MALACOLOGIA, 2014, 57(2): XXX–YYY

# NUPTIAL GIFTS IN *POMACEA CANALICULATA* (AMPULLARIIDAE, CAENOGASTROPODA): EXPERIMENTAL AND FIELD EVIDENCE ABOUT THEIR FUNCTION

Silvana Burela[1,2*] & Pablo Rafael Martín[1,2]

## ABSTRACT

*Pomacea canaliculata* is a South American apple snail that shows a multiple mating behavior. The copulations are frequent and long lasting, and consequently the males have to face strong sperm competition. The outer gland at the base of the penis sheath secrets drops of mucus that females eat during copulation. These mucus drops are nuptial gifts, and the occurrence of them is the only known instance of this behavior in molluscs. We investigated three possible functions of the gift-giving behavior in *P. canaliculata* based on three hypotheses: prowler deterrence, male mating effort and paternal investment. We also quantified the frequency of nuptial gifts in two populations of *P. canaliculata* and its possible role in male competition. We found no aversive reaction neither in females nor in males, but females were attracted to the mucus secretion. The consumption of artificial nuptial gifts (homogenates of the outer sheath gland) had no effect on the copulation duration nor on the total number of eggs and egg masses laid by females. In the field, the frequency of nuptial gifts was almost ten times greater in the population with the highest density of snails, indicating a much higher rate of production of nuptial gifts. The proportion of couples with both nuptial gifts and a prowler males attached was significantly higher than expected by chance in the population with the highest population density. Even though our results give no support for the three hypothesized functions for the nuptial gifts in *P. canaliculata*, this study revealed a possible different role in male competition: the enticement of the female to remain in copulation when the other males are trying to gain access.

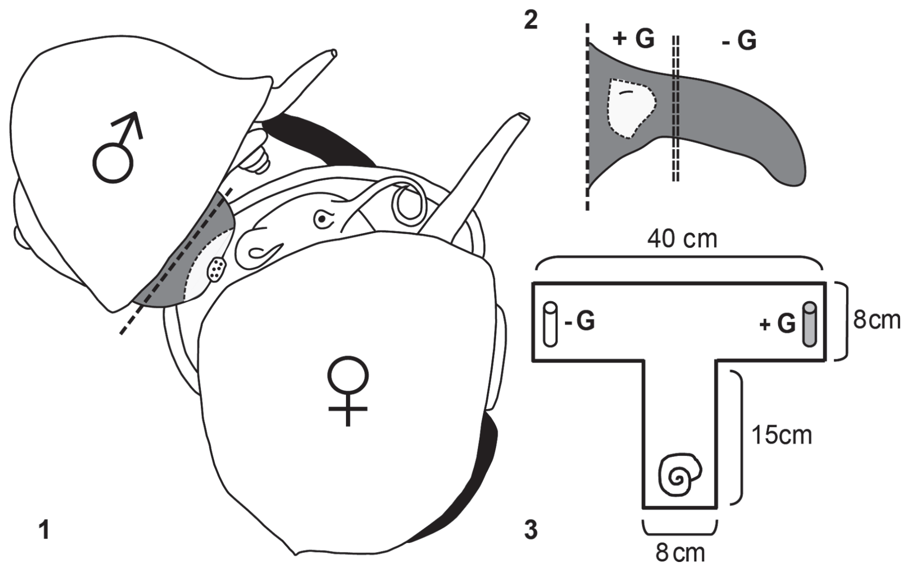
Key words: apple snail, copulation, male competition, paternal investment, mating effort.

## INTRODUCTION

*Pomacea canaliculata* (Lamarck, 1819) is a South American snail in the Ampullariidae. It is well known as crop pest and listed among 100 of the "World's Worst Invasive Alien Species" (Lowe et al., 2000). It exhibits a multiple-mating behaviour with frequent (2–3 times per week) and long lasting copulations (12–18 hours) (Albrecht et al., 1996; Burela & Martín, 2011). The sperm provided by a male tends to be replaced by the following ones (Yusa, 2004, 2007). The number of viable eggs that a female can lay after a single copulation is positively related to copulation duration (Burela & Martín, 2011). Males have a bipartite copulatory apparatus consisting of a slender penis that runs along an open channel of the penial sheath (Gamarra-Luques et al., 2006; Giraud-Billoud et al., 2012). The penial sheath presents a conspicuous outer sheath gland (OSG) that during copulation secrets mucus drops eaten by the female (Fig. 1; Burela & Martín, 2007, 2009). Andrews (1964) observed the mucus drops but not their ingestion by the female and hypothesized that their function was to repel prowler males during copulation, although this remained untested until now.

