment 1

Two mated males of similar size were located in each cylinder + a mated male of similar size located in one of the additional plastic containers and an unmated male located in the other additional plastic container + a receptive test female. In this case, our expectation was that a test crab may spend more time in the cylinder that received putative chemical signals from the unmated male.

2.2.2. Experiment 2

Two unmated males of similar size located in each cylinder + an unmated male of similar size located in one of the additional plastic containers and a mated male of similar size located in the other additional plastic container + a test receptive female. In this case, our expectation was that test crab may spend more time in the cylinder that received putative chemicals signals from the two unmated males, showing that test crabs could detect a chemical signal quantity.

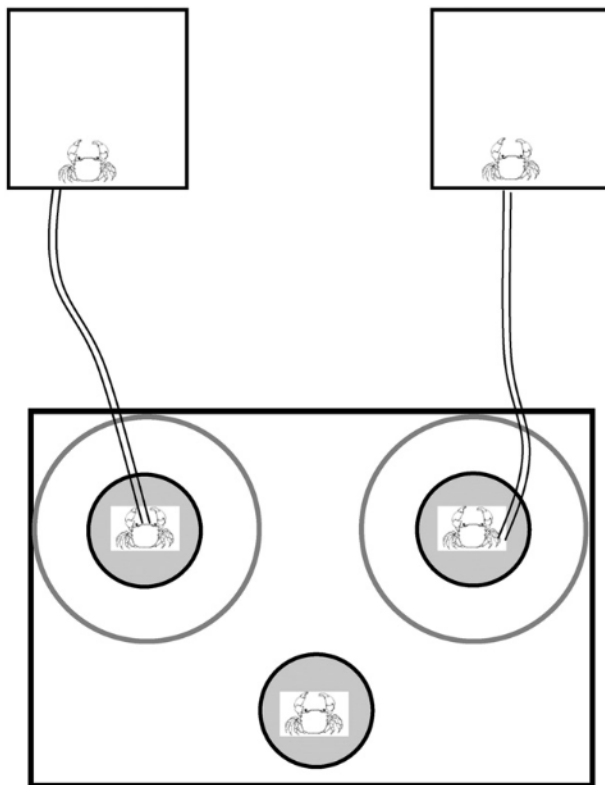


Fig. 2. Schematic representation of [Experimental design 2](#). The experimental plastic aquarium containing the two cylinders with perforations of 2 mm^2 (gray color), each one surrounded by drawn lines at a distance of 6 cm. Above test container, at a distance of 1 m, the two additional plastic containers could be visualized (white color), each one connected to the cylinders through a plastic hose (4 mm diameter) with its own flow.

In both experiments only opaque cylinders with perforations of 2 mm^2 and a seawater level of 3 cm (test of waterborne chemical signals) were used. Crabs inside the cylinders and the additional plastic containers were maintained a minimum of 2 h before the experiment started. The test female was acclimated 15 min before the experiment started. Once the experiment began, the cylinder containing the test crab was removed allowing the crab to move inside the experimental plastic aquarium, and the hose taps were opened, producing a continuous flow of seawater occurred between the additional plastic containers and the cylinders. As described for [Experimental design 1](#), the time spent on the drawn line or inside the circle was considered the dependent variable. The duration of the experiment and the number of replicates were the same as [Experimental design 1](#).

To determine whether receptive test females chose the experimental unmated males and detected a quantity of the putative chemical signal, for each treatment, paired *t*-tests were performed in order to compare the time spent by the test receptive females within the two circles.

3. Results

3.1. Experimental design 1

3.1.1. Experiment 1: large versus small male + receptive test female

In the experiment in which only visual signals were provided to females, no differences were found in the time spent by the test receptive female in the vicinity of large and small males ($t = 1.49$, $df = 9$, $P = 0.17$, $n = 10$, [Fig. 3A](#)). By contrast, differences in the time spent by females in the circles of large versus small males were found regarding the chemical signals: test receptive females spent more time in the circles of the large males compared to small ones when both chemical signals were tested, with ($t = 3.61$, $df = 9$, $P < 0.01$, $n = 10$, [Fig. 3B](#)) and without contact ($t = 4.63$, $df = 9$, $P < 0.01$, $n = 10$, [Fig. 3C](#)).

