

SEQUENTIAL PATHWAYS IN THE MATING BEHAVIOR OF THE APPLE SNAIL
POMACEA CANALICULATA (CAENOGASTROPODA: AMPULLARIIDAE)

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INTRODUCTION

Pomacea canaliculata is the most studied ampullarid in many aspects of reproductive biology, such as environmental factors triggering reproduction (Albrecht et al., 1999, 2005), functional properties of eggs (Heras et al., 2007), reproductive strategy and effort (Estebenet & Cazzaniga, 1993; Estoy et al., 2002b), and size and age at which maturity is reached (Estoy et al., 2002a; Tamburi & Martín, 2009). Surprisingly, though, the current knowledge on the details of mating behavior is scarce.

Males of *Pomacea canaliculata* have a complex copulatory apparatus of pallial origin. It comprises a bulky and muscular penis sheath with a deep channel on its inner face, along which a slender penis runs during copula (Andrews, 1964; Gamarra-Luques et al., 2006). Knowledge on mating behavior of *P. canaliculata* comes mostly from non-systematic observations: matings are long lasting (up to 20 h), occur under water at any time of the day or night, and females can crawl freely but the males cannot and therefore depend on the females' movements to reach the water surface and ventilate their lungs (Andrews, 1964; Albrecht et al., 1996; Burela & Martín, 2007). During mating, the male habitually secretes mucus from the outer gland of the penial sheath, and the female feeds on it, a phenomenon that was interpreted as a nuptial feeding (Burela & Martín, 2007). At least under laboratory conditions, both males and females show a multiple mating behavior (Albrecht et al., 1996). Several authors observed the mating behavior in other ampullarids (Guimarães, 1981; Bahl, 1928; Nono & Mane, 1931; Demian & Ibrahim, 1971; Berthold, 1989, 1991). All of the descriptions agree on the basic pattern. Matings are sub-aquatic, and the male's foot adheres to the right side of the last whorl, while gripping the rim of the female's shell with the penis sheath.

Beyond the practical aspect of saving time of observation in experimental reproductive studies (Trigwell et al., 1997), the recognition of a snail's intention to mate or, contrarily, to avoid it, is an important aspect in the study of sexual preferences and mate choice. The aim of this study is to describe the sequence of actions or states that can eventually lead, but not necessarily does, to ova fertilization, and to quantify the frequency of the alternative pathways in the mating behavior of *Pomacea canaliculata*.

MATERIAL & METHODS

Both adult snails and egg masses were collected in the Curamalal stream at Pasmán town, Buenos Aires Province, Argentina (37°14'31"S, 62°08'04"W), from a population that has been the subject of previous studies (Martín & Estebenet, 2002; Estebenet & Martín, 2003). Egg masses were incubated at room temperature until hatching. Hatchlings and field snails were kept in aquaria of appropriate size in a rearing room at 25 ± 2°C, under 14 h light per day and fed with lettuce *ad libitum*; water was changed and aquaria cleaned once a week. Field snails were sexed by the shape of the shell aperture and operculum (Estebenet et al., 2006); hatchlings were examined weekly to sex them as soon as possible, carefully seeking evidence of the testicle through the translucent shell. Females and males were reared separately.

Observation trials were performed in 3 liter glass aquaria, with water at 24–26°C and permanent illumination, in order to observe the mating behavior of isolated couples. One male and one female were put at the same time in the aquarium, without food, usually at 8:00 A.M. In each trial, up to 15 aquaria were simultaneously set up and the snails in them were observed continuously for 24 h.

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During the observation period each single state adopted or action performed by one or both mates (e.g. female passive, male crawling over the female's shell, mate probing) was recorded and chronometered. The frequency (%) of transition between two successive actions or states was estimated on the basis of the number of snails or mates that performed or adopted the first action or state; hence the sum of the frequencies of the alternative paths after an action or state is always 100%. When an action was repeated, the number of repetitions (mean \pm SD) was recorded.

In order to schematize the mating behavior of *P. canaliculata*, the set of states and actions was divided in four phases (adapted from Trigwell et al., 1997) to account for apple snail particularities): precourtship, courtship, copula and poscopula. The precourtship was operatively defined in our study as the period from the moment when snails were paired in the aquarium to their first encounter and contact. Courtship begins when the male mounts the female shell and ends when the male adopts the copula position in order to insert its penis sheath. The copula starts when the male inserts the penis sheath into the female's pallial cavity and ends when it withdraws it. The postcopula phase was defined as the period in which the male remains on the female without genital contact. The duration (mean \pm SD) of each phase was estimated on the basis of the snails or mates that completed that phase.