Lewis & South (2012) defined nuptial gifts (NGs) as "…materials beyond the obligatory gametes that are transferred from one sex to another during courtship or mating" and if eaten by the females they can be considered as oral NGs (Gwynne, 2008). The behaviour observed in *P. canaliculata* is so far the only reported case of oral NG-giving or nuptial feeding in molluscs

[1]Laboratorio de Ecología, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670 (8000), Bahía Blanca, Argentina
[2]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Ciudad Autónoma de Buenos Aires, Av. Rivadavia 1917, Argentina
*Corresponding author: silvana.burela@gmail.com




FIGS. 1–3. *Pomacea canaliculata*. FIG. 1: Scheme of a copulating pair of *P. canaliculata*; FIG. 2: Scheme of the penis sheath (dorsal view); FIG. 3: Scheme of the T-maze employed in the aversion-attraction experiment. Shells and soft parts are in white; opercula in solid black, penis sheath in dark grey, outer sheath gland (OSG) in light gray and nuptial gift (NG) in dotted white. Dashed lines indicate the incision made to sever the penis sheath from the mantle edge and the double dashed line indicates the cut executed to separate the portion of the penis sheath containing the OSG (+G) from the rest of it (-G).

(Burela & Martín, 2007). Most observations on NGs are derived from laboratory experiments, with the exception of several studies on insects (Svensson et al., 1990).

The functions of NGs have been explained under two non-mutually exclusive hypotheses (Vahed, 1998). The paternal investment hypothesis states that NGs increase the fitness or the number of offspring fathered by the gift-giving male. The male mating effort hypothesis states that the NGs attract the females to facilitate copulation or entice the females to remain in copulation for long periods, thus increasing the sperm transference and reducing the chance of sperm competition by other males. Another form of male mating effort is to give direct benefits to the females (e.g., nutritional) that increase their reproductive output after copulation. Among insects there are examples that illustrate these hypotheses: males that can manipulate the copulation duration with the size of the NG (Svensson et al., 1990) or can increase the egg production of females which consume the male's salivary secretion during copulation (Engqvist, 2007).

Within this evolutionary context we may expect similar functions of oral NGs in *P. canaliculata*. To test this we addressed three questions regarding the function of the male secretion during copulation. (1) Does it act as a repellent or attractant for males or females? (2) Does it serve as an enticement for females to remain in copulation for a longer period? (3) Does it improve the female reproductive output in the short term? Finally, we quantified the occurrence of NGs in the field and its relationship with competition among males.

## METHODS

### Experimental Approach

To control the release and the frequency of the NGs in the laboratory, we tested several approaches without success (e.g., cauterization of the OSG pore and adhesion of mechanical barriers to the shell to impede the consumption of NGs). For some trials, we needed large amounts of NG but obtaining the required quantities of naturally released NG was unpractical, because the males release no more than four drops during 13.31 (± 3.5 h) of copulation (Burela & Martín, 2007).

To mimic the effects of OSG secretion, we used tissue macerates obtained from the OSG portion of the penis sheath (Fig. 2). From each penis sheath, the portion with the OSG (+G) was dissected; an equivalent piece of sheath without the OSG was obtained (-G, Fig. 2) to be used as control of aversive reactions to conspecific's hemolympha (Aizaki & Yusa, 2010; Ichinose, 2002).