3.1.2. Experiment 2: receptive versus unreceptive female + unmated test male

No differences were found in the time spent by the test unmated males in the vicinity of the receptive and unreceptive females regarding the use of visual ($t = 0.21$, $df = 9$, $P = 0.83$, $n = 10$) and both chemical signals, with ($t = 1.07$, $df = 12$, $P = 0.31$, $n = 10$) and without contact ($t = 0.08$, $df = 10$, $P = 0.94$, $n = 10$).

3.1.3. Experiment 3: mated versus unmated male + receptive test female

Receptive females were attracted towards the vicinity of the unmated males compared to the mated ones using both chemical signals, with ($t = 3.52$, $df = 9$, $P < 0.01$, $n = 10$, [Fig. 4A](#)) and without ($t = 2.99$, $df = 9$, $P < 0.05$, $n = 10$, [Fig. 4B](#)) contact between partners.

3.1.4. Experiment 4: receptive versus unreceptive female + mated test male

No differences were found in the time spent by the test mated males in the vicinity of the receptive and unreceptive females regarding the use of both chemical signals, with ($t = 0.05$, $df = 9$, $P = 0.96$, $n = 10$) and without contact ($t = 1.18$, $df = 9$, $P = 0.27$, $n = 10$).

[Experiments 1 and 3](#) were used to analyze whether differences in the intensity of the response to both chemical signals exist since differences were found in the time spent by the test crab in the vicinity of both cylinders. No differences were found regarding the response of test receptive females using both types of chemical signals to select large ([Experiment 1](#): $t = 1.44$, $df = 9$, $P = 0.16$, $n = 10$) and unmated males ([experiment 3](#): $t = 0.11$, $df = 9$, $P = 0.91$, $n = 10$).

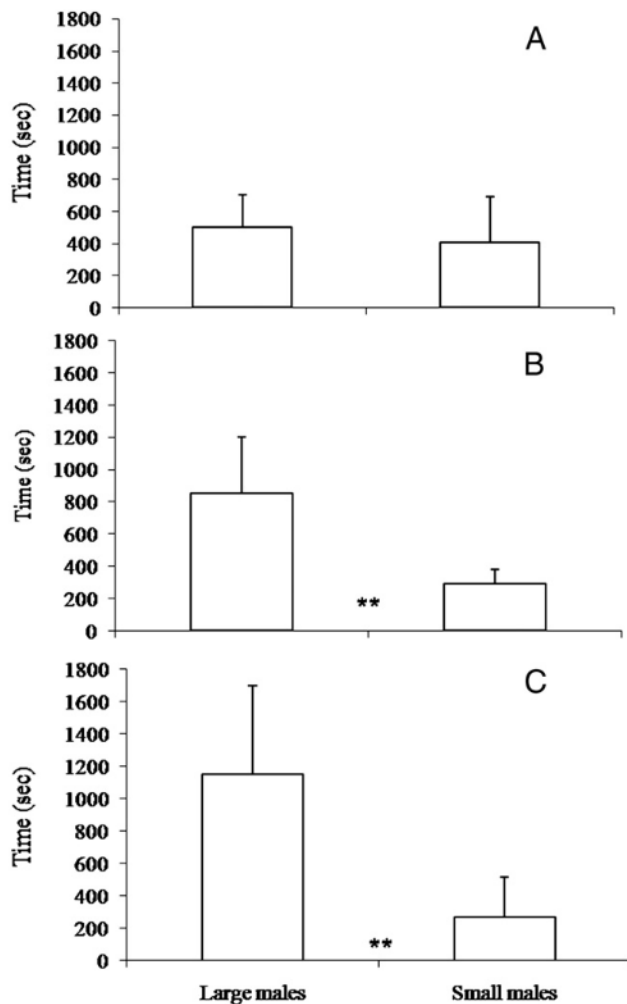


Fig. 3. Experimental design 1. Response of test receptive *Neohelice granulata* females selecting large versus small males in treatments: (A) 1 (visual signal), (B) 2 (chemical with contact signal), and (C) 3 (chemical without contact signal). Error bars indicate the standard deviation. (** $P < 0.01$).

