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# Habitat-related differences in the pre-copulatory guarding and copulation behavior of *Neohelice granulata* (Brachyura, Grapsoidea, Varunidae)



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## ABSTRACT

Mating behavior strategies of brachyuran crabs are flexible depending on the habitat and social context. We evaluate pre-copulatory guarding in *Neohelice granulata* and describe the unusual copulatory behavior. Laboratory experiments were conducted to test whether factors such as male size, presence of burrows, a male-biased operational sex ratio (OSR) and female mating history affect the copulation duration in two different study areas. Females initiated a searching behavior approximately 20–26 h before they became receptive by assessing large male burrows and displaying a courtship behavior (“flirting”) in its entrance. Once the female entered inside the male burrow, pre-copulatory guarding was observed until the female became receptive and successful copulation occurred immediately after. This species shows some very unusual copulatory behavior involving eye-stalk grasping not seen in any other varunid crab. The copulation duration was independent of male size and the presence of burrows, although it was dependent on the study area, the OSR and the female mating history. Thus, copulation duration is a flexible character in this species which varies with habitat and biological factors such as mating history and sex ratio.

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## 1. Introduction

The mating system of a species is characterized by the sex specific strategies used to maximize its fitness in a particular habitat (Jormalainen, 2007). In decapod crustaceans with seminal receptacles the fitness of females may be maximized by having their seminal receptacles filled with as much sperm as possible, while male fitness may be maximized by occupying as much female seminal receptacle space as possible with their sperm (McLay and López Greco, 2011; Van den Brink and McLay, 2010). Mating systems are modulated by the reproductive strategies developed by females and males in order to increase their fitness, resulting in inter- and intra-sexual competitions that reflect differences in the seminal receptacle structure.

Intra-sexual competition for mate acquisition is a fundamental characteristic of sexual reproduction and copulatory behavior (Trivers, 1972). Specifically in grapsoid crabs intra-sexual competition occurs commonly among males and is related to their size and habitat (Brockhoff and McLay, 2005a). Male size is a common character which determines reproductive success (Abele et al., 1986; Backwell

and Passmore, 1996; Jivoff, 1997; Kamio et al., 2003; Moriyasu and Comeau, 1996; Sainte-Marie et al., 1997). Large males monopolize more and large females, thus, achieving greater fertilization rates (Forbes et al., 1992; Ridley and Thompson, 1985; Stein, 1976; Stevens et al., 1993). Moreover, dominant individuals can obtain and maintain greater control over critical ecological resources (Ellis, 1995). Ecological resources used for mating purposes, such as the presence of burrows, influence reproductive behavior. In ocyropid crabs male burrows are used for copulating and burrow form affects their selection of females (Backwell and Passmore, 1996; Christy, 2007 and references therein; De Rivera, 2005). By contrast, in grapsoids few studies have recorded whether mating can occur on the surface or inside burrows (Brockhoff and McLay, 2005a; Seiple and Salmon, 1982) and the importance of burrows to the copulating behavior and the mating success has rarely been assessed.

In addition to typical male–male competition, males can also compete by means of sperm competition when multiple mating occurs and sperm from numerous individuals occupy the female seminal receptacles (Parker, 1970). Diesel (1991) characterized the female brachyuran seminal receptacle into dorsal and ventral morphological types, and thus, the first or the last male to inseminate the female could have an advantage in fertilizing the oocytes. Moreover, the development of specific reproductive behaviors by males such as pre- or post-copulatory guarding assures the paternity of the offspring and

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excludes other males from copulating with females until fertilization of the oocytes, thereby diminishing the risk of sperm competition (Grafen and Ridley, 1983; Smith, 1984).

Pre- and post-copulatory guarding behaviors are flexible and influenced by the socio-sexual context (Rondeau and Sainte-Marie, 2001; Sainte-Marie et al., 2008). Pre-copulatory guarding behavior occurs frequently in species in which females are only receptive during a limited period. This behavior may be developed to monopolize a female prior to her becoming receptive, assuring copulation with the female (Grafen and Ridley, 1983; Jivoff, 1997; Parker, 1974). Specifically in decapods with dorsal type seminal receptacles as defined by Diesel (1991), pre-copulatory guarding may be an advantage for the male mating first with the female. The advantage for females of being pre-copulatory guarded by males is related to avoidance of predation risks (especially in species with mating linked to molting, Jivoff and Hines, 1998), and also it allows females to assess and determine which males are successful by resisting males during pre-mating interactions (e.g. Berrill and Arsenault, 1984; Jormalainen and Merilaita, 1993). The synchrony of the receptive period in a population modifies the duration of the optimal guarding behavior (Yamamura and Jormalainen, 1996). The benefit of developing a pre-copulatory guarding behavior is related to the monopolization of a female when the probability of finding a receptive female is low (Parker, 1970). Additionally, the probability of finding a receptive female depends on the operational sex ratio (OSR, defined as the relationship between the number of fertilizable (i.e. receptive) females and the number of sexually mature males in a determined space and time, Emlen and Oring, 1977) (Parker, 1974). Although past studies predict well developed pre-copulatory guarding behavior in species with male-biased OSR or when the receptive period is limited and females become receptive asynchronously (Emlen and Oring, 1977; Grafen and Ridley, 1983), its occurrence in Grapsoidea has been rarely characterized.

The mating system of *Neohelice granulata* (Dana 1851) is based on the defense of the resource “burrows” constructed by the large males and used as a sheltered/protected copulatory chamber or “boudoir” (Sal Moyano et al., 2012a). Copulatory chambers have different forms: closed (closet) chambers in the upper or median part of the burrow when they are constructed in habitats with muddy fine sediments, or open (exposed) chambers in the entrance of the burrow when they are constructed in habitats with gravel coarse sediments (see Sal Moyano et al., 2012a). By contrast, small males construct burrows without chambers and their copulations occur more frequently on the surface by interception of receptive females or less frequently in empty burrows of large males (Sal Moyano et al., 2012a). Thus, the presence of burrows may affect copulatory behavior. The burrow form varies among study areas according to the different sediment characteristics, thus influencing the reproductive behavior in each particular habitat (Sal Moyano et al., 2012a). *N. granulata* females search and select large and unmated males using chemical signals (Sal Moyano et al., 2014). Females have ventral type seminal receptacles and become receptive and copulate when in hard-shell condition (López Greco et al., 1999; Sal Moyano et al., 2012b). Females become receptive several times (2–4) during the reproductive season, although for a limited period each time. Thus, the OSR is frequently male-biased and females may mate with several males (Sal Moyano et al., 2012b). Consequently, the OSR and the capacity of females to mate with multiple males could affect copulatory behavior. The duration of the receptive period varies between two types of receptive females: [1] those with short receptivity (1–5 days) and [2] those with extended receptivity (7–12 days) (Sal Moyano et al., 2012b). The seminal receptacles are expandable storage chambers and receptivity duration is dependent on seminal receptacle weight and reproductive history: females with short receptivity have recently copulated and have heavier seminal receptacles ( $0.059 \pm 0.01$  g), whereas extended receptivity females have not recently copulated and have lighter seminal receptacles ( $0.037 \pm 0.01$  g) (Sal Moyano et al., 2012b).