Aversion-Attraction Experiment

To test the aversive or attractive effects of the mucus secretion, we employed the tissue from the penis sheath of 20 males larger than 35 mm of shell length (SL, measured from the apex to the farthest extreme of the aperture) collected in the field (Pigüé-Venado Channel, 37°09'59''S, 62°40'28''W, Buenos Aires Province). The males were killed by submersion in a bath of water with cubs of ice at -20°C, and their penis sheaths were dissected. The +G and -G portions of the penis sheaths were pooled and macerated separately with an electric microblender for five min. Each macerate was diluted with deionised water to obtain a suspension of 0.05 g tissue/ml. Doses of 1 ml of both suspensions were placed in Eppendorf tubes and frozen at -20°C.

The aversion-attraction trials were conducted in two T-mazes of polystyrene foam that were coated with a polyethylene film before each trial in order to avoid any trace left by the previous experimental snail. The T-mazes were filled with tap water at 25°C. The experimental snails (50 females and 50 males larger than 42 mm SL, sexed by observation of the shape of the shell aperture and of the operculum; Estebenet et al., 2006) were collected from the same population in Pigüé-Venado Channel. Males and females were maintained in separate collective tanks at 25°C and fed with lettuce *ad libitum*. We observed simultaneously a male and a female, each one in a different T-maze. Snails were positioned at the base of the T-maze (Fig. 3) and frozen +G or -G Eppendorf tubes were randomly placed at the opposite arms of the T-maze. The experimental snails were observed for 30 min after they became active (moving the operculum, extending the tentacles, crawling, etc.) and their behaviour and choice of the arms (-G or +G) were registered. Choice data were analyzed with Chi-square tests.

Copulation Duration Experiment

To evaluate if the NGs prolong the copulation duration, we conducted an experiment with 40 females (mean SL = 43.95 ± 3.56 mm) and 40 males (mean SL = 41.44 ± 2.34 mm) collected in the Pigüé-Venado Channel. To mimic the NGs, the females were fed during copulation with artificial nuptial gifts (ANG): the same +G or -G suspensions described for the previous experiment that in order to facilitate the feeding were jellified with commercial red jelly (Royal ®), placed in plastic canulae and stored at -20°C.

The females and males were randomly paired in 3 L aquaria filled with tap water at 25°C. The treatments were applied randomly to copulating pairs (n = 29), and the females were fed with ANG that came out from the tip of the canula with the help of a syringe. Two hours after copulation began, one group of females (n = 10) was fed with (+G) ANG and another one (n = 10) with doses of (-G) ANG; every 30 min the females were fed with a dose of their respective treatment until the copulation ended. A third group of copulating pairs (n = 9) was used as a control of the ANG method, in which females were not fed with ANG but, in order to equate with the rest of the treatments, empty canula were submerged every 30 min. The copulation durations were recorded and analyzed with a one-way Kruskal-Wallis test.

Female Reproductive Output Experiment

An experiment with four different ANG treatments was conducted in order to study a possible effect of the oral NG on the female reproductive output. Thirty-seven females (SL = 47.74 ± 4.10 mm) were collected from Pigüé-Venado Channel in the middle of the reproductive season (February; Estebenet & Martín, 2002), so we assumed that they all have already copulated. They were acclimatized during one week in individual 3 L aquaria with tap water and kept under controlled conditions of light (14 h light) and temperature (25 ± 2°C).

After acclimatization females were randomly assigned to different ANG feeding treatments (in addition to lettuce *ad libitum*): (1) no ANG (controls), (2) females that were fed with 1 ml of suspension (-G) ANG, (3) females that were fed with 1 ml of suspension (+G) ANG; (4) females that were fed with 3 ml of suspension (+G) ANG. The ANGs were dosed in Eppendorf tubes in the same way as for the aversion-attraction experiment; the ANGs were provided in a single dose at the beginning of the experiment (day 0). The females were kept isolated in the same aquaria for 25 days and fed with lettuce *ad libitum*. The egg masses laid on the aquarium walls were gently separated and submersed in water until the individual eggs separated to count the number of eggs. The total number of eggs and egg masses per female were analyzed with one-way Kruskal-Wallis tests.

