

Receptivity of female *Neohelice granulata* (Brachyura, Varunidae): different strategies to maximize their reproductive success in contrasting habitats

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Abstract The extent of the receptive period may determine the mating strategies employed by female crabs to obtain mates. Here, we studied the receptivity of female *Neohelice granulata* (Dana, 1851) in the laboratory, including the form of the vulvae and the anatomy of the seminal receptacle (SR). We examined the factors that influence the duration of receptivity by comparing two populations inhabiting contrasting habitats: Mar Chiquita Coastal lagoon (MCL), which is an oligo-polyhaline estuary, and San Antonio Oeste (SAO), which is an eu-hyperhaline marine bay. Non-receptive females have immobile vulva opercula, while receptive females have mobile opercula. Histological sections of the SR showed that the degree of epithelium secretions was associated with the receptive stage of females, and they may be involved in the maintenance of viable sperm and in the dehiscence of spermatophores. The existence of a special tissue at the junction of the oviduct and the SR was described and proposed as an internal mechanism influencing the timing

of ovulation. The duration of receptivity was dependent on the SR load and the capacity to lay eggs. Thus, females with empty SR exhibited longer receptivity and did not lay eggs, while those with full SR exhibited shorter receptivity and always laid eggs. Interpopulation differences showed that females from SAO had shorter receptivity and heavier SR and laid eggs more frequently than females from MCL. Based on our results, we suggest that *N. granulata* females can adjust the duration of their receptivity and control the moment of fertilization according to different internal mechanisms related to the morphology of the vulvae, the fullness of the SR and anatomical attributes of the SR. An important consequence of this control is greater sperm competition. The extent of the receptive period and the number of times that a female could become receptive in a single reproductive season may also depend on the habitat characteristics.

Keywords Female receptivity · Vulva · Seminal receptacle · Interpopulation differences · Sperm competition · Grapsoids

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Introduction

Mating systems depend on a set of ecological conditions characterized by limited abundance and distribution of resources and mates in space and time, which determine the environmental potential for polygamy (Emlen and Oring 1977; Shuster and Wade 2003). Emlen and Oring (1977) considered the temporal and spatial distribution of receptive females as important elements shaping mating systems. In addition, mating systems are the result of complex mating strategies employed by both males and females. Mating decisions of both sexes appear to be based

on the availability of mating opportunities, mate quality and environmental conditions (Thiel and Duffy 2007). Female decisions may be of equal importance to those of males in molding mating systems of crustaceans (Brockerhoff and McLay 2005a). Several studies have been conducted in order to clarify the mating strategies developed by males to ensure mating success (e.g., Goshima et al. 1996; Jivoff 1997; Rondeau and Sainte-Marie 2001; Sato et al. 2006), but fewer have focused on the strategies employed by females (e.g., Brockerhoff and McLay 2005b; Sato and Goshima 2007; Sainte-Marie et al. 2008).

It has been assumed that females optimize their reproductive success by mating with several males, thus increasing the genetic diversity of their offspring (Andersson 1994; Jennions and Petrie 2000), although more recent studies have shown that females and their offspring benefit by being monandrous and choosing a male of ‘good genes’ (Hunt et al. 2004; Jennions et al. 2007). Moreover, the female strategy may be to maximize the probability of egg fertilization by maintaining a viable sperm supply and keeping the seminal receptacle (SR) as full as possible (McLay and López Greco 2011). In the case of males, their fitness is maximized by reproducing with as many females as possible, and the achievement of this requires his sperm occupy as much SR space as possible and to ensure that it is in the best position in the SR to maximize fertilization chances (McLay and López Greco 2011). The timing and duration of receptivity can have an impact on male mating strategies because males may choose whether to invest time by staying with a receptive female, defending or guarding her, or to search for other receptive females so as to achieve maximum reproductive success (Parker 1970). Depending upon SR structure, it is sometimes better to be first while for others it is better to be last to copulate (McLay and López Greco 2011).

Regarding the timing and duration of female receptivity, mating in Brachyura can (1) be restricted to a certain period of time when females are morphometrically able to mate as in the post-molt mating of portunids and cancrids, or when the vulvae opercula of females becomes temporarily mobile during the intermolt period as in some grapsoids and ocypodids, or (2) it can be unrestricted, and females are able to mate any time as in some majoids (McLay and López Greco 2011). The timing and duration of female receptivity is important as it determines the operational sex ratio (OSR, defined as the ratio of fertilizable females to sexually mature males at any given time, Emlen and Oring 1977). In some crustacean species, most females become receptive synchronously, reducing the opportunity for males to mate with multiple females (Sastry 1983). By contrast, females of other species become receptive asynchronously, resulting in a male-biased OSR promoting strong competition between males

for obtaining mating pairs and increasing the potential for sexual selection (Orensanz et al. 1995; Correa and Thiel 2003).

The duration of female receptivity depends on external factors such as the temperature and/or lunar cycle (Zimmerman and Felder 1991; Caubet et al. 1998; Flores and Negreiros-Fransozo 1998; Henmi and Murai 1999) or internal factors such as the development of the ovaries and molt (DeKleijn 1998). Moreover, there is evidence that female crustaceans can control their receptivity (Cowan and Atema 1990; Thiel and Hinojosa 2003; Brockerhoff and McLay 2005b). Regarding the internal factors, the form of the vulvae has important implications in the type of mating and in the extent of the receptive period. In brachyuran females, there are three possible forms of vulva closure: (1) simple muscle action, (2) a non-hinged cover or (3) a calcified hinged operculum (Hartnoll 1968, 2006; Thompson and McLay 2005). Additionally, the reproductive state of females, including the stage of development of ovaries and the characteristics of the SR, is an important factor determining receptivity. The SR is the site where sperm competition must necessarily take place when sperm from different males are stored (Orensanz et al. 1995; Sainte-Marie 2007). Morphological attributes of the SR can favor the dehiscence of spermatophores or maintenance of viable sperm (Anilkumar et al. 1996, 1999), and thus they are involved in sperm competition. The study of the vulva form, the duration of receptivity and the factors affecting it, and the reproductive state, are important elements in the understanding of mating behavior, and the female strategies employed to maximize their reproductive success.