In this context, the aims of the present paper were to study the reproductive behavior of *N. granulata*, specifically to [1] test for the existence of pre-copulatory guarding behavior, [2] describe the copulatory behavior, and [3] analyze the effect of different factors such as “male size”, “presence of burrows with copulation chambers”, “a male-biased OSR” and “female mating history” (recently versus not recently mated females) in two different habitats.

## 2. Materials and methods

### 2.1. Study areas

The study was carried out in two coastal areas: one at Mar Chiquita (MCL), which is a coastal lagoon located in Buenos Aires Province, Argentina ( $37^{\circ}45'S$ ,  $57^{\circ}19'W$ ), and the other one at San Antonio Oeste (SAO), a bay located in the northwest of San Matías Gulf, Río Negro Province, Argentina ( $40^{\circ}46'S$ ,  $64^{\circ}50'W$ ). Mar Chiquita is a body of brackish water ( $46 \text{ km}^2$ ) with low tidal amplitude (ca. 1 m) permanently connected to the sea (Reta et al., 2001). The upper intertidal zone is a mixed *Spartina densiflora* and *Sarcocornia perennis* marsh (Isacch et al., 2006), and the mid- and lower intertidal zones are mudflat. Crabs occupy the upper and mid-intertidal zones. By contrast, the other area, SAO, is a marsh lacking freshwater input, except for the scarce rainfall (200 mm/year), with a semidiurnal macrotidal (ca. 9 m) regime. The upper intertidal is a *S. perennis* marsh, and the middle intertidal divides into a *Spartina alterniflora* marsh (Isacch et al., 2006) and a mudflat. *N. granulata* crab burrows occupy both the upper and mid-intertidal zones (Bas et al., 2005). In both areas, burrows ameliorate the harsh physical conditions, when salt marsh and mudflat are uncovered by water, by diminishing the range of variation of temperature and/or salinity (Luppi et al., 2013; Silva et al., 2009). Burrows holding the copulatory chambers have closed chambers in its upper or median part in MCL and open chambers in its entrance in SAO (Sal Moyano et al., 2012a).

### 2.2. Individuals used in experiments

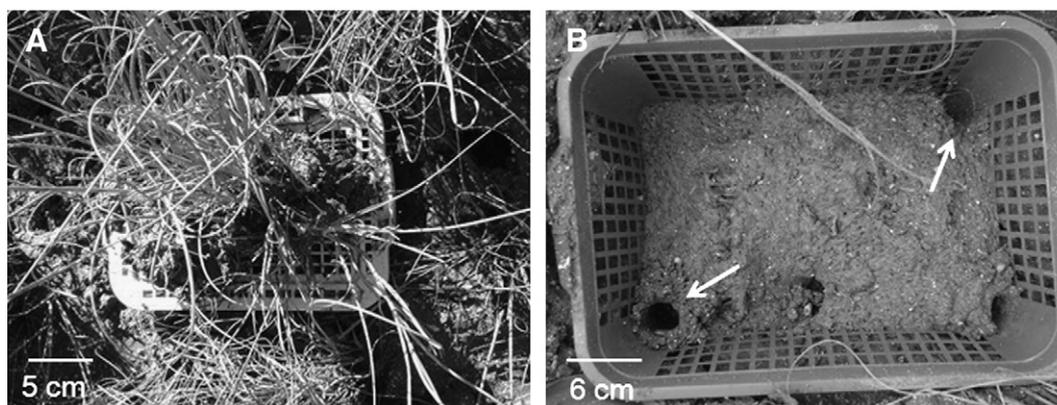
All individuals were measured with a caliper accurate to 0.1 mm. Only morphologically and physiologically mature crabs of both sexes were used in the experiments ( $>19$  mm of carapace width (CW); López Greco and Rodríguez, 1998). Based on the size of the mating partners observed copulating in the field, the size difference between males and females never exceeded 4 mm.

### 2.3. Pre-copulatory guarding behavior

#### 2.3.1. Do males use pre-copulatory guarding?

Experiments were carried out in field during the reproductive season of 2010/2011 in MCL. Exclusion cages ( $19 \times 28 \times 15$  cm) constructed of plastic mesh ( $1 \text{ cm}^2$  opening) were buried 18 cm deep and located on transects in the middle intertidal, in the transition between the mudflat and the salt marsh so they were covered by water during high tide (Fig. 1a). In order to allow males to construct burrows with copulation chambers in the upper part of the burrow, exclusion cages were filled with sediment from surrounding areas at a level of 20 cm following Sal Moyano et al. (2012a) (Fig. 1b). Thus, if copulation occurred inside chambers it could be observed from the surface. Above the exclusion cages a clump of *Spartina* was added in order to mitigate the effects of high temperatures during low tide.