### Nuptial Gifts in the Field

Field observations of occurrence of NGs in mating pairs were registered in two natural populations with contrasting abundances of *P. canaliculata*: El Huáscar stream (36°55'50"S, 61°35'48"W, Buenos Aires Province) during one day (December 27, 2011) and Piedras Moras Reservoir (32°10'23"S, 64°14'57"W, Córdoba Province) during a complete reproductive season (one day per month, from October 2011 to March 2012). Both water bodies have transparent water (which is odd for *P. canaliculata* habitats; Martín et al., 2001) that allowed us a good view of mating pairs on the bottom; the gentle slopes also allowed their easy capture by wading. This permitted the rapid examination of the mating couples to check for the presence of NG near the pore of the OSG before the snails retract deep into their shells. For each of the collected mating pairs, we recorded the presence of NG, the sizes (SL) of each male and female, and the presence of prowler males firmly attached to the shell of one of the mates; we also recorded whether or not the penis sheath of the prowler was inside the pallial cavity of either member of the mating pair.

Due to the great differences in abundance in the two locations, different methods were used to estimate the density of *P. canaliculata*. In the denser population, it was estimated by counting the number of snails in ten quadrates (96 x 48 cm) deployed along the shore (Saveanu & Martín, 2013), and in the less dense population by counting the snails collected along a wading path at least 32 m-long and 3.0 m-wide (assuming a maximum visual detection distance of 1.5 m on each side of the path) in each sampling date. The association of prowler males and the presence of NGs in copulating pairs was analyzed using $X^2$-tests. The sizes of copulating males were compared with those of prowler males using a paired t-test.

## RESULTS

### Aversion-Attraction Experiment

Seventy-one out of 100 tested snails chose an arm of the T-maze with no significant difference in the behaviour between sexes ($X^2_1$ = 1.47, $P$ = 0.306; Fig. 4). However, the number of females that entered the (+G) arm was significantly higher than the number that entered in the (-G) arm, while the males showed no deviations from the expected by random crawling. Five out of 26 females that chose the (+G) arm inserted the mouth inside the Eppendorf tube and fed on its content; none of the males or the females that entered in to the (-G) arm did this.

### Copulation Duration Experiment

Despite slightly shorter copulation durations in the treatment (+G), there were no significant differences among the two treatments with ANG or the control without them ($X^2_2$ = 5.226, $P$ = 0.073; Fig. 5).

### Female Reproductive Output Experiment

The total number of eggs laid was highest for females fed with three doses of (+G), but it was the lowest for those fed only 1 dose of (+G) (Fig. 6). There were no significant differences among the treatments in the total number of eggs ($X^2_3$ = 1.151, $P$ = 0.765) and egg masses ($X^2_3$ =1.410, $P$ = 0.703).

### Nuptial Gifts in the Field

Both NGs (Fig. 7) and prowler males (Fig. 8) were observed in mating pairs from the two populations. The estimated densities were six times higher in El Huáscar stream than in Piedras Moras Reservoir (Fig. 9). In Piedras Moras Reservoir, 156 mating couples were observed, but only four couples (2.56%) had NGs; prowler males were found in only three mating couples (1.92%), so the data from this population were not analyzed further. In El Huáscar stream, 63 of 269 mating couples (23.42%) had NGs on the OSG and 44 of 269 (31.82%) had a prowler male; one couple had two prowler males. No female was found in prowler position. The frequency of mating couples with both NG and a prowler male was higher than expected by chance ($X^2_1$ = 4.914,