Observations of precourtship, courtship and the first part of copula phase (up to the moment in which the female enters a passive state after sheath intromission; Fig. 1) were performed with virgin snails reared in the laboratory. Males (n: 50, 30–35 mm of shell length, SL) and females (n: 50, 35–40 mm SL) were randomly paired; if they did not mate after 2 h of observation, they were reused after at least 24 h. A total of four hundred pairs were arranged during the trials; the pairs that ultimately engaged in copula were separated after pre-fixed times as part of a study on sperm transference, which will be published elsewhere.

Observations of the last part of copula and postcopula were performed with adult snails that presumably had mating experience: field females (n: 15, 40–50 mm SL) and males reared in the laboratory (n: 40, 31–43 mm SL) that had previously had contact with females (i.e. shared the same aquarium). Males and females were randomly paired and reused

after at least 24 h; a total of 32 couples commenced sperm transference after the females had entered the passive state. All the behaviors registered during the first set of observations (virgin snails) were also observed in the second one (experienced snails), but only these 32 couples were used in the calculations of transition frequencies in the last part of copula and poscopula (Fig. 1).

RESULTS

Of a total of 400 pairs formed in the observation aquaria for the precopulatory behavior, only 191 (47.75%) engaged in some kind of interaction, initiating the precourtship phase (Fig. 1). Mate encounter comprises all the movements of the snails in the aquarium up to the moment of the first physical contact; in most cases, it was the male that approached the female and this occurred both when the later was moving or laying motionless. In one half of the mate encounters, the male mounted the female shell directly. In the other half, mate probing was observed: contacts with cephalic tentacles, labial palps or feet, radular scraping on shells or snouts, etc. This probing was generally performed by the males. The mean duration of precourtship was very variable: from a few seconds (if no probing occurred) to 2.29 h.

Only 147 out of the 191 pairs (76.96 %) reached the courtship phase, with the male mounting the female shell (i.e., the male's foot completely lost its hold on the aquarium); in many cases, the males crawled counter-clockwise over the female shell (viewed from the female apex; Fig. 2A), but this circling behavior was not quantified. After this, the male frequently dismounted spontaneously from the female's shell. Otherwise, the male began the positioning in order to gain foothold on the right (sutural) side of the female's last whorl and to near the sutural side of its aperture to that of the female. The male's positioning commonly elicited a swinging reaction by the female (Fig. 2B): the female rotated its shell several times in a counter- and clockwise way, with the foot adhered to the substratum. After a short pause, this swinging reaction could be repeated up to four times by the female. The female shook off the mounting male in almost 60% of the cases. If not, after the swinging the female entered a passive state (motionless and firmly adhered to the aquarium) with the male still mounted.