The burrowing and semiterrestrial crab *Neohelice granulata* is a key species in South American salt marshes, mudflats and estuaries, ranging 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, generating extensive burrowed areas (Spivak et al. 1994). Burrows can reach high densities, with up to 60 burrows m⁻² (Iribarne et al. 1997). Previous studies of *N. granulata* have compared two populations inhabiting contrasting habitats: Mar Chiquita lagoon (MCL), an oligo-polyhaline estuary located in Buenos Aires Province, Argentina (37°45'S; 57°19'W), and San Antonio Oeste (SAO) bay, an eu-hyperhaline habitat located in Río Negro Province, Argentina (40°46'S; 64°50'W), where differences related to the population structure (Bas et al. 2005), reproductive biology (Ituarte et al. 2006), number, size and composition of eggs and embryonic development (Bas et al. 2007, 2008) were found to be linked to the type of habitat and the environmental conditions. Those studies demonstrated that the reproductive season is longer in MCL than in SAO, extending from

September to February and from November to January, respectively, although the differences in the reproductive traits were not associated with a latitudinal temperature gradient; instead, the quality and quantity of benthic food resources and productivity may be responsible for the observed reproductive differences (Ituarte et al. 2006; Bas et al. 2007). Further, recent studies have demonstrated that burrows are places used for copulation, although differences in the form and size of the burrows between the two habitats promote differences in successful post-copulatory guarding behavior (Sal Moyano et al. 2012). During the reproductive season, *N. granulata* individuals can spawn several batches of eggs (Ituarte et al. 2004, 2006), although the extent of the receptive period was not assessed before the present study. It is expected that females from these populations have different reproductive strategies, regarding their receptive period, associated with habitat differences.

The objectives of the present study were to (1) characterize the morphology of the vulvae of receptive and non-receptive females using scanning electron microscopy (SEM), (2) study the anatomy of the SR of receptive and non-receptive females, (3) determine the duration of female receptivity and evaluate different factors that influence it such as female size, the presence of males or the fullness of the SR in two populations inhabiting contrasting environments and (4) evaluate different mechanisms involved in the dehiscence of male spermatophores.

Materials and methods

Sampling was carried out during the reproductive seasons of 2008/2009, 2009/2010 and 2010/2011 in MCL, and 2009/2010 and 2010/2011 in SAO. Crabs were measured with calipers using the maximum carapace width (CW) as the reference variable. Mature crabs (>19 mm CW, López Greco and Rodríguez 1998) were taken to the laboratory. In the laboratory, crabs were placed in individual aerated seawater aquaria (0.3 × 0.35 × 0.25 m, 3 cm of seawater), maintained under natural daylight conditions, and temperature was checked, ranging between 23 and 27 °C (mean 24.5 °C). Individuals were fed daily with pet food pellets, and water was changed weekly. The receptive period of females was monitored in the laboratory by checking operculum mobility: The pleon was lifted, and the two opercula were gently probed daily using fine forceps under a binocular microscope. When the opercula became mobile, it could be pushed inwards like a trapdoor, and females were receptive (Brockerhoff and McLay 2005c). The duration of female receptivity is defined as the time from the first day a female has mobile opercula up to the day it becomes immobile again (during the period of

mobility, females can mate and lay eggs or opercula can become immobile again without egg laying).

Morphology of the vulvae

For the description of the vulvae, the area surrounding the vulva of 14 receptive (ranging from 22.9 to 29.7 mm CW) and 9 non-receptive females (ranging from 20.3 to 31.2 mm CW) was dissected and examined by SEM. Samples were fixed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer, at pH 7.2–7.4. Samples were dehydrated in a graded alcohol series, dried in hexametildisilazane replacing the critical point, coated with gold–palladium and viewed with a JEOL 6460 microscope.

Anatomy of the seminal receptacle

For histological studies, two groups of females collected from the field and maintained under laboratory conditions were differentiated and used: 26 receptive females that had laid eggs (ranging from 23.4 to 28.3 mm CW) and 20 non-receptive females, immature or females that had recently become non-receptive without egg laying (ranging from 16.7 to 29.4 mm CW), were killed by placing them in a freezer at –15 °C for approximately 30 min. Right and left SRs were dissected. The stage of maturity of the ovary was classified according to its morphology and color: Being mature ovaries, they covered the entire hepatopancreas with granular consistency and violet color (stage 5, Ituarte et al. 2004). Tissue samples were fixed in Bouin's solution during 24 h, dehydrated through a series of graded alcohol solutions, cleared in xylene and embedded in paraffin. Serial sections (5–7 μm) were cut with a Minot microtome. Sections were mounted on glass slides and stained with standard hematoxylin–eosin and Masson's trichrome for observation under a light microscope.

Duration of receptivity: factors involved

Receptivity of females from MCL (ranging from 20.2 to 30.6 mm CW) and SAO (ranging from 21.4 to 28.2 mm CW) was ascertained under laboratory conditions. Crabs were collected from the field one month before the beginning of the reproductive season in each study area, transported to laboratory and arranged in two treatments: (1) four females and (2) three females and one male. When a female became receptive, it was marked with dots of colored nail polish and examined daily until the opercula became immobile again. Timing of fertilization was recorded.

Two types of studies were done: short and long term. In short-term studies, duration of receptivity was related to four variables: (1) whether females laid eggs or not

($N_{MCL} = 123$, $N_{SAO} = 78$), (2) the size of females ($N_{MCL} = 101$, $N_{SAO} = 65$), (3) the presence of males ($N_{MCL} = 118$, $N_{SAO} = 68$) and (4) the SR load: Immediately after the opercula became immobile at the end of the first receptive period recorded, females were killed by placing them in a freezer at $-15\text{ }^{\circ}\text{C}$ for approximately 30 min. Right and left SRs were dissected and weighed; an average of both weights was calculated ($N_{MCL} = 63$, $N_{SAO} = 47$). In long-term studies, 16 females from MCL and 17 females from SAO were collected from the field one month before the beginning of the reproductive season (August for MCL and October for SAO) and maintained under laboratory conditions during the entire reproductive season, September–February and November–January, respectively, in the two treatments described above. The number of times that single females became receptive was recorded.

Normality and homogeneity of variances were checked prior to performing parametric tests. When data did not meet the assumptions of normality and homogeneity of variances, log transformations were performed or non-parametric tests were used. In short-term studies, a two-way ANOVA was performed in order to test for the differences in the duration of receptivity (dependent variable) in relation to two independent factors: female reproductive status (egg-laying and non-egg-laying females) and the study area. A two-way ANCOVA was conducted in order to test whether the duration of receptivity (dependent variable) was related to the covariable female size and two independent factors: type of female (with short or extended receptivity, see Results) and the study area. A three-way ANOVA was used to test whether the duration of receptivity (dependent variable) was related to three independent factors: presence of males, type of female and the study area. A two-way ANCOVA was conducted in order to test whether the duration of receptivity (dependent variable) was related to the covariable weight of the SR and two independent factors: type of female and study area. A posteriori two-way ANOVA was used to test for the differences in the weight of the SRs (dependent variable) in relation to two independent factors: type of female and study area. Differences in the proportion of females that laid eggs or not between study areas were compared using a chi-square test: If differences were encountered between them, the observed and the expected frequencies were compared. For females from MCL, the differences in the duration of receptivity among the months September to February of the reproductive seasons 2008/2009, 2009/2010 and 2010/2011 were tested using a Kruskal–Wallis test. Those females corresponded to the ones used in the short-term trials, which were followed during one single receptive period and then killed immediately after the receptive period. After a significant

Kruskal–Wallis test, a Student–Newman–Keuls test was conducted in order to identify the months that contributed to the difference. In long-term studies, since a low number of females were maintained during the entire reproductive season, the number of times that single females became receptive and laid eggs from the two treatments was pooled and compared between study areas using a t-test. It should be noted that some females were in the same container; thus, these data were not independent, and this could affect behavior (Cowan and Atema 1990). Results are reported as mean \pm SD.