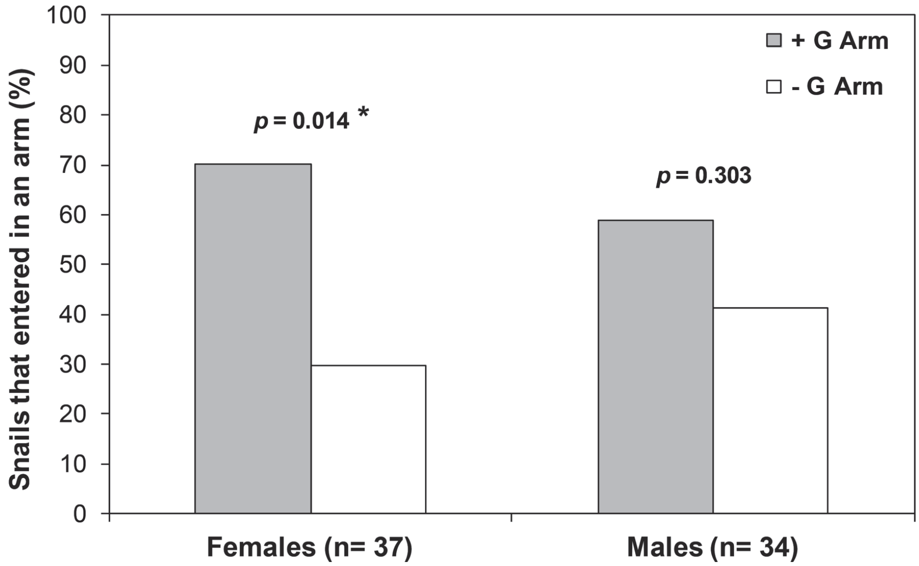



FIG. 4. Percentage of females and males that entered in the arms (+G) and (-G) in the T-maze experiment of aversion-attraction.

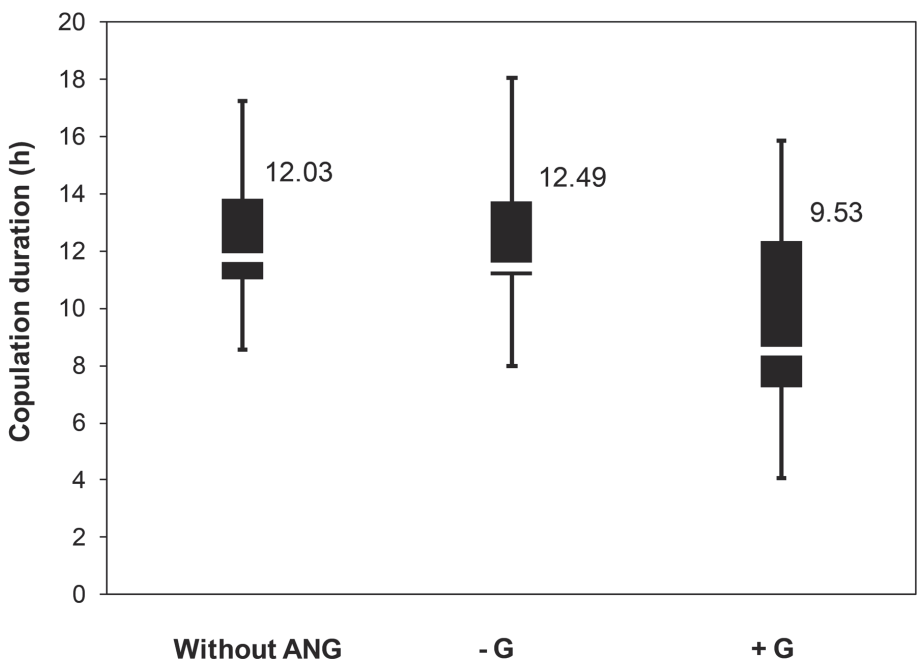



FIG. 5. Copulation durations in the experiment with ANG. Boxes and bars indicate quartiles and extreme values (maximum and minimum) respectively. White lines indicate the median and numbers indicate the mean values.