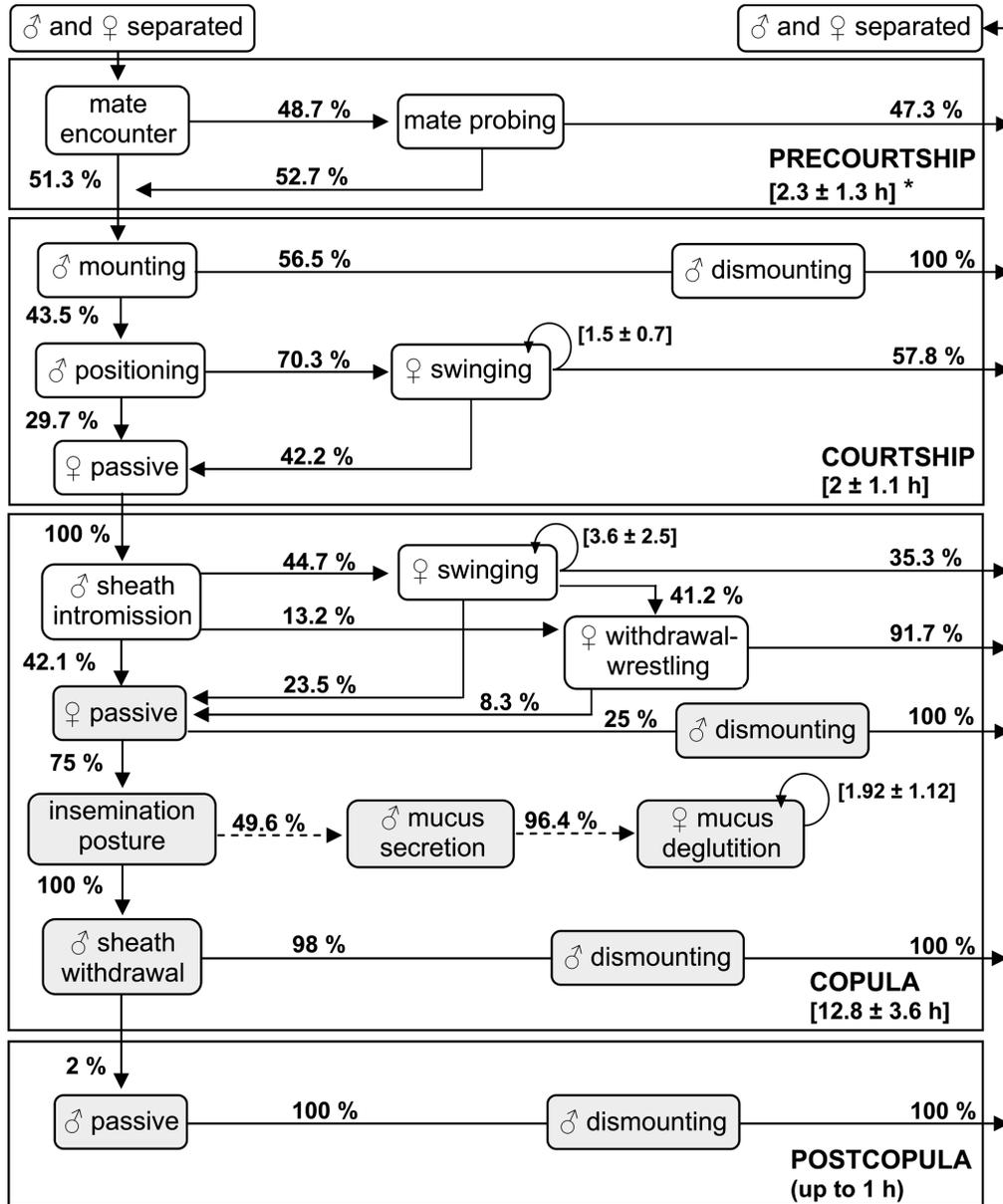


FIG. 1. Descriptive scheme and statistic summary of mating behavior in *Pomacea canaliculata*. Rounded boxes represent single states or actions performed by one or both mates. Percentages are the frequencies of the different pathways that snails followed after each action or state. Solid arrows connect successive actions or states; hatched arrows join two actions that can occur simultaneously and curved arrows indicate repeated actions (number of repetitions [mean ± SD]). Straight boxes include the different actions or states of each phase; phase durations [mean ± SD] are shown (* calculated only for the cases where mate probing occurred). White boxes and the arrows arising from them correspond to virgin snails whereas gray boxes and their arrows correspond to snails with mating experience.