Spermatophore dehiscence experiments

Males and females were collected before the beginning of the reproductive season (1 month) in MCL and maintained separately under laboratory conditions. Ten receptive females (ranging from 23.3 to 30.5 mm CW), 10 non-receptive females (ranging from 24.9 to 32.3 mm CW) and 20 males (ranging from 25.4 to 33.1 mm CW) were killed by placing them in a freezer at $-15\text{ }^{\circ}\text{C}$ for approximately 30 min. The SR and ovary of females and the vas deferens of males were dissected and weighed. The mean weight of tissue samples was $0.04 \pm 0.0056\text{ g}$. The ovaries and the vas deferens were cut into sections in order to obtain free oocytes or spermatophores (SPF), while a squash of the SR was used. Females can store sperm so the SR could contain spermatozooids from the previous ejaculates, although the presence of SPF is unlikely because females were maintained under laboratory conditions isolated from males before the beginning of the reproductive season (Sal Moyano, unpubl. data). Dehiscence experiments were conducted using the following treatments: (1) a control of SPF alone, (2) incubation of SPF and the SR of a receptive female, (3) incubation of SPF and the SR of a non-receptive female, (4) incubation of SPF and oocytes of a receptive female (5) incubation of SPF and oocytes of a non-receptive female. All treatments were conducted in a 5-ml glass beaker containing 80 % filtered seawater and shaken by hand for 10 min. A sample of 0.2 ml was taken with a pipette, mounted on glass slides, stained with methylene blue and observed under a light microscope. Slides were divided into 75 quadrants of similar size ($0.5 \times 0.5\text{ cm}$) in order to facilitate the quantification of SPF. The primary sperm-packaging unit in *N. granulata* is the spermatophore. These were recognized by their ellipsoidal shape and were surrounded by a thin membrane containing many spermatozooids (Fig. 1). The number of intact SPF per quadrant was quantified and added, and compared between treatments. Differences in the quantity of SPF between treatments were tested using a Kruskal–Wallis test due to data not meeting normality and homogeneity of variance criteria. After a significant Kruskal–Wallis test, a

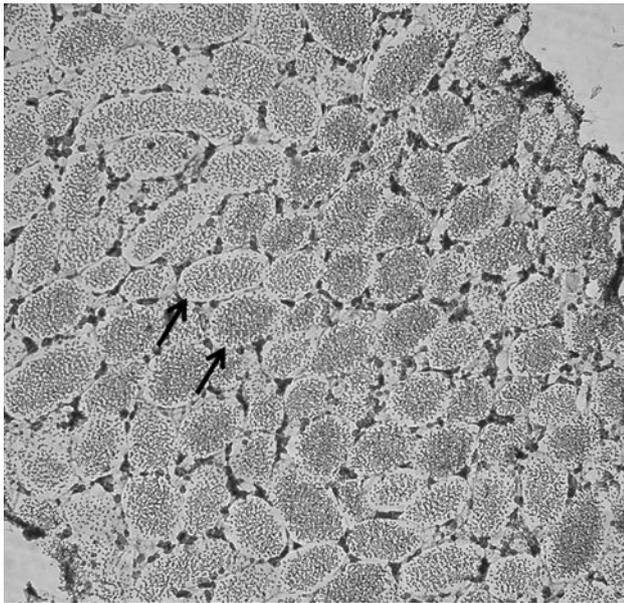


Fig. 1 Spermatophores (SPF) of *Neohelice granulata* males quantified in the dehiscence–SPF experiments. Single SPFs were surrounded by a thin membrane (arrows) and many spermatozoa could be observed inside them

Student–Newman–Keuls test was conducted in order to identify the treatments that differed. Ten replicates were obtained for each treatment. This methodology was designed following Sarker et al. (2009).

Results

Morphology of the vulvae

The vulva of receptive and non-receptive females consists of two portions: an operculum and a membrane or hinge (Fig. 2a). The skeleton around the vulva edge is raised and encircles the hinged operculum, which is semilunar in shape. When the vulva is closed, the operculum seals the opening and projects beyond the edges of the sternum (Fig. 2a, d). Both the hinge and the operculum are used to close the opening. The operculum margin opposite the hinge is concave, thereby allowing it to be pushed into the vagina by the male gonopod during copulation. On the operculum, the presence of a few dispersed simple conical setae with infracuticular insertion was observed (Fig 2b). These setae could be covered and surrounded by secretions (Fig. 2c). Differences between the hinge lines of non-receptive and receptive females were found. In non-receptive females, the membrane had a smooth surface and could not be stretched by prodding (Fig. 2a). In receptive females, the membrane had a wrinkled surface that could stretch allowing the operculum to be displaced backwards, when the male gonopod is inserted during copulation

(Fig. 2d). Following the withdrawal of the male gonopod, the membrane had enough elasticity to return the operculum back in place and close the entrance, as it was observed in killed females that have recently mated with a male.

Anatomy of the seminal receptacle

Macroscopically, the SR looked like a small inflated balloon and was of the ventral type, as the short tube-like oviduct connected to the ventral part of the SR near the vagina. In histological sections, the SR showed the presence of two chambers, dorsal and ventral, surrounded by connective and muscle tissue layers (Fig. 3a). A constriction separating dorsal and ventral chambers could not be distinguished, only two different kinds of tissue linings (Fig. 3a). The dorsal chamber had a multi-layered epithelium of secretory cells, whose thickness changed according to the ‘receptive’ or ‘non-receptive’ state of the female. In receptive crabs, it had one or two layers of secretory cells (Fig. 3b), while in non-receptive crabs it was multi-layered with secretory cells showing a high activity level due to the quantity of secretions produced (Fig. 3c). The ventral chamber presented a simple epithelium of columnar cells, which is folded in virgin females (Fig. 3d), but stretched in females that have copulated. This simple epithelium of the ventral chamber was internally lined with cuticle (Fig. 3e). In immature or mature females whose opercula have recently become immobile without egg laying, the presence of a special tissue of densely packed cells was encountered at the junction of the oviduct with the ventral chamber (Fig. 3f–h). In receptive females that had laid eggs, this special tissue was absent, and the oviduct connected freely with the ventral chamber of the SR. The oviduct was characterized by a single-layered epithelium of flattened cells (Fig. 3h). All dissected receptive females had ovaries in the ultimate stage of development with eggs ready to be ovulated, characterized by a granular consistency and a burgundy to dark violet color, while all receptive females that had laid eggs presented spent ovaries characterized by a translucent appearance.