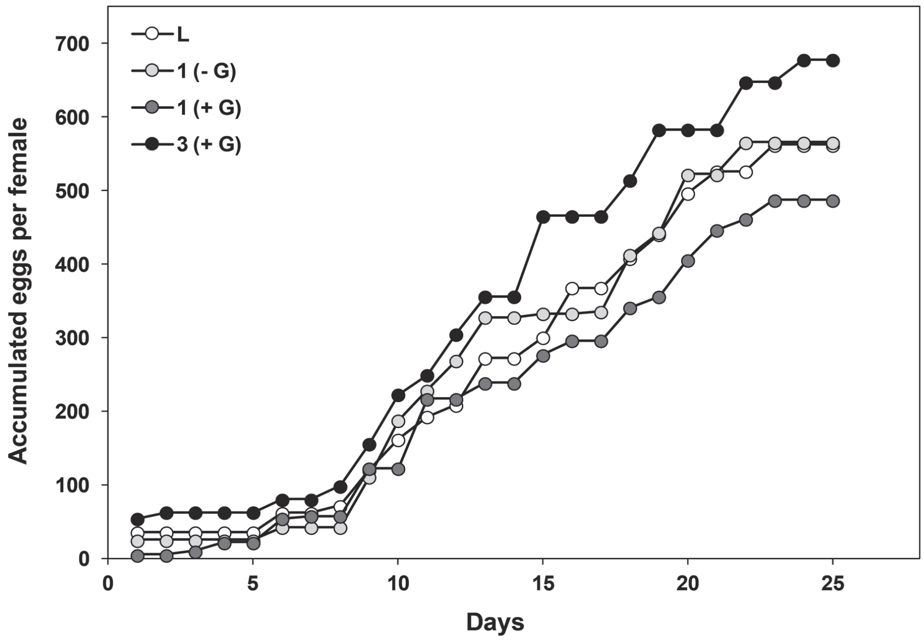
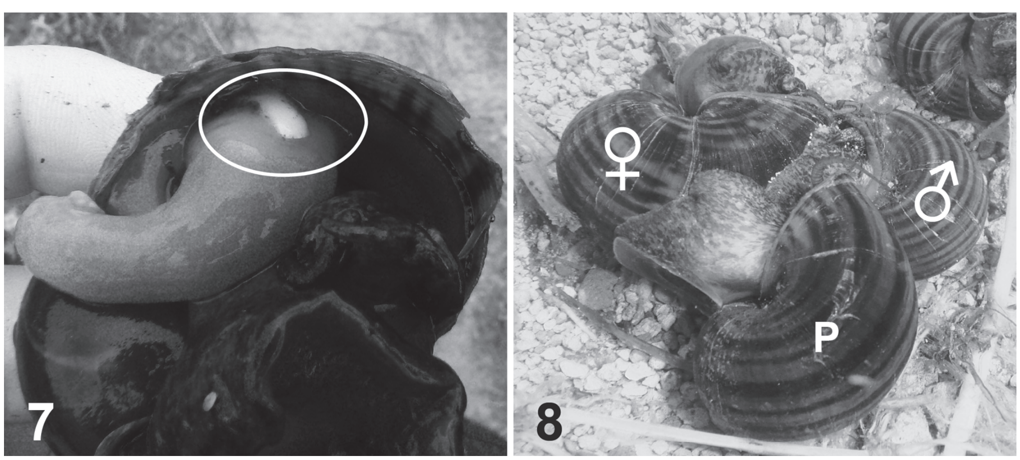



FIG. 6. Cumulative number of eggs per female during a period of 25 days for the different treatments: L = lettuce only, 1(+G) = 1ml of +G suspension, 1(-G) = 1 ml of -G suspension, 3(+G) = 3ml of +G suspension).

*P* = 0.025). The penis sheath of the prowler was inserted in the pallial cavity of one of the mating snails in 31.82% of the cases; in 78.57% of these cases, it was inserted in the pallial cavity of the copulating male. The mean SL of prowler males was significantly smaller than that of the copulating males (*t* = 3.009, *P* = 0.004, n = 44).




FIGS. 7, 8. Copulating pairs of *Pomacea canaliculata* in the field. FIG. 7: *P. canaliculata* male with a mucus secretion drop on his penis sheath (ellipse), after being separated from the female in copulation, from El Huáscar stream; FIG. 8: Mating couple (♀ and ♂) from El Huáscar stream with a prowler male (P) attached to the female shell.