The experiments consisted of two treatments (T): [1] an unreceptive non ovigerous female (CW  $> 24.5$  mm) + a large male (LM, CW  $> 27.5$  mm) and, [2] two unreceptive non ovigerous females. Individuals in each treatment were identified by marking with dots of colored nail polish and these marks were reinforced three times a week. Exclusion cages were monitored daily for 5 min in order to see whether individuals constructed burrows, the burrow position



**Fig. 1.** Exclusion cages ( $19 \times 28 \times 15$  cm) located in the middle intertidal in Mar Chiquita Coastal lagoon to evaluate the existence of precopulatory behavior in *Neohelice granulata*. (A) Exclusion cage covered by a clump of *Spartina* to provide shade and avoid high temperatures during low tide. (B) Exclusion cage showing the burrows (arrows) constructed by the individuals in the experiments.

and whether burrows were being maintained by their owners. Receptivity of females was recognized by the detection of mobile vulvae opercula which could be pushed inwards like a trapdoor when examined 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). Experiments started the day the individuals were located in the exclusion cage and ended when a receptive female was found. The receptive female position was scored as: on surface, inside its burrow, inside the male burrow (T1), or inside the other female burrow (T2). If a non receptive female became receptive inside the male burrow in T1, and the male showed overt guarding behavior such as holding a female pereiopod with his chela, caged within his legs or simply stayed in contact, we considered this to be a successful pre-copulatory behavior. The time between when the female was found unreceptive and subsequently became receptive was recorded.

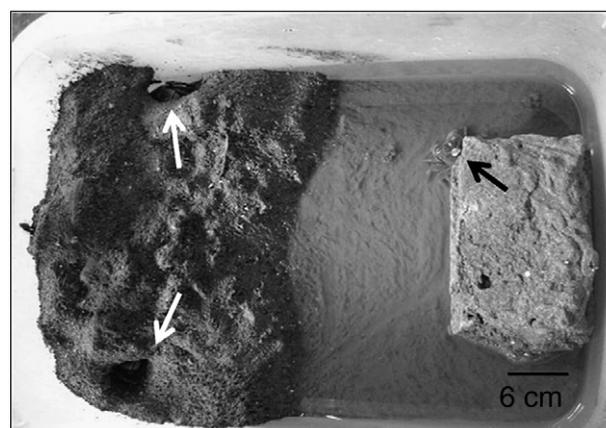
For statistical analyses, the null hypothesis of independence between the number of receptive females found on the surface, inside their burrow or the other burrow, and the treatment, was tested using a G independence test. When differences between treatments were found, a Z-test of comparison between treatments of proportions was used to test for differences regarding the place where the receptive female was found. Fifteen replicates were obtained for each treatment.

### 2.3.2. Do receptive females initiate a pre-copulatory guarding?

Based on a previous study showing *N. granulata* female attraction to LM chemical signals (Sal Moyano et al., 2014), a second experiment was carried out to test whether receptive females search and select large male burrows, possibly initiating a pre-copulatory guarding behavior. Given that unreceptive females which are close to becoming receptive could not be morphologically recognized, females the first day they became receptive were used in the experiments, whereas ovigerous females were used as a control because they are not close to becoming receptive (until egg hatching). Thus, the searching behavior of receptive females was compared to that of ovigerous females when exposed to a LM in a burrow. The experiment consisted of studying the searching activity of ovigerous females, and females with short (1–5 days) and extended receptivity (7–12 days) (for details see Sal Moyano et al., 2012b). Unreceptive ovigerous and non ovigerous females and LM were captured in the field during the reproductive season of 2011/2012 in MCL and transported to the laboratory. Here they were maintained in aerated seawater aquaria ( $0.3 \times 0.35 \times 0.25$  m, containing 3 cm of seawater), and sexes were held separately. 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).

Experiments were conducted in aerated plastic aquaria ( $0.4 \times 0.5 \times 0.2$  m) containing 20 cm of sediment obtained from MCL in one side, and a rock (approximately  $10 \times 20 \times 6$  cm), as an alternative refuge, on the other side (Fig. 2). Given that only LM constructs burrows with copulating chambers (Sal Moyano et al., 2012a), two LM were placed in the aquaria and left for 1–3 days until the construction of a burrow with a viewable copulating chamber in its upper part was completed.

Experiments started when a receptive or ovigerous female was added to the aquaria. The female ambulatory activity was recorded as a “searching behavior”; counting the time in seconds during which the females were observed moving inside the aquaria (a minimum displacement of 1 cm was considered as evidence of “searching activity”). The female behavior described included whether females sampled male burrows, contacted and copulated with males, or stayed alone and buried in the sediment. To determine whether the receptivity duration was short or extended, after the experiment ended, the female was isolated in an aquarium until its operculum became immobile. Based on the number of days that the operculum was mobile the female was classified as having short (1–5 days) or extended receptivity (7–12 days) following Sal Moyano et al. (2012b). The duration of the experiment was 30 min, starting with the introduction of the female after a period of 5 min of acclimation. Ten replicates were obtained for ovigerous and receptive females. In the case of receptive females, the first ten short and extended receptive females obtained were considered. An ANOVA was performed to test for differences in the activity among three female



**Fig. 2.** Experimental plastic aquaria ( $0.4 \times 0.5 \times 0.2$  m) containing sediment in one side to allow the construction of male burrows (white arrows), and a rock as an alternative refuge in the other side. Two males inside each burrow and a receptive female close to the rock (black arrow) can be observed.

types. Where significant differences were found a posterior Tukey-test was conducted to identify which differed.

#### 2.4. Copulating behavior and factors affecting it at MCL and SAO

*N. granulata* crabs were collected by hand from the field one month before the beginning of the reproductive seasons of 2009/2010, 2010/2011 and 2011/2012 in MCL and SAO and transported to the laboratory. In the laboratory individuals were maintained under the same conditions described above and experiments were conducted in the aerated plastic aquaria. Experimental aquaria contained sediment obtained from each study area. To describe the copulation behavior and evaluate the factors affecting it, we carried out four experiments with two treatments each. In each experiment the copulation behavior was noted and we recorded the “copulation time”, defined as the duration of copulation in minutes (the time since the mating pair was observed in the sternum–sternum position with both male and female abdomens open until they separated), as the dependent variable.

Experiment 1 was conducted during September–December of the 2009/2010 reproductive season. This experiment tested the factor “male size”. Treatment 1 consisted of adding a small male (SM, MCL: 20.7–26.3 mm CW, SAO: 21.4–26 mm CW) + a receptive female ( $20 < CW < 26$  mm), while treatment 2 consisted of adding a LM (MCL: 27.8–33.6 mm CW, SAO: 27–31.6 mm CW) + a receptive female ( $26 < CW < 32$  mm) (MCL:  $n = 20$ , SAO:  $n = 15$ ). Since no differences in the copulating time were found between LM and SM (see Results), experiments 2, 3 and 4 were performed using indiscriminately selected LM and SM of similar range sizes as those used in experiment 1 and simply referred to as “males”. These experiments were conducted simultaneously during December–February of the reproductive season of 2009/2010, and September–February of the reproductive seasons of 2010/2011 and 2011/2012.