Duration of receptivity: factors involved

Short-term studies showed that the duration of receptivity ranges from 2 to 16 days in females from MCL, and from 1 to 12 days in females from SAO. The two-way ANOVA showed the differences in the duration of receptivity between the factors female reproductive status, egg laying or non-egg laying, and the study area: egg-laying females had shorter receptivity than non-egg-laying ones, and females from SAO had shorter receptivity than females from MCL (Table 1; Fig. 4, $N_{\text{MCL laying eggs}} = 68$,

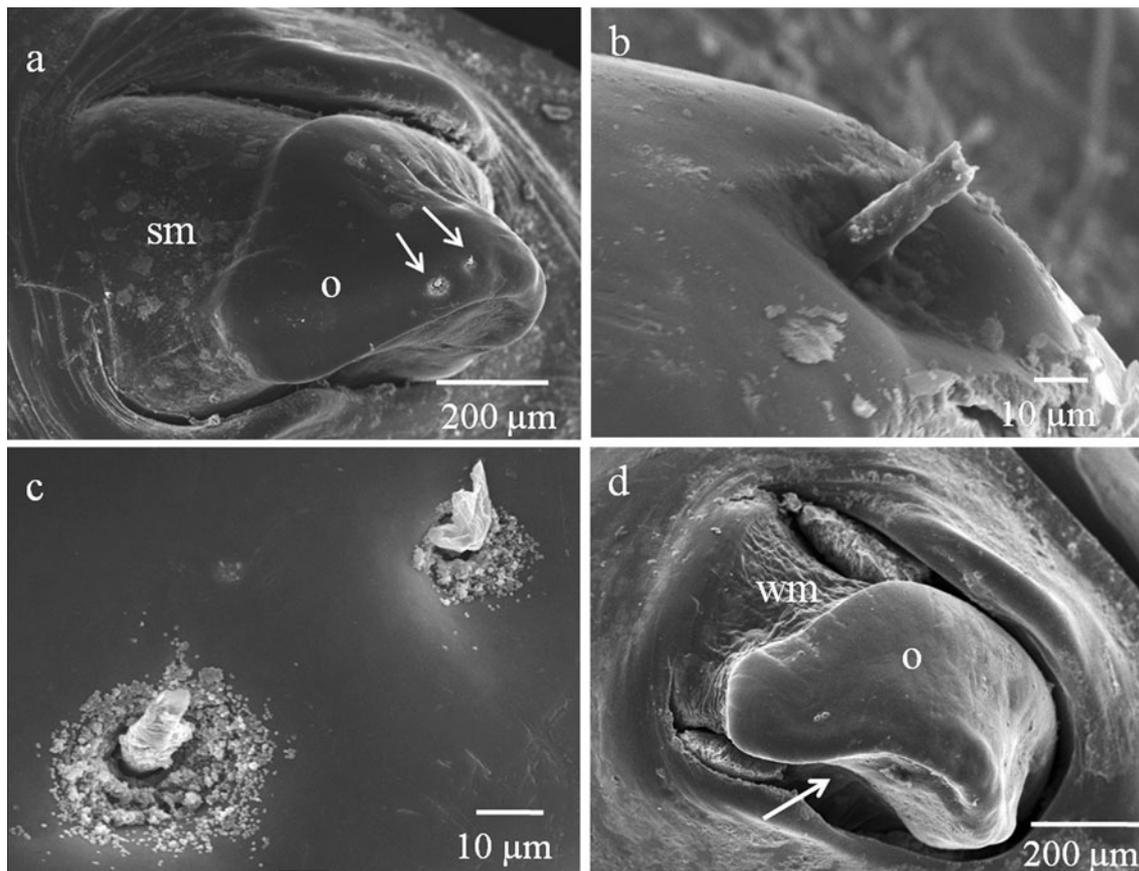


Fig. 2 Vulva of *Neohelice granulata* females. **a** Non-receptive female characterized for the presence of an opercula (*o*) and a smooth membrane (*sm*). Note the setae in the tip of the opercula (*arrows*). **b** Detailed view of simple conical setae with infracuticular insertion. Note that the tip of the setae on the picture was broken off.

c Detailed view of two simple conical setae covered and surrounded by secretions. **d** Receptive female with mobile opercula. Note the opercula (*o*) and a wrinkled membrane (*wm*) allowing the opercula mobility, which could be displaced backwards by the male gonopod insertion during copulation (represented by an *arrow*)

$N_{\text{MCL non egg-laying}} = 55$, $N_{\text{SAO laying eggs}} = 62$, $N_{\text{SAO non egg-laying}} = 16$). Thus, two types of females were recognized for each study area: (1) females that always laid eggs had short receptivity lasting 2 and 6 days (3.81 ± 1.3) in MCL and between 1 and 4 days (2.17 ± 0.97) in SAO; and (2) non-egg-laying females had longer receptivity lasting 7 and 16 days (9.65 ± 3.08) and between 5 and 12 days (6.55 ± 1.81), respectively. In receptive females, both opercula became mobile simultaneously, and subsequently, both of them became immobile again.

A two-way ANCOVA showed that the duration of female receptivity was dependent on the type of female (with short receptivity and egg-laying or extended receptivity without egg laying) and on the study area, although it was not related to female size (Table 2). The three-way ANOVA showed that the receptivity duration was not related to the presence of males, although it was dependent on the type of female and the study area (Table 3). The significant interactions between the independent factors 'type of female' and 'study area' shown in Tables 2 and 3,

indicated that females of extended receptivity from MCL had a longer receptivity than the ones from SAO. A two-way ANCOVA showed that the duration of female receptivity was related to the type of female, the study area and the weight of the SR (Table 4). A posteriori two-way ANOVA showed differences in the weight of the SR between both types of females and the study area: Females with short receptivity had similar SR weights, but these were heavier than in females with extended receptivity in both study areas (Fig. 5, Table 5). The interaction between both independent factors showed that females with extended receptivity from SAO had heavier SRs than the ones from MCL (Fig. 5; Table 5). All dissected females had ovaries in their ultimate stage of development characterized by its granular consistency and violet color, independently of the extent of the receptive period.