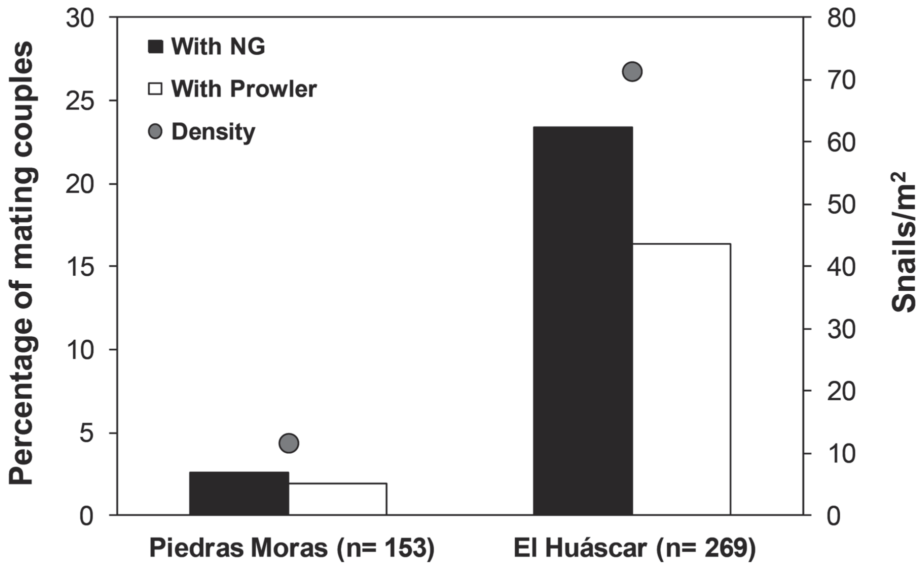



FIG. 9. Percentage of mating couples with prowler males and NG registered in Piedras Moras reservoir and El Huáscar stream.

## DISCUSSION

No aversive reactions were observed in the trials with penis sheath suspensions in spite of the fact that *P. canaliculata* snails usually display alarm responses to chemical cues released from injured conspecifics (e.g., Aizaki & Yusa, 2010; Ichinose, 2002). *Pomacea canaliculata* snails are attracted by water-borne odours from aquatic plants and conspecifics from short distances (Estebenet, 1995; Takeichi et al., 2007). Tests for aversive-attractive effects demonstrated that the males were indifferent to the presence of NGs. Moreover, the copulating males of *P. canaliculata* frequently produce NGs even if other males are not present in the same aquarium (Burela & Martín, 2007), so the repellent effect on prowler males suggested by Andrews (1964) seems not to be a plausible function. In fact, in the field, prowler males were associated more frequently to couples in which males were releasing NGs than to those without them.

Conversely, *P. canaliculata* females are strongly attracted to the mucus secretion of the OSG even when it is not offered within a sexual context during the experiment. Possibly, the attractive effect is due to phagostimulant substances in the mucus, as occurs in some species of insects in which males produce NGs rich in free aminoacids (i.e., candymaker hypothesis; Warwick et al., 2009).

In some snails, the transfer of bioactive substances like allohormones during courtship or copulation can manipulate physiologically the female's reproductive output, influencing the numbers of deposited eggs or affecting the re-mating rate (Koene, 2005; Koene & ter Maat, 2001). However, in *P. canaliculata* the total number of eggs and egg masses laid by the females of different treatments was practically the same in the short term, therefore not supporting the paternal investment hypothesis. Perhaps, the *ad libitum* feeding of females could have masked the differences in the oviposition among treatments if the paternal investment is relevant only under trophic deprivation. However, the amount of material transferred (one to four NG of 9 $mm^3$; Burela & Martín, 2007) is very small relative to the volume of eggs per egg mass produced during the experiment (9.9 ± 0.2 $mm^3$ per egg times 101.0 ± 6.8 eggs per egg mass, Albrecht et al., 1996), which points against a direct trophic significance of NGs. Although empirically more difficult to test, it is possible that the NG-giving males can increase the number of eggs that they fertilize and thus achieve a greater genetic representation in the progeny (Gwynne, 2008; Vahed, 1998).