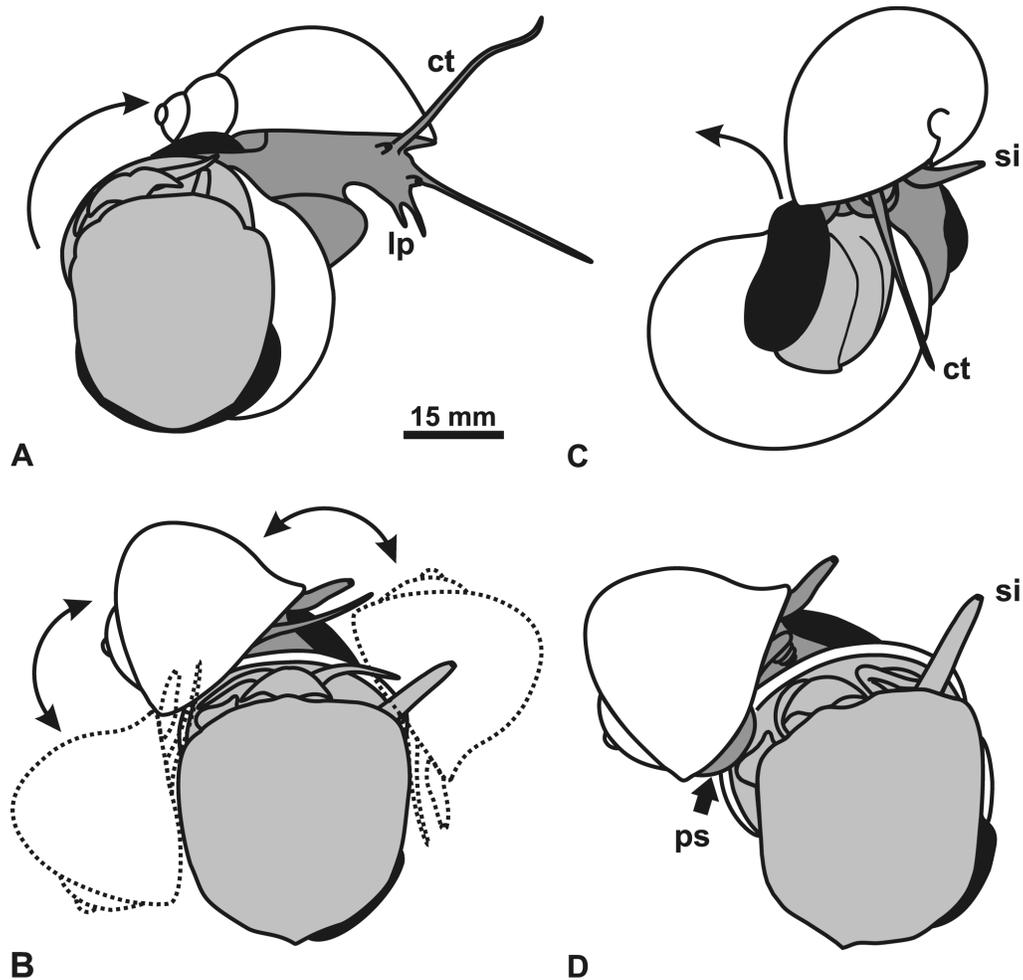


FIG. 2. Schemes of actions or states that occur during the mating behavior in *Pomacea canaliculata* (drawn on the basis of photographs taken in aquaria; water surface is located towards the upper side in all cases and the female's foot is adhered to the aquarium wall, except in C). A: Circling; B: Swinging; C: Withdrawal-wrestling; D: Insemination posture. Female body (light gray), male body (dark gray), shells (white) and opercula (black), si (siphon), ct (cephalic tentacle), lp (labial palps) and ps (penis sheath); curved arrows indicate the direction and sense of movement.

The duration of the courtship was quite variable, ranging from 0.32 to 4.77 h; on the whole, only 38 pairs (19.89%) completed the courtship phase and began copula.

The intromission of the penis sheath initiates the copula phase; this action elicited strong reactions in 57.90% of females. Most of them performed several swinging events that shook off the male in a third of the cases. A 13.16% of the females that suffered the intromission of the sheath reacted immediately loosening

its foothold on the aquarium, falling to the bottom, withdrawing the cephalopodium inside the shell, and trying to close the aperture with the operculum; if the male did not retrieve the sheath, the female engaged in a wrestling behavior, trying to push the sheath out of the pallial cavity with the operculum (Fig. 2C). A 41.18% of the females that showed swinging also performed the withdrawal-wrestling behavior afterward. Almost all of the females that performed this behavior interrupted the copula. On

the whole, in 73.96% of the cases, the female finally entered a passive state, tightly adhered to the aquarium, after the sheath intromission. In a few cases, after 20–60 minutes, the male spontaneously retrieved the sheath and then dismounted the female shell, without any apparent cause.

In cases when spontaneous quitting did not occur, the male also entered a passive state similar to the female's while tightly adhered to its shell. Both members of the couple usually then remained in this position with their cephalic tentacles coiled (insemination posture, Fig. 2D), and apparently sperm transference began. A direct observation of this process (including the contact of the tip of the penis with the female gonopore) was not possible although it certainly occurred, since laying of fertilized eggs commenced a few days later. During sperm transference, the female was usually motionless but occasionally moved around to ventilate the lung; sometimes the male took advantage of this last behavior to do the same. After a minimum of four hours, in many cases it was possible to observe that the outer sheath gland began to secrete a whitish mucus that accumulated in the form of a small drop on the gland opening; in most cases, the mucus drop was deglutated at irregular intervals by the female. After 12.80 hours on average (ranging from 4.13 to 19.78 h), the male slowly retrieved the sheath, dismounted the female shell and the copula ended. Only in a few cases, the male remained on the female's shell for up to one hour with the sheath folded in resting position inside its own pallial cavity (postcopula phase).

DISCUSSION

The observations on mating behavior of *Pomacea canaliculata* reported on here are in agreement with those of previous non-systematic studies (Andrews 1964; Berthold, 1989, 1991; Schnorbach, 1995; Albrecht et al., 1996); however, a number of new behaviors, especially those that can be interpreted as mate-rejection actions by the female, are reported for the first time.