Experiment 2 tested the factor “presence of burrows with copulation chambers”. Treatment 1 consisted of adding a male ( $20 < CW < 34$  mm) in a burrow + a receptive female ( $20 < CW < 32$  mm), whereas treatment 2 consisted of adding a male without a burrow + a receptive female (MCL and SAO:  $n = 10$ ). Aquaria were conditioned similar to the “pre-copulatory experiments”: one side of the aquarium was filled with sediment obtained from both study areas at a depth of 20 cm (MCL) and 10 cm (SAO) based on burrow characterized in field, whereas the other side contained a rock as an alternative refuge. As detailed above, a LM was added to the aquaria for the construction of burrows with closed copulation chambers in its upper region (MCL) or open copulation chambers in its entrance (SAO) so that we could observe copulation from the surface. Because SM could be found inside burrows constructed by LM at MCL (Sal Moyano et al., 2012a), SM were used in this treatment. In these cases, the LM was removed without modifying the form of the burrow, and the SM was added. In treatments where burrows were not needed we provided sediment that was a 2 cm depth from MCL and SAO was provided.

Experiment 3 tested the factor “male-biased OSR”. Treatment 1 consisted of adding three males of similar size ( $20 < CW < 34$  mm, with a maximum difference of 0.5 mm among the three males) + a receptive female ( $20 < CW < 32$  mm) (MCL:  $n = 13$ , SAO:  $n = 12$ ). We measured the copulation time of the first male that copulated with the female. For treatment 2 we used the data obtained in experiment 1 corresponding to the pooled copulation time of LM and SM (since no effect of the male size on the copulation time was found, see Results), considering that number to represent the copulation time when the OSR was 1:1.

Experiment 4 tested the factor “female mating history”. Treatment 1 consisted of adding a female ( $20 < CW < 32$  mm) which had copulated recently with another male ( $20 < CW < 34$  mm) + a male similar in size to the male that recently copulated with the female (MCL and SAO:  $n = 10$ ). Females with a known mating history were obtained after placing a receptive female (the first day it was found receptive)

and a male in an aquarium and successful copulation was observed. Copulation was considered to have occurred when both the male and female pleons were observed open and the male gonopods were inserted into the female vulva. Immediately after copulation ended and the pair separated, the female was used for the “female mating history” experiment. As in treatment 2 we used the data obtained in experiment 1 corresponding to the pooled copulation time of LM and SM, considering it as the copulation time of females that became receptive without copulating recently.

The statistical analyses consisted of performing four two-way ANOVAs to test the effects of the independent factors “study area”, “male size” (experiment 1), “study area”, “presence of burrows with copulation chambers” (experiment 2), “study area”, “OSR” (experiment 3), and “study area”, “female mating history” (experiment 4), with respect to the dependent variable “copulation time”.

### 3. Results

#### 3.1. Precopulatory guarding behavior

##### 3.1.1. Do males show pre-copulatory guarding?

In both treatments, crabs constructed independent burrows. The upper part of male burrows contained a copulation chamber. Each time the experiment was monitored, the crabs were consistently found inside the constructed burrows and rarely occurred on the surface or in the burrow of the experimental partner. In T1, unreceptive females were found inside male burrows 20–26 h ( $20.8 \pm 3.4$ ) before they became receptive, whereas in T2 receptive females were found inside their own burrows in the period prior to becoming receptive (approximately 1 day). When females were found receptive their location depended on the treatment ( $G = 13.61$ ,  $P < 0.01$ ) (Fig. 3). Differences were found in the amount of time that a receptive female was encountered on the surface (20% versus 73.3% for T1 and T2 respectively,  $Z = 2.56$ ,  $P < 0.05$ ), and in another burrow (66.7% versus 6.7% for T1 and T2 respectively,  $Z = 3.03$ ,  $P < 0.01$ ), whereas no differences were found in the females found inside their burrows (13.3% versus 20% for T1 and T2 respectively,  $Z = 0.002$ ,  $P = 0.99$ ). When females were inside male burrows (T1), they were found always in contact with the male, sometimes caged within his legs, pereopod held with his chela or with the male blocking the entrance of the burrow thereby preventing her escape. All these male behaviors were considered as pre-copulatory guarding.

##### 3.1.2. Do receptive females initiate pre-copulatory guarding?

Each LM constructed a burrow with an upper copulation chamber in each corner of the aquaria with sufficient sediment to allow burrow construction (Fig. 2). Differences in female searching activity were found among ovigerous females and females with short and extended receptivity (ANOVA  $F = 3.48$ ,  $P < 0.05$ ). The searching activity of females with short and extended receptivities differed (Tukey-test,

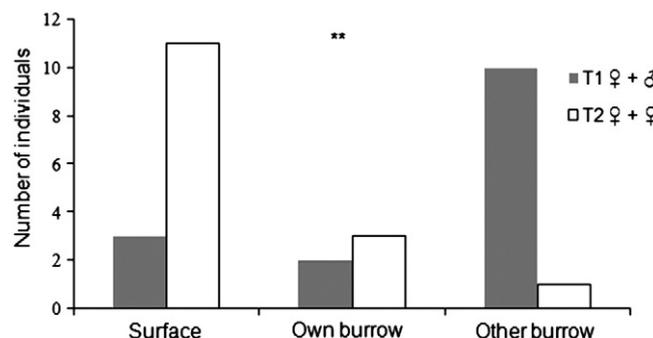


Fig. 3. Number of receptive *Neohelice granulata* females found on the surface, inside its own burrow or inside the burrow constructed by the other individual in the treatment: T1 (a male + a female), T2 (two females).