3.2. Experimental design 2

3.2.1. Experiment 1: two mated males versus an unmated male, a mated male + a receptive test female

Females spent more time in the vicinity of the circles where putative chemical signals were supposed to be released by one mated and one unmated male versus two mated males ($t = 3.49$, $df = 9$, $P < 0.01$, $n = 10$, Fig. 5A).

3.2.2. Experiment 2: two unmated males versus a mated male, an unmated male + a receptive test female

No differences were found regarding the time spent by test receptive females in the vicinity of the circles where putative chemical signals were supposed to be released by two unmated males versus one mated and one unmated male ($t = 0.23$, $df = 9$, $P = 0.82$, $n = 10$, Fig. 5B).

4. Discussion

The form of chemical communication mediated by sexual pheromones needed for mate recognition and assessment vary according to the mating system of each species (Bauer, 2011). In *N. granulata* the mate choice conducted by receptive females using chemical signals could be explained by the mating system of this species, which is based on the defense of the resources (burrows) constructed

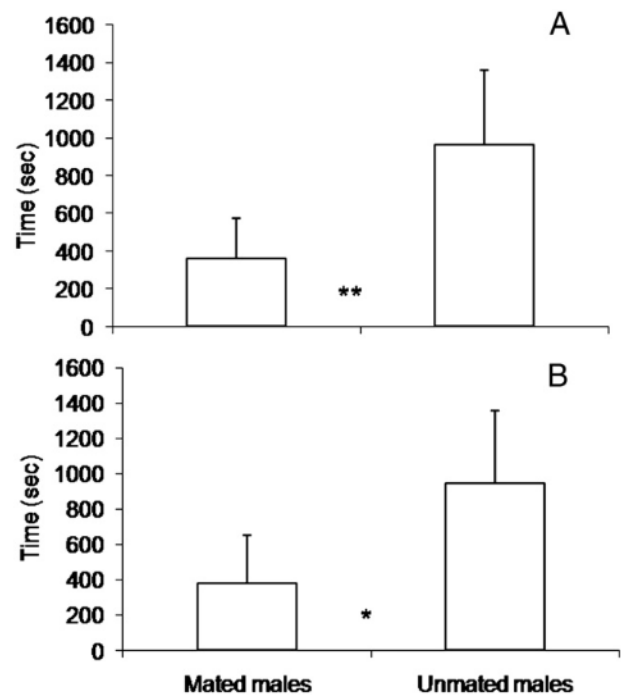


Fig. 4. Experimental design 1. Response of test receptive *Neohelice granulata* females selecting mated versus unmated males in two treatments: (A) 2 (chemical with contact signal), and (B) 3 (chemical without contact signal). Error bars indicate the standard deviation. (* $P < 0.05$, ** $P < 0.01$).

by the large males (Sal Moyano et al., 2012a). According to the results obtained here, since males do not seemed to select females using the signals tested in the different experiments, and the mating system type described for this species (Sal Moyano et al., 2012a), it is proposed that large males would invest their energy in the construction of high quality burrows for mating purposes, which would be searched and selected by receptive females. In this way, females would be the “searching” sex, detecting the signals emitted by males. Moreover, *N. granulata* female’s shows a courtship behavior which consists in sampling male burrows by getting inside and outside burrows and contacting each time their chelae and legs with the male ones before choosing the male burrow in which they will copulate (M.P. Sal Moyano unpublished data), thus supporting the idea that females are the selecting sex. This strong link between sexual communication, mate choice and the mating system was also described for *Rhynchocinetes typus* (Díaz and Thiel, 2004).

The female mate choice observed in *N. granulata* was also registered in many crustacean species. In the lobster *Homarus americanus* pre or inter-molt females would have the capacity to find male burrows through the chemical signals emitted by them (Cowan and Atema, 1990). In the portunid *Callinectes sapidus* a male pheromone released to water initiate the female searching reproductive behavior (Bushman, 1999; Teytaud, 1971). In the lobster *Jasus edwardsii* females chose large males through a combination of visual, tactile and chemical signals (Raethke et al., 2004). A particular case constitutes the one of the shrimp *Rhynchocinetes typus* since receptive females select the “robustus” type males using chemical signals, while males select receptive females using visual signals (Díaz and Thiel, 2004).