Differences in the proportion of females with short receptivity that laid eggs and with extended receptivity without egg laying were found between both study areas ($\chi^2 = 13.33$, $P < 0.001$, $N_{\text{MCL}} = 123$, $N_{\text{SAO}} = 78$): the

Fig. 3 Seminal receptacle of *Neohelice granulata* females. **a** General view of the dorsal chamber (*dc*) with a secretory epithelium and the ventral chamber (*vc*) with a simple epithelium. **b** Receptive female, the multi-layered secretory epithelium (*ep*) of the dorsal chamber and its secretions (*s*), surrounded by a connective tissue layer (*ct*). **c** Non-receptive females, the multi-layered secretory epithelium (*ep*) of the dorsal chamber and its secretions forming vesicles (*v*), surrounded by a connective tissue layer (*ct*). Note the spermatozooids (*spz*) surrounded by the secretions. **d** Ventral chamber of the seminal receptacle of an immature female. Detailed view of the folded ventral epithelium (*ep*) of columnar cells surrounded by connective (*ct*) and muscle (*mu*) tissue layers enclosing the seminal receptacle secretions (*s*). **e** Detailed view of the simple folded epithelium of the ventral chamber internally lined with cuticle (*arrow*). **f** Ventral chamber of the seminal receptacle of an immature female. The ovary (*ov*) connects to the oviduct (*od*) which ends in a mass of densely packed cells forming a special tissue (*st*). The ventral chamber has an epithelium (*ep*) of columnar cells and it is full of seminal receptacle secretions (*s*). **g** Mature female that has recently become unreceptive without egg laying. Densely packed cells forming a special tissue (*st*) are observed at the junction of the oviduct (*od*) and the ventral chamber (*vc*). **h** Detailed view of the densely packed cells of the special tissue (*st*) at the junction of the oviduct (*od*) and the ventral chamber. Note the columnar epithelium (*ep*) of the ventral chamber and the oviduct epithelium of flattened cells (*arrows*)

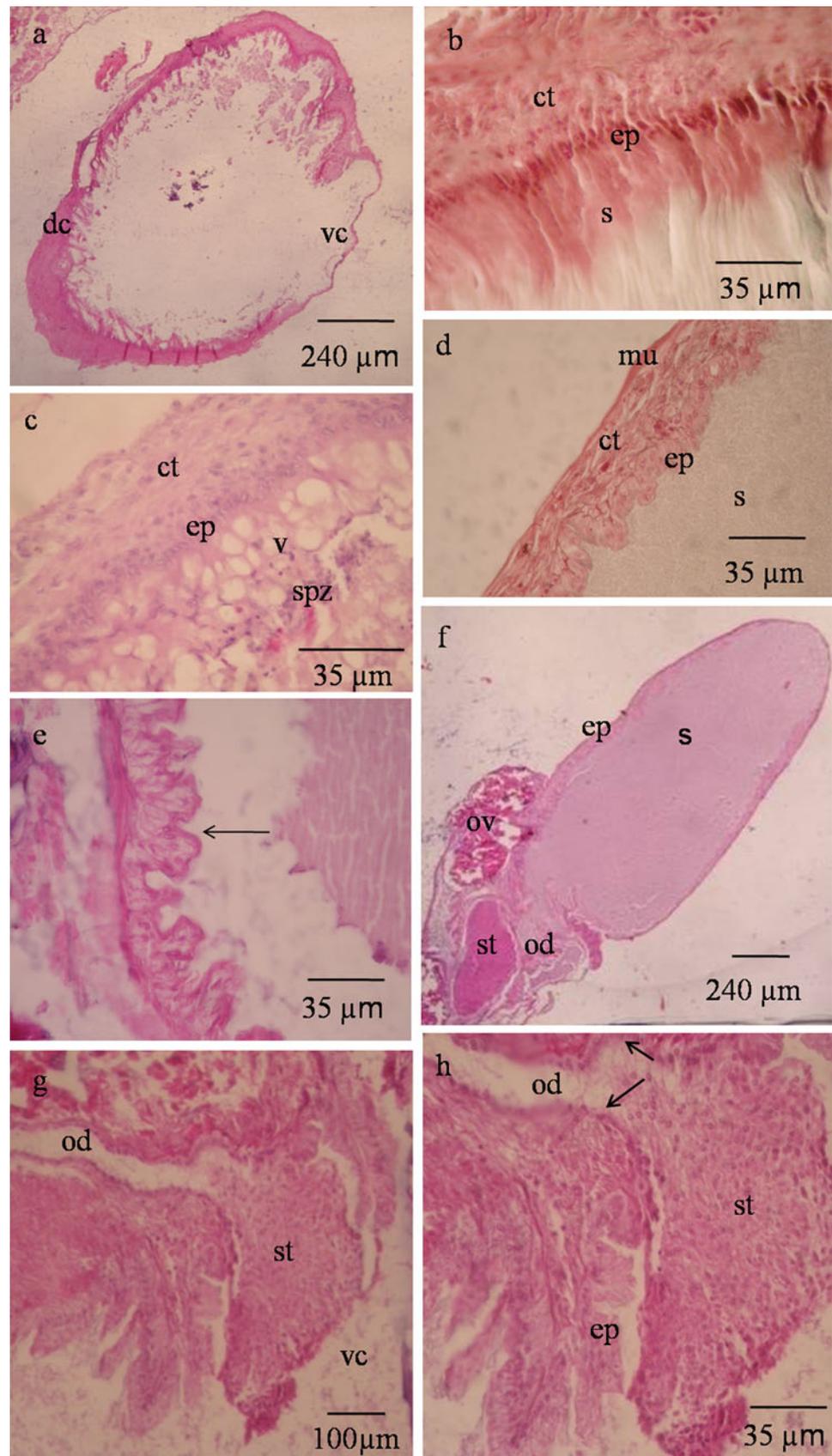


Table 1 Two-way ANOVA showing the effect and interactions between the dependent variable, duration of receptivity, with respect to the independent factors: study area and female reproductive status (egg laying or non-egg laying)

Effect	SS	Degree of freedom	MS	F	P
Intercept	0.2681	1	0.2681	2,198.318	***
1. Study area	0.001	1	0.001	53.343	***
2. Female reproductive status	0.0104	1	0.0104	447.684	***
1 × 2	0.0009	1	0.0009	1.654	ns
Error	0.0197	106	0.0002		