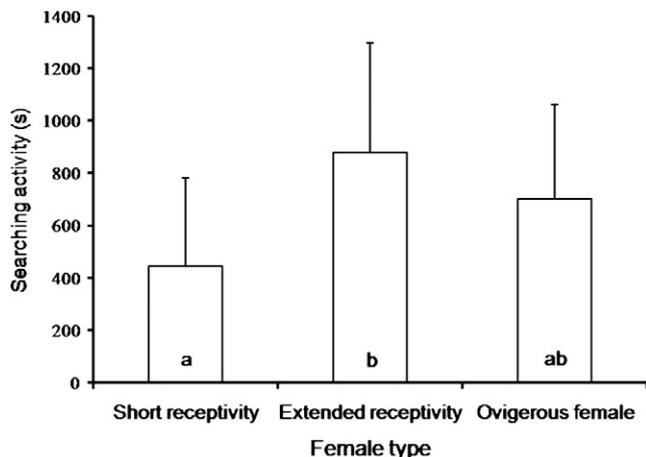


Fig. 4. Searching activity of *Neohelice granulata* females in seconds (s) in Mar Chiquita Coastal lagoon: short receptivity females, extended receptivity females and ovigerous females. Different letters indicate significant differences after a Tukey-test ( $p < 0.001$ ).

$P < 0.05$ ), while no differences were found between ovigerous females and females with short or extended receptivity (Fig. 4).

Short receptivity females showed little searching activity ( $334.2 \pm 337.7$  s), and they generally remained under the rock or buried in the sediment. By contrast, extended receptivity females showed much higher searching activity ( $727.4 \pm 417.5$  s), moving all around the aquaria and displaying a “search and sampling behavior” of male burrows. This behavior consisted of the female approaching the burrow entrance of one and or the other male, and making contact each time with the owner. Contact involved the dactyls of the last three legs and consisted of short movements of contact and separation analogous to feeling each other. Females “visited” each male burrow between 3 to 5 times. This behavior ended once the female completely entered one of the male burrows or when one of the males emerged from its burrow, captured the female with his chelae and took her inside the burrow. Ovigerous females showed either high or little activity ( $582.5 \pm 357.6$  s), hiding under the rock, burying in the sediment or moving around the aquaria or entering male burrows but avoiding capture. Neither the ovigerous female nor the male showed much interest in each other.

### 3.1.3. Copulating behavior and factors affecting it at MCL and SAO

Mating pairs were formed by both LM and SM and copulation occurred in all experimental aquaria. Copulation occurred inside burrows or on the surface when burrows were absent. The copulation behavior was similar between LM and SM in both study areas. Immediately after the male captured the female, it positioned the female rostrum-to-rostrum and immediately assumed a sternum-to-sternum position (Fig. 5a, b). The male opened his abdomen and that of the compliant female and inserted his gonopods inside the female vulva, in which case copulation was recorded as successful. During approximately the first 15 min, while copulating, the male held the female eyestalks with his chela (Fig. 5b). During the next 15 min the male opened one of his chelae and continued to hold the female eyestalk with the other chela (Fig. 5c). During the next 15 min the male opened both chelae, thereby releasing the female (Fig. 5d). Copulation ended when both individuals separated and, immediately after, the male held the female by her chelae or legs or caged her within his legs (Fig. 5e, f).

In experiment 1 copulation duration was independent of male size ( $F = 0.06$ ,  $df = 1$ ,  $SS = 12.4$ ,  $P = 0.81$ ). However, copulation time differed between study areas ( $F = 4.89$ ,  $df = 1$ ,  $SS = 994.5$ ,  $P < 0.05$ ), lasting longer in MCL compared to SAO (MCL:  $47.9 \pm 15.9$  min, SAO:  $40.9 \pm 11.7$  min) (Fig. 6). There was no interaction between male size and study area ( $F = 0.38$ ,  $df = 1$ ,  $SS = 79.2$ ,  $P = 0.54$ ).

In experiment 2 copulation duration was independent of the presence of burrows with copulation chambers ( $F = 0.54$ ,  $df = 1$ ,

$SS = 62.5$ ,  $P = 0.47$ ). However, copulation time differed between study areas ( $F = 4.97$ ,  $df = 1$ ,  $SS = 577.6$ ,  $P < 0.05$ ), lasting longer in MCL compared to SAO (MCL:  $48.0 \pm 13.2$  min, SAO:  $41.2 \pm 6.9$  min) (Fig. 7). There was no interaction between presence of burrows and study area ( $F = 0.08$ ,  $df = 1$ ,  $SS = 10$ ,  $P = 0.77$ ).

In experiment 3 copulation duration differed between females that copulated in the presence versus absence of competing males in both study areas ( $F = 74.1$ ,  $df = 1$ ,  $SS = 9381.6$ ,  $P < 0.001$ ): the copulation time lasted longer when the OSR was 1:1 (MCL:  $47.9 \pm 15.9$  min, SAO:  $40.9 \pm 11.7$  min, data from T1) compared to a 3:1 OSR (MCL:  $19.6 \pm 3.11$  min; SAO:  $20.1 \pm 3.85$  min) (Fig. 8). When the OSR was male-biased, interactions between the copulating male and the competing males were observed where they contacted and tried to separate the mating pair, although competing males were never successful in taking over the receptive females. No differences in copulation time were found between study areas ( $F = 2.41$ ,  $df = 1$ ,  $SS = 305.4$ ,  $P = 0.13$ ). There was no interaction between the OSR and the study area ( $F = 3.05$ ,  $df = 1$ ,  $SS = 385.9$ ,  $P = 0.086$ ).