Selection based on female choice may be associated with different independent characteristics such as: (1) the male size, (2) the form of the burrow, since females of *Uca annulipes* choose large males and high quality burrows (Backwell and Passmore, 1996), and (3) the “unmated” condition of males with respect to the mated ones in order to avoid the risk of sperm limitation (Backwell and Passmore, 1996). In this study, *N. granulata* females preferred the large and

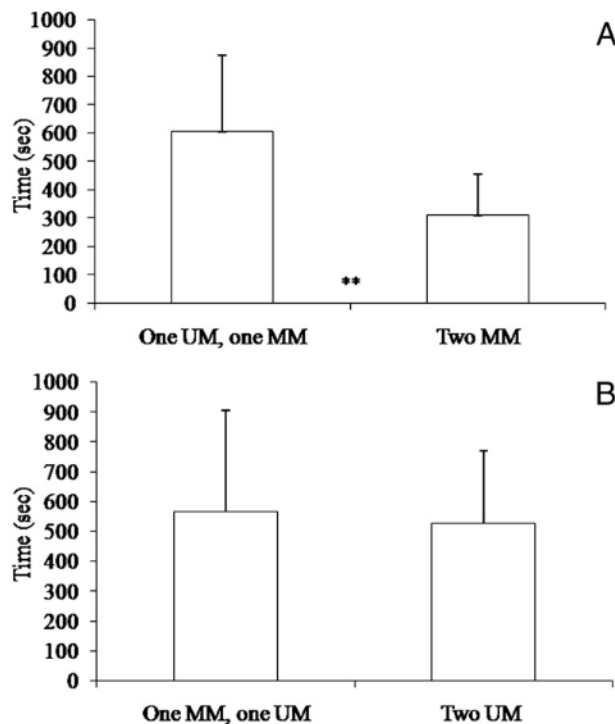


Fig. 5. Experimental design 2. Response of test *Neohelice granulata* receptive females selecting mated versus unmated males using chemical signals without contact between partners. (A) Two unmated males (UM) were located in each additional plastic container + an unmated male located in a cylinder + a mated male (MM) located in the other cylinder. (B) Two mated males (MM) were located in each additional plastic container + a mated male located in a cylinder + an unmated male (UM) located in the other cylinder. Error bars indicate the standard deviation. (**P < 0.01).

unmated males. Given that only large *N. granulata* males construct chambers for underground mating in their burrows (Sal Moyano et al., 2012a), female mate choice for large males may benefit from avoiding predation and agonistic interactions during mating. The choice of the dominant or larger males by receptive females was commonly recorded for different species of crustaceans (Atema and Steinbach, 2007; Díaz and Thiel, 2004; Karplus et al., 2000). Moreover, since *N. granulata* females preferred also the unmated males, it is suggested that they would select males based on different criteria. In the latter case the selection could be based on the capacity of males to retain and transfer a larger amount of spermatophore saturating the capacity of the seminal receptacle and avoiding sperm limitation. The selection of unmated males by females was also described for the stone crab *Haplogaster dentata* (Sato and Goshima, 2007). The flexible *N. granulata* and *H. dentata* female mate choice based on the presence of mated versus unmated males may be related due to the replenishment of male sperm reserves which last about 10 (P. Silva unpublished data) and 20 (Sato and Goshima, 2007) days after the previous mating, respectively. By contrast, in species such as *C. sapidus* females show no mate choice in response to the risk of sperm limitation (Kendall and Wolcott, 1999), although large males require approximately 9–20 days to fully recover their sperm reserves (Kendall et al., 2001).