Significant *P* values: *** *P* < 0.001, *ns* non-significant

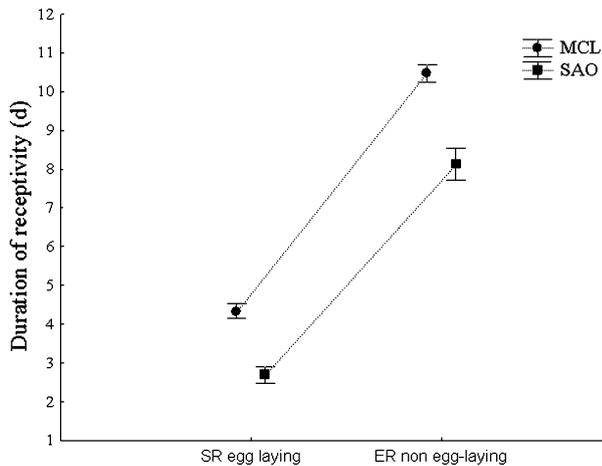


Fig. 4 *Neohelice granulata* females. Duration of receptivity in days (d) for females that laid eggs and non-egg-laying females, and study areas, Mar Chiquita Coastal Lagoon (MCL) and San Antonio Oeste (SAO): females with egg laying had shorter receptiveness (SR) than the ones without egg-laying and extended receptivity (ER), and females from SAO had shorter receptiveness than females from MCL

proportion of females that laid eggs was higher in SAO ($\chi^2 = 33.64$, $P < 0.001$), while similar proportions of both female types were found in MCL ($\chi^2 = 0.92$, $P = 0.34$) (Fig. 6).

Differences in the duration of receptivity among the different months of the reproductive season were found in

MCL (Kruskal–Wallis, $H = 43.31$, $P < 0.001$, $N_{\text{total}} = 118$). The results from the Student–Newman–Keuls test showed that the duration of receptivity shortened during the reproductive season: During September–November, females had long receptive periods of 7–12 days (8.16 ± 3.2), during November–December females had intermediate receptive periods of 6–8 days (6.6 ± 2.6), and during December–February females had short receptive periods of 3–5 days (4.9 ± 2.1) (Fig. 7).

The long-term study of captive females showed that a single female could become receptive and lay a batch of eggs up to four (3.56 ± 0.67) or two (1.62 ± 0.48) times in a single reproductive season for females from MCL and SAO, respectively. These differences between study areas were significant ($t = 7.49$, $P < 0.0001$, MCL: $N_{\text{with males}} = 5$, $N_{\text{without males}} = 11$, SAO: $N_{\text{with males}} = 7$, $N_{\text{without males}} = 10$).

Spermatophore dehiscence experiments

Differences in the quantity of intact SPF between treatments were encountered (Kruskal–Wallis, $H = 30.24$, $P < 0.001$). A Student–Newman–Keuls test showed differences in treatments 2 and 3 (where SPF were incubated with the SR of receptive or non-receptive females, respectively) with respect to the rest of treatments 1, 4 and 5 (where SPF were incubated alone or with oocytes of

Table 2 Two-way ANCOVA showing the effect and interactions between the dependent variable, duration of receptivity, with respect to the covariable size of female, and two independent factors: type of female and study area

Effect	SS	Degree of freedom	MS	F	P
Intercept	42.237	1	42.237	7.2768	**
1. Size of females	1.822	1	1.822	0.3139	ns
2. Type of female	1,118.181	1	1,118.181	192.6440	***
3. Study area	214.525	1	214.525	36.9591	***
2 × 3	47.296	1	47.296	8.1483	**
Error	934.507	161	5.804		

Significant *P* values: ** *P* < 0.01, *** *P* < 0.001, *ns* non-significant

Table 3 Three-way ANOVA showing the effect and interactions between the dependent variable, duration of receptivity, with respect to the independent factors: type of female, presence of males, and study area

Effect	SS	Degree of freedom	MS	F	P
Intercept	5,619.262	1	5,619.262	1,218.410	***
1. Type of female	1,291.111	1	1,291.111	279.948	***
2. Presence of males	2.560	1	2.560	0.555	ns
3. Study area	333.352	1	333.352	72.280	***
1 × 2	1.197	1	1.197	0.259	ns
1 × 3	114.206	1	114.206	24.763	***
2 × 3	9.445	1	9.445	2.048	ns
1 × 2 × 3	7.144	1	7.141	1.549	ns
Error	820.930	178	4.612		

Significant P values: *** P < 0.001, ns non-significant

Table 4 Two-way ANCOVA showing the effect and interactions between the dependent variable, duration of receptivity, with respect to the covariable weight of the seminal receptacle (SR), and two independent factors: type of female and study area

Effect	SS	Degree of freedom	MS	F	P
Intercept	550.4207	1	550.4207	225.4025	***
1. Weight of the SR	60.1019	1	60.1019	24.6123	***
2. Type of female	287.3699	1	287.3699	117.6807	***
3. Study area	93.9718	1	93.9718	38.4824	***
2 × 3	0.0356	1	0.0356	0.0146	ns
Error	256.4043	105	2.4419		

Significant P values: *** P < 0.001, ns non-significant

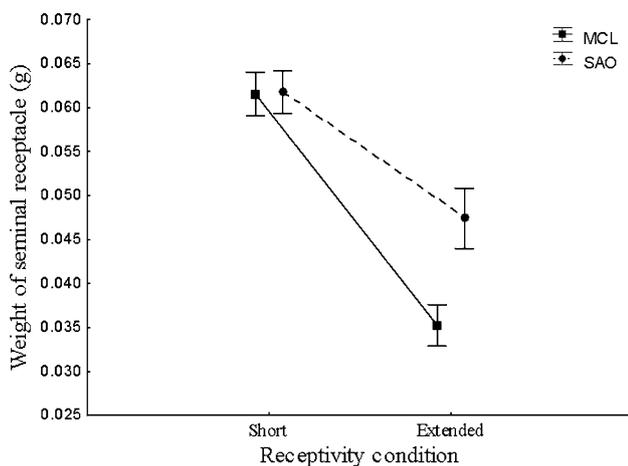


Fig. 5 Differences in the weight of the seminal receptacles in grams (g) between females with short and extended receptivity from the two study areas, Mar Chiquita Coastal Lagoon (MCL) and San Antonio Oeste (SAO): females with short receptivity had heavier seminal receptacles than the ones with extended receptivity at both study areas, and females with extended receptivity from SAO had heavier seminal receptacles than the ones from MCL

receptive or non-receptive females, respectively) (Fig. 8). In the former, few SPF and many free spermatozooids were encountered, while in the latter, many more intact SPF

were found. Thus, the SR secretions of both receptive and non-receptive females may be involved in the dehiscence of spermatophores, while oocytes seem not to be.