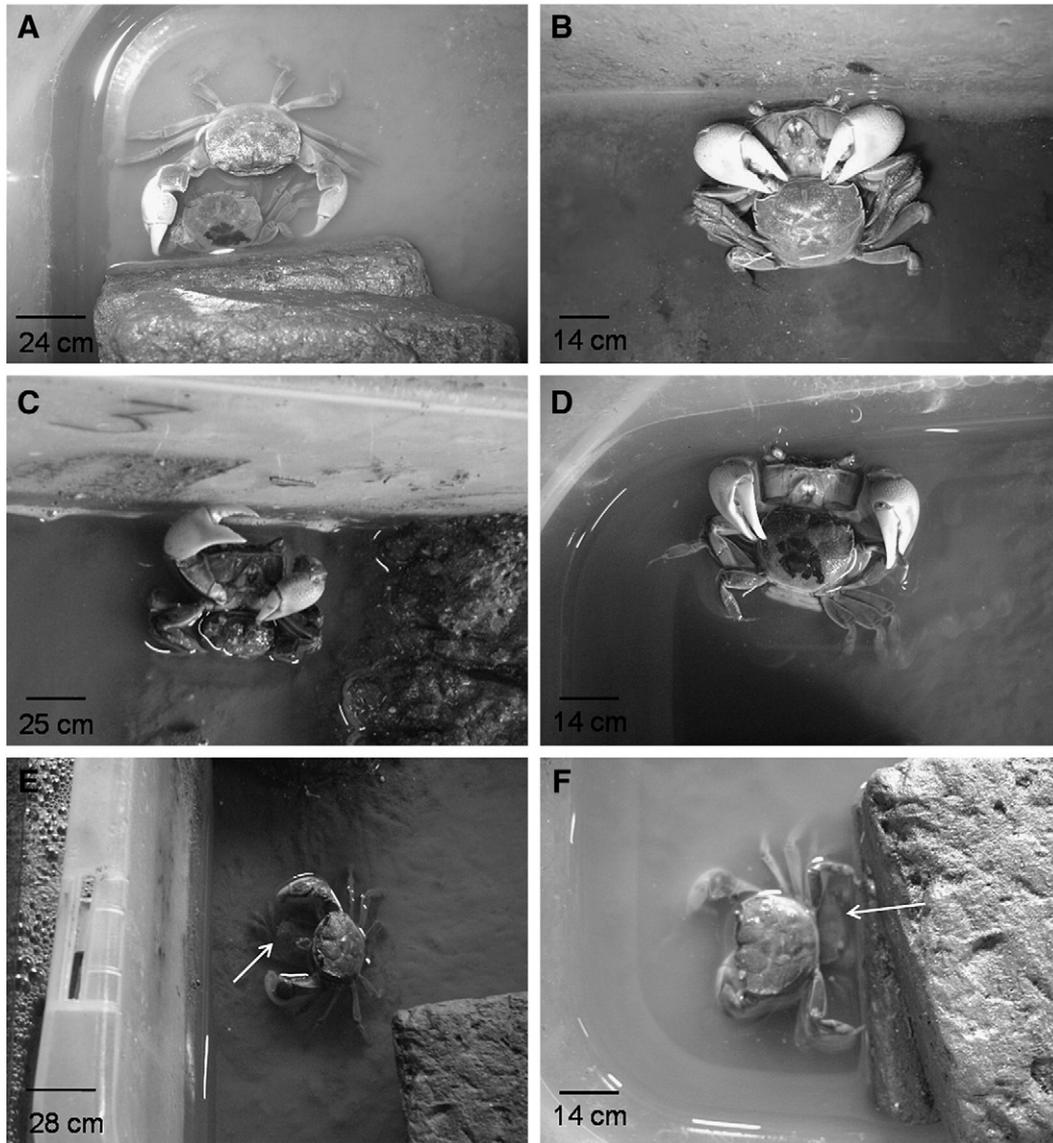
In experiment 4 differences were found in copulation duration between multiple copulated females and females that copulated for the first time immediately after they became receptive in both study areas ( $F = 52.1$ ,  $df = 1$ ,  $SS = 7561.5$ ,  $P < 0.001$ ): the copulation time of females that copulated only one time (MCL:  $47.9 \pm 15.9$  min, SAO:  $40.9 \pm 11.7$  min, data from T1) lasted longer compared to females that copulated twice (MCL:  $19.18 \pm 6$  min; SAO:  $21.6 \pm 5.43$  min) (Fig. 9). The copulation time did not differ between study areas ( $F = 0.67$ ,  $df = 1$ ,  $SS = 185.6$ ,  $P = 0.42$ ). There was no interaction between the “female mating history” factor and the study area ( $F = 3.06$ ,  $df = 1$ ,  $SS = 444.7$ ,  $P = 0.086$ ).

## 4. Discussion

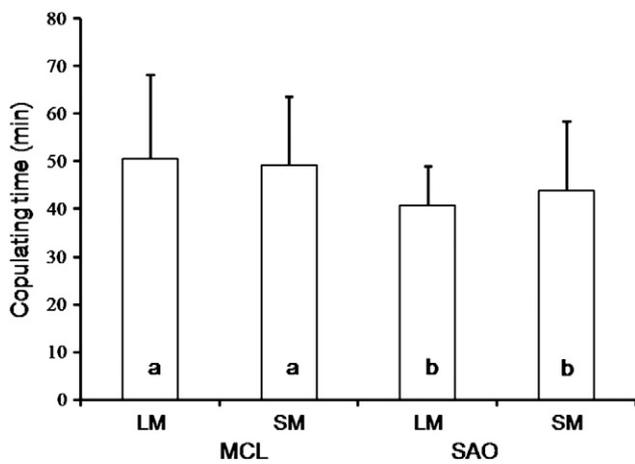
### 4.1. Pre-copulatory guarding behavior

The development of pre-copulatory guarding behavior is predicted in species with male-biased OSR (Emlen and Oring, 1977) and when mating is limited to a short period (Grafen and Ridley, 1983). In *N. granulata* mating is restricted to a limited period when the female is receptive, thus the OSR is male-biased, and consequently, the existence of the pre-copulatory guarding behavior described here corresponds with theoretical expectations. By contrast, in other Varunidae such as the genus *Hemigrapsus*, pre-copulatory guarding was not found (Brockerhoff and McLay, 2005a). More studies of the family are necessary to evaluate the frequency of the existence of pre-copulatory guarding. In *N. granulata* mate-searching consisted of females abandoning their burrows, approximately 24–26 h before they become receptive, to look for large male burrows where they become receptive and copulation occurs. Thus, the pre-copulatory guarding begins when they find the male burrow and ends when females become receptive. These results demonstrate that females are the searching sex in *N. granulata*, selecting males and their burrows. This type of mating system in which females are mobile and select males and their burrows has also been described for many Ocypodidae (see Christy, 2007 and references therein; Jormalainen, 2007).