For the NGs to function as a paternal investment, the benefits to the progeny must appear before the sperm of a subsequent mate begins to fertilize the ova. In *P. canaliculata* females, the replacement of the sperm of the first mate by the sperm belonging to the second mate occurs on average within the first 3.6 egg masses and during the first 19.8 days (Yusa, 2004). The frequent copulation of *P. canaliculata* relative to egg mass laying (2.9 vs. 1.4 times a week; Albrecht et al., 1996) probably does not favour the evolution of such paternal investment. However, the minimum time needed for sperm replacement could be in turn modified by the consumption of NGs, which could reduce the re-mating rate in some insects (Gwynne, 2008). The lack of evidence of direct benefits of consuming the NG in *P. canaliculata* is coincident with recent studies that provide little support to the beneficial effects of NGs in females and give more strength to the negative or manipulative effects of the male donations, reducing the female re-mating rate even below its optimum (Engqvist, 2007; Gwynne, 2008).

The consumption of ANGs had no effect on the copulation duration of *P. canaliculata* couples relative to those that received no ANGs. As it was not possible to impede the spontaneous release of NGs and their ingestion, NG probably occurred in all treatments. Perhaps the addition of ANG had no additive effect on copulation duration due to the fact that the males already attained their optimum duration with their own NGs. In decorated crickets, the males produce NGs large enough to reach a complete sperm transfer from the spermatophore to the female spermatheca (Sakaluk, 1984).

Except for the T-maze experiment, the doses of macerated penis sheath tissues were jellified and coloured in order to achieve a better administration. The processing of penis sheath tissues perhaps diminished or cancelled the putative manipulative effect (possibly allohormonal, *sensu* Koene & ter Maat, 2002) of the mucus secretion in the copulation duration experiment (e.g., Benke et al., 2010), but this seems less plausible for the supposed nutritional effects in the reproductive output experiment (e.g., Gwynne, 2008).

The production of NGs in the field is reported here for the first time in two natural populations of *P. canaliculata*. Laboratory observations (Burela & Martín, 2007, 2009) on the interval between the start of release of NGs and their consumption (ca. 30 seconds), the frequency of NGs per copulation (maximum four) and the copulation duration (mean 12.8 h) indicate that NGs are detectable only during a very short part of the duration of the copulation (ca. 0.25%). Hence the frequency of detection of NG in the field is probably an underestimation of the proportion of snails that produce them; even under continuous observation in the lab, NGs were observed only in 19.91% of the mating couples that reached the insemination phase (Burela & Martín, 2009). On the other hand, the frequency of snails with NGs in the field is probably positively correlated to the rate of NGs production. The frequency of NGs was almost ten times higher in the population with the highest density of snails (El Huáscar stream), indicating a much higher rate of production of NGs. The proportion of couples with prowler males was also higher in El Huáscar stream, and moreover, the proportion of couples with both NGs and prowler males attached was significantly higher than expected by chance in the population with the highest population density.

The evidence gathered here supports a possible role of NGs in the male-to-male competition for ova. NGs are a common in the long copulations of *P. canaliculata* (Burela & Martín, 2007, 2009), and they are released even in the absence of other males. However, the copulating males that perceive a male competitor or risk of male competition probably deliver more NGs during copulation to entice the female to remain in copulation through a sensory exploitation (discussed below). Perhaps, a non NG-giving copulating male cannot achieve its optimal sperm transference if the interference of a competitor male prompts the female to disengage prematurely. Thus, a good NG-giving male would have a competitive advantage to face male competition, but this explanation needs to be tested in the future.

The present results provided little support for the hitherto tested functional explanations of the gift-giving behaviour in *P. canaliculata*. However, the explanations related to the increase in copulation duration and reproductive output cannot be absolutely discarded with our experimental approach using ANGs, as we could not impede natural NGs. Until now, the paradoxical association of NG production with prowler males and the female attraction to the mucus secretion are the only clues to the significance of this behaviour in *P. canaliculata*.

## ACKNOWLEDGEMENTS

We wish to thank to Daniela Miloch, Nicolás Cecchetto, Lucía Saveanu and Juan Francisco Tellarini by their assistance with field work. This work was funded by grants from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, PIP 112 200901 00473) and UNS (Universidad Nacional del Sur, PGI 24/B185 and PGI 24B/144). S. B. and P. R. M. are researchers in CONICET.

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Revised ms. accepted October 11, 2013