Discussion

Our results suggest that to a certain extent *Neohelice granulata* females can adjust the duration of their receptivity and can choose when to lay eggs or not. Thus, females could control the moment of fertilization according to different internal mechanisms related to the morphology of the vulvae, the fullness of the SR and anatomical attributes of the SR.

The duration of receptivity is unknown for most grapsoid crabs, although two major groups can be distinguished: females that are morphologically always able to mate and females whose sexual receptivity is restricted morphologically to certain times when opercula are mobile (see Brockerhoff and McLay 2005c). Captive *N. granulata* females could become receptive and lay eggs several times in a single reproductive season, but only for a restricted period each time; thus, they belong to the latter group. The presence of mobile opercula advertizing the receptivity of

Table 5 Two-way ANOVA showing the effect and interactions between the dependent variable, weight of the seminal receptacle (SR), with respect to two independent factors: study area and type of female

Effect	SS	Degree of freedom	MS	F	P
Intercept	5,890.34	1	5,890.34	1,440.048	***
1. Study area	142.933	1	142.933	5.189	*
2. Type of female	1,199.56	1	1,199.56	56.018	***
1 × 2	4.432	1	4.432	4.857	*
Error	527.857	197	2.679		

Significant *P* values: * *P* < 0.05, *** *P* < 0.001

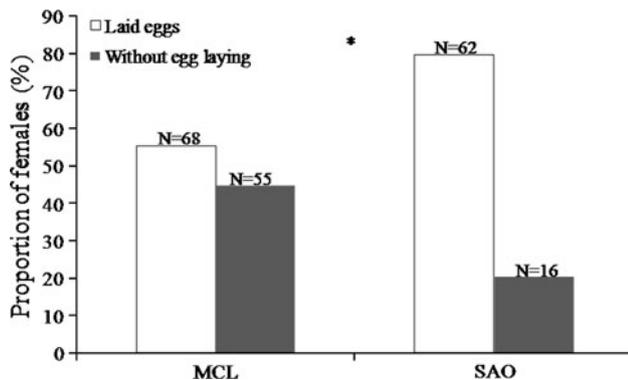


Fig. 6 Proportion of *Neohelice granulata* females (%) that laid eggs or without egg laying in Mar Chiquita Coastal Lagoon (MCL) and San Antonio Oeste (SAO). *Differences were found between both study areas

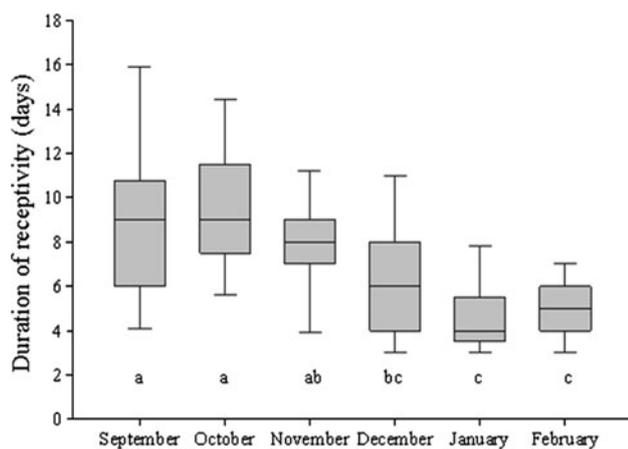


Fig. 7 Receptivity duration of *Neohelice granulata* females during the months of the reproductive seasons of 2008–2010 from Mar Chiquita Coastal lagoon. Different letters indicate significant differences after a Student–Newman–Keuls Test

N. granulata females is similar to those described for other grapsoids (Hartnoll 1965, 1968; Seiple and Salmon 1982; Brockerhoff and McLay 2005a, b, c). The capacity of the opercula to become movable is usually attributed to a decalcification process before mating (Hartnoll 1969; Henmi and Murai 1999).

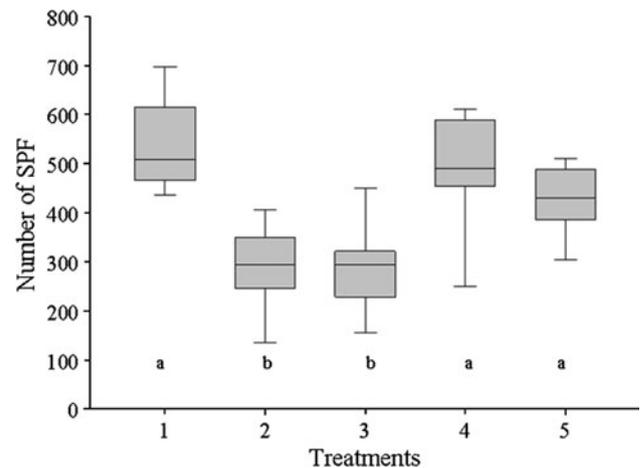


Fig. 8 Number of spermatophores (SPF) quantified in treatments, 1 a control of SPF alone, 2 SPF + seminal receptacle of a receptive female, 3 SPF + seminal receptacle of a non-receptive female, 4 SPF + oocytes of a receptive female and 5 SPF + oocytes of a non-receptive female. Different letters indicate significant differences after a Student–Newman–Keuls Test

Regarding the presence of setae on the opercula of the *N. granulata* vulva, their morphology is similar to the cuspidate setae described by Beninger et al. (1991) for the first male gonopod of *Chionoecetes opilio*, by Garm (2004) for the maxilla of *Cherax quadricarinatus* and by Scelzo et al. (2010) for the female gonopore of *Pagurus exilis*. A probable role in mechanoreception was suggested due to the infracuticular insertion of this setae type, allowing movements of gonopods during ejaculation (Beninger et al. 1991). A similar function could also be suggested for the opercula setae of *N. granulata* females: Setae might sense and help to position the male gonopod thereby facilitating copulation.

The SR of *N. granulata* was first described by López Greco et al. (1999) who indicated that it belonged to the ‘ventral type’ described by Diesel (1991). Here, we provide more detailed information about the cells encountered in the dorsal secretory chamber and in the ventral one, which corresponds to the anatomy described for other Brachyura (Ryan 1967; Johnson 1980; Beninger et al. 1988; Diesel

1989; Sainte-Marie and Sainte-Marie 1998). Differences between receptive and non-receptive *N. granulata* females were found in the secretory function of the dorsal chamber epithelium and the presence of a special tissue at the junction of the oviduct and the SR. Regarding the secretions produced by the gland cells of the dorsal chamber, differing functions have been proposed, for example sperm maintenance (Subramoniam 1993; Anilkumar et al. 1996), dehiscence of spermatophore wall (Ryan 1967; Diesel 1989; Anilkumar et al. 1999), or facilitating the removal of degraded sperm (Sal Moyano et al. 2010). In this study, secretions were more abundant and formed vesicles in non-receptive females. This might suggest that secretions may be involved in maintaining viable sperm during the non-receptive stage, because *N. granulata* females could spawn at least one batch of eggs without remating in a single reproductive season (López Greco et al. 1999). According to the results obtained in the dehiscence experiments, secretions of the dorsal epithelium may also be involved in the bursting of spermatophores, although no differences were encountered between secretions from receptive and non-receptive females.