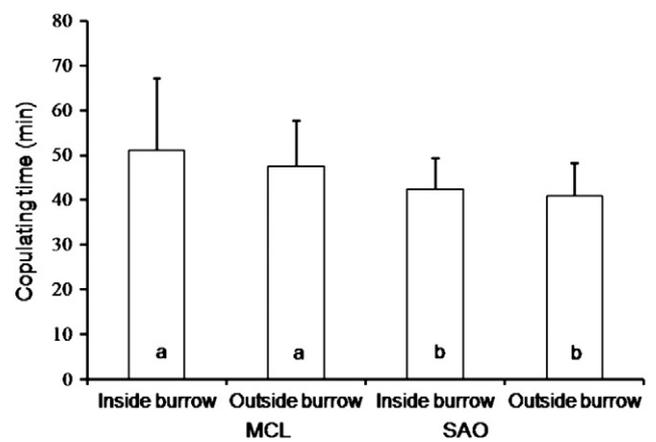
To initiate mate-searching and pre-copulatory guarding the present results suggest that *N. granulata* females may have the capacity to detect when they are close to becoming receptive and thus initiate searching behavior. This strategy of females being able to detect when they will become receptive has been described for many caridean species (see Bauer, 2011). In the case of *N. granulata* males, they would have the opportunity to recognize that females are close to becoming receptive and, consequently, accept them inside their burrows. Similarly, in the isopod *Asellus aquaticus* males recognize receptive females at a distance of 5 cm or less (Bertin and Cezilly, 2005). Given that the guarding duration is critical for the reproductive success of males, they would be expected to have the sensorial capacity to recognize the time remaining until



**Fig. 5.** Copulating behavior of *Neohelice granulata*. (A) A male holding a receptive female using its chelae prior to copulation. (B) The mating partners in the sternum–sternum position during copula, with the male holding the female eyestalks with his chelae. (C) The male opened one of his chelae and kept holding the female with the other chela continuing taking it through her eyestalk. (D) The male with both chelae open during copulation. After copulation the male retained the female (arrows): (E) using his chelae, or (F) caging the female within his legs.



**Fig. 6.** Copulation time in minutes of *Neohelice granulata* individuals: large (LM) and small males (SM) in both study areas, Mar Chiquita Coastal lagoon (MCL) and San Antonio Oeste (SAO). Different letters indicate significant differences ( $P < 0.05$ ).



**Fig. 7.** Copulation time in minutes of *Neohelice granulata* individuals that copulated inside and outside burrows in both study areas, Mar Chiquita Coastal lagoon (MCL) and San Antonio Oeste (SAO). Different letters indicate significant differences ( $P < 0.05$ ).

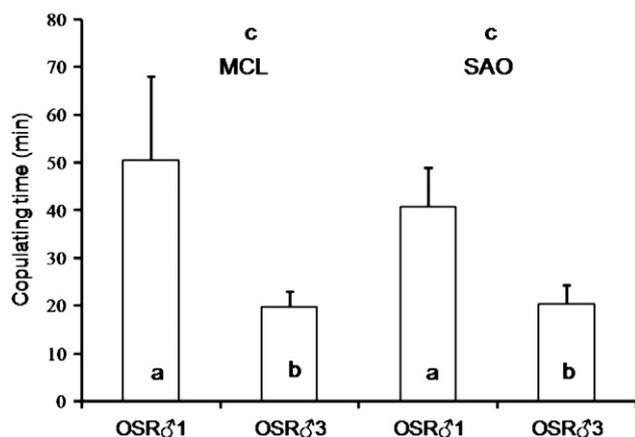


Fig. 8. Copulation time in minutes of *Neohelice granulata* individuals that copulated in the absence (OSR♂1) or presence of alternative males (OSR♂3) in Mar Chiquita Coastal lagoon (MCL) and San Antonio Oeste (SAO). Different letters indicate significant differences ( $P < 0.001$ ).

females become receptive (Jormalainen, 1998, 2007). In the ocypodid crab *Uca paradussumieri* males can detect when females are close to the period of ovulation using chemical signals (Murai et al., 2002). It is important to note that although *N. granulata* females are not morphologically receptive because their vulvae operculums are immobile, they might be physiologically receptive in the previous period until the operculum becomes mobile, and this condition may be detected by males, possibly through the emission of a chemical signal. Previous work demonstrated female attraction to large and unmated males using both distance and contact chemical signals for *N. granulata* (Sal Moyano et al., 2014). The behavior of the female approaching the entrance of the male burrow and contacting the male repeatedly described in the present work was considered to be female courtship behavior, because females appeared not only to assess males, but also to “flirt” with males in order to be accepted by them to enter their burrows.

The results obtained in the female searching activity experiment showed that *N. granulata* females with extended receptivity were the most active, searching and selecting male burrows through the described courtship behavior. Given that females with extended receptivity have light seminal receptacles, likely containing few sperm, while short receptivity females have heavy seminal receptacles, probably with many sperm (Sal Moyano et al., 2012b), we predict that the former look more actively for male burrows in which to copulate and fill their seminal receptacles. Short receptivity females, in contrast, may prefer

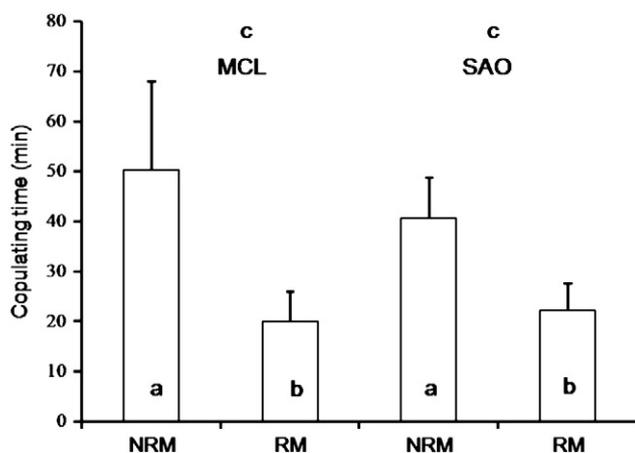


Fig. 9. Copulation time in minutes of *Neohelice granulata* individuals: males that copulated with recently mated (RM) females or males that copulated with not recently mated (NRM) females in Mar Chiquita Coastal lagoon (MCL) and San Antonio Oeste (SAO). Different letters indicate significant differences ( $P < 0.001$ ).

to remain inside their burrows without copulating because their seminal receptacles may contain sufficient sperm. Thus, avoiding predation risks or other hazards associated with mating has been described in some species (Benhalima and Moriyasu, 2001; Elwood and Neil, 1992).

Successful pre-copulatory guarding depends on the size of males when males compete for access to females (Wada et al., 1999). In *N. granulata*, males showing pre-copulatory guarding may depend indirectly on their size because burrows with copulation chambers where this guarding occurred are constructed only by large males. In the case of SAO, no experiments were conducted to test for the existence of pre-copulatory guarding behavior, however, because of the form of the burrows with open copulation chambers in the entrance (Sal Moyano et al., 2012a). We suggest that the interactions with other males may be favored by the burrow form, and thus, may impair the guarding behavior of the resident male. Similarly, interactions between the resident and competing males in *N. granulata* open burrows of SAO were observed to affect post-copulatory guarding behavior (Sal Moyano et al., 2012a).