Regarding the type of signal involved in mate assessment and selection, many studies conducted on crustaceans indicate the importance of chemical signals mediating the reproductive behavior interactions (Bouchard et al., 1996; Díaz and Thiel, 2004; Dunham, 1988; Jones and Hartnoll, 1997; McCleese et al., 1977). For females of many species, the chemical signals are the most important used in recognition and location of potential mates and to determine male quality (Bushmann and Atema, 2000; Díaz and Thiel, 2004). In *N. granulata*, it was demonstrated that chemical signals were the

ones mediating female choice, while the visual ones seemed not to be involved, although it is a semi-terrestrial species. However, communication via visual signals in connection with the display of elaborate courtship behaviors is more commonly represented among terrestrial and semi-terrestrial crustacean species than those that live in aquatic environments (Backwell et al., 1998; Burggren and McMahon, 1988; Pope, 2000; Salmon, 1983). Specifically in cyclopoids the visual signals are commonly used since males station themselves on the burrow surface and display a courtship behavior by waving their chela to visually attract females (Christy, 2007; Christy and Rittschof, 2011 and references therein). By contrast, *N. granulata* males are located inside their burrows, being not visible from the surface, since females are the searching sex and sample male burrows by getting inside and outside them, contacting males inside burrows and initiating the courtship behavior (M.P. Sal Moyano unpublished data). Moreover, in spite of the sexual dimorphism that exists between *N. granulata* females and male's chela (Luppi et al., 2004), visual signals did not influence mate choice. However, the larger male chela size may have an indirect effect on mating selection since it favors the construction of the burrow chambers used for mating and also favor the winning of male–male contests.

Regarding the type of the chemical signal, in *N. granulata* both types, those released to water not involving contact between partners and those requiring physical contact, were used in mate choice with a similar intensity. Since copulation occurs during high and low tides, water-borne chemical signals would be preferentially used during high tide and contact chemical ones during low tide. Similarly, in the crab *Telmessus cheiragonus* two kinds of pheromones were involved in mating behavior: a “distance pheromone” which would induce the pre-copulating behavior, and a “contact pheromone” which would induce the copulating behavior (Kamio et al., 2002).

The results showing that *N. granulata* males were not attracted towards receptive females would indicate that females could conceal their reproductive condition. This may favor the selection of the best male burrow in which to copulate while sampling burrows and might avoid being detected by small males during the searching process (it is suggested that since small males do not construct burrows with chambers where copulation occur, their strategy is to intercept and copulate receptive females on the surface while females are searching male burrows, Sal Moyano et al., 2012a). This capacity of females to conceal their reproductive condition to males was also registered in the shrimp *Palaemonetes pugio*, although in this case was proposed to prevent harassment from males (Bauer and Abdalla, 2001). The concealment of female reproductive condition could be related to the moment of the release of the chemical signals. The females control over the release of the chemical signal could be supported by the hypothesis that chemical signals are emitted through the urine. The idea indicating that pheromones are released in the urine was proposed by several authors (Bushmann, 1999; Bushmann and Atema, 2000; Gleeson et al., 1984; Ryan, 1966; Salmon, 1983).

The amount of the chemical signal released by males could depend on their size, thus being an indicator of their quality (Sato and Goshima, 2007). Studies conducted in fish demonstrated that during agonistic interactions, the amount of pheromone released in the urine is related to dominance: dominant males store and release larger quantities of pheromones than non-dominant ones (Barata et al., 2007). In *N. granulata*, the results found in Experiment 2 of the Experimental design 2 (where receptive females did not show a preference for the cylinder that received putative chemical signals from two unmated males with respect to the cylinder that received putative pheromones from one unmated and one mated male) indicate that the amount of the chemical signal liberated by unmated males may not be concentrated since we did not observe a bigger effect due to the presence of two unmated mated versus only one. Another possibility is that the experimental plastic aquarium, a closed space, was saturated with chemical substances from the three unmated males before the start of the experiment and for that reason no differences were found. Thus,

females may be capable of detecting the presence of the putative chemical signals although not its amount or distinct type of males may be releasing different kind of pheromones or small and mated males are not emitting any signal. Future experiments will help to elucidate this concern.

5. Conclusions

The results obtained here demonstrate that in *N. granulata* mate selection is conducted by females using chemical signals and not visual ones. Since copulation occurs during high and low tides, water-borne chemical signals would be preferentially used during high tide, while contact ones during low tide. Males did not show a preference for receptive females using chemical neither visual signals. Females preferred large and unmated males detecting the presence of the chemical signals emitted by them although not its amount. By contrast, small and mated males may be not liberating any signal, or they would be releasing different kind of pheromones, not attractive to females.

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