Regarding the presence of a special tissue at the junction of the oviduct and the SR, this was first described for the grapsoid *Eriocheir sinensis* and named as a valve-like tissue (Lee and Yamazaki 1990). During egg laying this tissue might hinder sperm from entering the oviduct and favor entrance of oocytes into the SR, as suggested for ocytopodid crabs (Lautenschlager et al. 2010). More recently, this kind of tissue was also reported for pinnotherid crabs and named as a ‘holocrine transfer tissue’ from which oocytes have to pass through holocrine secretion at ovulation (Becker et al. 2011). It is proposed that this tissue may function as an alternative female mechanism to choose whether or not to fertilize the oocytes: if females could delay fertilization, while continuing to mate, more sperm from different males accumulate in the SR, thus leading to sperm competition.

Few studies have been conducted in order to elucidate the factors influencing the duration of female receptivity. In different species of grapsoid crabs such as *Hemigrapsus sexdentatus* it was demonstrated that in the absence of males, females could extend their receptiveness (Brockerhoff and McLay 2005a, b); while in *Cyclograpsus lavauxi* and *Austrohelice crassa* the duration of receptivity was independent of female size and of the presence of males in short and long-term trials (Brockerhoff and McLay 2005c). In *N. granulata* it was demonstrated that the duration of receptivity was independent of female size and of the presence of males, although it was dependent on the SR load: females with empty SRs have extended receptive periods while females with full SRs exhibit short receptivity. It is possible that females of many decapod species can assess how much sperm they have stored (Sainte-Marie

2007), as appears to be the case in some insects (e.g. Sakurai 1998; Wedell 2005). It was suggested that female lobsters can monitor their sperm load and solicit matings when they need sperm during a long intermolt period (Atema and Steinbach 2007). In our study, it is expected that females with empty SRs exhibit an extended receptive period to copulate with new males to (re)fill their SRs where sperm can be mixed resulting in sperm competition. Sperm storage may afford females the opportunity to accumulate sperm from several males (e.g. Diesel 1991; Jensen et al. 1996; Gosselin et al. 2005; Sainte-Marie 2007), resulting in genetically diverse progeny (Eberhard and Cordero 1995; McLay and López Greco 2011). In the case of *N. granulata*, females with full SRs would benefit by having a short receptive period to avoid predation risk while being guarded or mated by males and to avoid being injured during male-male contests. Sperm storage may reduce female mating frequency and thus decrease her exposure to physical stresses or predators when searching for mates (Morgan et al. 1983; Koga et al. 1998), to male inflicted injuries or microbe infection (Elner and Beninger 1995).

The differences between *N. granulata* females that lay eggs or not may be related to the duration of receptivity, SR load and sperm competition: in females with short receptive periods and full SRs sperm mixing may well have occurred, so they do not need to copulate to replenish them, and they can lay eggs ensuring fertilization of all embryos; while in females with extended receptive periods the SRs are not full, they may not lay eggs because they may be unable to fertilize all the embryos. It is likely that these females would copulate with several males to replenish their SRs, leading to sperm competition. Since all receptive females, independently of their short or extended receptivity, had ovaries in their ultimate stage of development, indicates that ovary maturation is not just linked to egg laying. Although in this study females with extended receptivity did not lay eggs, under other experimental conditions, when maintained with more than one male, it might be possible that they copulate to fill SRs and consequently lay eggs, or they could shorten their receptivity duration. Future experiments will help to differentiate these alternatives.

For females from MCL, changes in the receptive period during the reproductive season could be explained as follows: during the first months, females are more likely to have few sperm remaining in their SRs left over from the previous mating season or they may not be viable; thus, they need to copulate with males to replenish supplies. It has been demonstrated that the SR of *N. granulata* shows seasonal variation, with the smallest size observed during autumn, before the beginning of the reproductive season (López Greco et al. 1999). In contrast, at the end of the

reproductive season, females have become receptive and laid eggs many times (four times, this study; and up to six times, Ituarte et al. 2004), thus they have probably copulated with several males each time, filling their SRs, so they exhibit a shorter receptive period. A similar strategy was observed in fiddler crabs of the genus *Uca*, in which it was demonstrated that early in the breeding cycle, after choosing a mate, females take longer to ovulate compared to females that mate at the peak of the cycle (Christy 2007).

The differences encountered in the number of times females became receptive between study areas could be due to the differing environmental conditions. Ituarte et al. (2006) demonstrated that the late start and short duration of the reproductive season at SAO (compared to MCL), was due to females not fully developing their ovaries during winter, probably caused by a limited food supply. Further, other studies showed that the duration of the embryonic development was similar in both study areas (Bas et al. 2008). Consequently, females from SAO become receptive fewer times (about half) in a single season since the reproductive season was considerably shorter than at MCL.

The results showing that females with extended receptivity from SAO had heavier SRs than the ones from MCL could be explained by the comparatively low fecundity of females from SAO (35–50 % lower than in females from MCL; Bas et al. 2007). Thus, females from SAO utilize less sperm stored in their SRs to fertilize the eggs extruded. On the other hand, the similar SR weights of females with short receptivity from both study areas indicate that females can fill their SRs up to the same capacity: although females from MCL have higher fecundity than females from SAO, they also have longer receptivity duration and can become receptive more times in a single reproductive season according to our results; thus, they might copulate with several males each time, thereby explaining the similar weights.

In conclusion, we propose that *N. granulata* females exhibit flexibility in the receptive duration and in the timing of oviposition according to their sperm load. Females with empty SRs could extend their receptivity copulating with new males, thus leading to sperm competition. In females with full SRs, sperm mixing from different males could have occurred, their receptive period is shorter, and females may omit mating, but they can still lay eggs. The morphology of the vulvae and anatomical attributes of the SR allow females to control the moment of fertilization. Additionally, the environmental characteristics also influence the extent of the receptive period and the number of times that a female could become receptive in a single reproductive season.

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Conflict of interest The authors declare that they have no conflict of interest.

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