#### 4.2. Copulation behavior and factors affecting it at MCL and SAO

Although the behavior leading up to mating in *N. granulata* is similar to other varunids in that the copulatory position is female over male, the male habit of grasping the female by her eyestalks is noteworthy. We are unaware of any other crab that does this. Male *Hemigrapsus* species normally wrap their chelae around the body of the female, embracing her carapace, and they may also interlock their walking legs with those of the female to restrain her (Brockerhoff and McLay, 2005b). The New Zealand species *Austrohelice crassa* most similar to *N. granulata* does not grasp the female by her eyestalks, but employs behavior much more like *Hemigrapsus*.

The copulation duration was similar between large and small males and independent of the presence of burrows with copulation chambers. Small males copulate more frequently on the surface by intercepting receptive females and might be more exposed to predation risks, whereas large males copulate inside burrows (Sal Moyano et al., 2012a). However, once small males intercept a receptive female, they would be expected to copulate for a similar time compared to large males inside their burrows, in order to transfer as many sperm as possible, thus possibly increasing their fertilization success. By contrast in the macrophtalmid *Hemiplax hirtipes* (formerly *Macrophthalmus*) the copulation duration lasted longer inside burrows compared to copulating on the surface, although we did not investigate a possible effect of male size (Jennings et al., 2000). Although differences in the amount of sperm transferred by small and large males were not quantified in *N. granulata*, studies in some taxa demonstrate that large males store and transfer at a faster rate than small males (Kwei, 1978; Markow et al., 1978; Pitnick, 1996; Wilber, 1987).

The copulation duration of *N. granulata* differed between study areas. A previous study demonstrated that females of the same size from SAO have low fecundity compared to those from MCL (35–50% lower, Bas et al., 2007). Thus, diminishing copulation duration could result from transfer of less sperm in males from SAO. Differences in the mating behavior in relation to habitat conditions were described in previous studies (Yamamura, 1986; Yamamura and Tsuji, 1989). In spite of differences in *N. granulata* copulation duration between MCL (~40 min) and SAO (~50 min), these values were intermediate compared to the copulation duration noted in other varunids such as *A. crassa* and *Hemigrapsus sexdentatus* (10–15 min, Brockerhoff and McLay, 2005b), and *Cyclograpsus lavauxi* (2 h, Brockerhoff and McLay, 2005a).

The presence of competing males in OSR experiments diminished copulation duration in *N. granulata*. Previous field study demonstrated agonistic interactions for burrows among *N. granulata* males (Sal Moyano et al., 2012a). Thus, although take-over of females was not observed in the present study, interactions between the mating pair and the alternative males were observed that could affect the copulation

time, diminishing its duration. Diminishing copulation time in the presence of competing males could be a deliberate male strategy. By contrast, studies on *Chionoecetes opilio* demonstrated that the volume of ejaculate transferred to females increases in the presence of rival males (Rondeau and Sainte-Marie, 2001). Avoiding displacement by a rival male during mating (Borgia, 1981; Elwood et al., 1987; Howard and Kluge, 1985), would favor males that extend the copulation duration and guarding behaviors, increasing the probability of fertilizing the female oocytes (McLain, 1980; Svard and Wiklund, 1988).

Males spend less time copulating with females that recently mated with other males. The seminal receptacle of *N. granulata* females is ventral, thus the last male copulating the female should have more chances of fertilizing the oocytes (López Greco et al., 1999; Sal Moyano et al., 2012b). However, histological sections of *N. granulata* seminal receptacles of females, which have copulated with different males, showed that sperm is mixed inside the seminal receptacle without differentiating sperm packets or layers corresponding to the different males, thus promoting sperm competition (Sal Moyano et al., 2012b). We propose that males may have the capacity to detect that females have been recently mated by another male, for example using some mechanical or chemical signal, and thus, decide not to invest energy in copulating with that particular female thereby avoiding sperm competition. Instate they may wait another female which has been receptive recently without copulating with other males, diminishing the risk of sperm competition. In this case, successful post-copulatory guarding would ensure paternity of the offspring. Another possibility is that females could resist an extended copula because they have full seminal receptacles and for that reason the copula duration would be shorter. By contrast, in *C. opilio* copulation duration was longer in multiparous than primiparous females because sperm competition may be greater in the former and males could transfer larger ejaculates to these females and increase the probability of fertilizing the oocytes, diminishing sperm competition (Rondeau and Sainte-Marie, 2001). Moreover, theory predicts that when two males copulate with a female, alternative mating strategies such as increased sperm allocation, sperm displacement, sperm ejection, or differences in sperm quality may be displayed (Parker et al., 1990; Simmons, 2001). In *N. granulata* the mating system is based on the defense of the resource “burrows with copulation chambers” constructed by large males (Sal Moyano et al., 2012a). Burrows are safer places to copulate than on the surface in order to avoid predation risks or take overs by competing males, and the burrow also provide protection against desiccation (especially at SAO) (Sal Moyano et al., 2012a). Because large burrows are constructed only by large males, male size could be a legitimate indicator of direct benefits such as sperm quality and quantity. Under natural conditions, males with chambered burrows likely mate more with females who have not mated recently than with recently mated females, thus avoiding sperm competition and assuring paternity of the offspring. By constructing chambers, males may gain access to females that have not recently been copulated (experiments demonstrate their longer copulation time). These females benefit from greater safety compared to recently mated females which may not gain from reduced predation risk because their copulation time is shorter. Small males do not construct burrows with chambers and often copulate with females during surface interception (Sal Moyano et al., 2012a), and they may therefore copulate more frequently with recently mated females.

## 5. Conclusions

Our study shows that *N. granulata* males develop pre-copulatory guarding of females approximately one day before females become receptive inside male burrows. Females search and assess burrows of males that display courtship behavior, allowing them to choose the male and the burrow in which to copulate. Copulation duration varies with habitat and biological factors. Although it is independent of male size and the presence of burrows, it does depend on the study area,

the OSR and female mating history. Thus, males may modulate sperm allocation as a function of population, individual and habitat factors.

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