Almost half of the virgin snails paired in the trials did not show any type of interaction even when they shared an aquarium for two hours. Probably some of these virgin snails, specially the females, were still immature, despite the fact that they were larger (35–40 mm SL) than the lower limit reported previously to begin egg laying (25 mm SL; Estebenet & Martín, 2002;

Tanaka et al., 1999). However, it has been recently reported that females reach maturity at different shell lengths and ages, depending on food availability: females taking 32 weeks to mature (the age of virgin females used in the present study) began to copulate at 40 mm of shell length (Tamburi & Martín, 2009).

The precourtship phase, especially the active mate search, was unnatural in our study, because mates were paired randomly in a small aquarium. Takeichi et al. (2007) provided evidence that in the laboratory *P. canaliculata* snails are able to detect conspecific's water-borne odors and also to follow their mucus trails. Darby et al. (1997) suggested that *P. paludosa* males can follow females' trails into funnel traps in a restored marsh in Florida. However, the existence of specific mate recognition mechanisms in *P. canaliculata* is unclear, because Albrecht et al. (1996) recorded males in copula with other males when maintained together without females for two weeks. In all-male rearing tanks, routinely maintained in our laboratory, such homosexual couples were frequently observed, either for virgin or for experienced males, but never in tanks where males and females were reared together.

At the beginning of the courtship phase many males performed a counterclockwise circling behavior over the female shell. Such behavior has been described for freshwater pulmonates and interpreted as an assessment of partner size (De Boer et al., 1996; Koene et al., 2007). Perhaps this behavior has the same meaning in *P. canaliculata*, because males prefer to mate with big females, at least under laboratory conditions (Estebenet & Martín, 2002).

Swinging behavior was elicited in the courtship phase when the male adopted the copula position on the female's shell but not when it mounted the shell; swinging was rarely observed in rearing tanks when a snail was mounted by other. Two possible explanations can be stated for this behavior: the female could perform the rotations to evaluate male mate quality (weight, vigor or motivation) or to reject a male that showed a clear intention to begin copula. At least an explanation based on male size seems unlikely because it is neither important in access to females (Estebenet & Martín, 2002; Estoy et al., 2002a) nor in female reproductive output (Tamburi & Martín, 2009). Mate rejection is the most commonly accepted explanation for swinging behavior in freshwater pulmonates (e.g., DeWitt, 1991; Trigwell et al., 1997; Facon et al., 2006); swinging is also used as an avoidance response to predatory

leeches in this group (e.g., Townsend & McCarthy, 1980; Wilken & Appleton, 1991) and also in small juveniles of *Pomacea bridgesii* (less than 13 mm; Aditya & Raut, 2005).

Swinging or withdrawal-wrestling behaviors, sometimes combined, were triggered when the male inserted the penis sheath in the pallial cavity of the female. Withdrawal-wrestling behavior can be considered a more definitive mate rejection, since it forced the male to quit in almost all cases. Nono & Mane (1931) described a similar female response to male mounting in the Philippine apple snail *Pila luzonica* and attributed it to a lack of sexual receptivity. Operculum closing is a defensive response to predatory leeches in some species of small freshwater prosobranchs (Kelly & Cory, 1987) and in juveniles of *P. bridgesii* (Aditya & Raut, 2005), but probably not in adults of *P. canaliculata* since they can harbor entire populations of leeches and temnocephalids in their pallial cavities (Vega et al., 2006). Operculum closing seems not to be merely an alarm response to the entering of a strange body inside the pallial cavity. When considered together with the expulsion of the sheath from the pallial cavity, it appears as a clear mate rejection response.

One feasible reason for a female to avoid copula is that it is physiologically ready to deposit an egg mass. This explanation does not apply in the present case, because all females were virgin, and virgin females do not lay eggs (Gamarra-Luques et al., 2006). Females of this species are able to store sperm and to lay viable eggs for up to 140 days and also after winter resting (Estebenet & Martín, 2002), so that the benefits of multiple matings for females are not clear. Forced matings have been reported in other invertebrate taxa (e.g., Cordero, 1999) but they would apparently be rare in *P. canaliculata*, considering the effectiveness of the withdrawal-wrestling behavior. Estoy et al. (2002a) reported that virgin females with immature albumen glands, and therefore not able to lay eggs, nevertheless copulate with mature males in the laboratory. Perhaps these immature females rejected the first mating attempts but eventually accepted the mates (i.e., entered in a passive state) due to the fact that they remained paired in the same aquarium during the entire experiment. Facon et al. (2006) noted that mate rejection behaviors are probably more effective in the field than in no-choice experiments where both partners are artificially maintained isolated together for extended periods.

In some cases, the male quitted spontaneously after the female entered the passive state. It could be that the male failed to contact the female gonopore with the T-shaped sulcus of the penis sheath and to adhere to the pallial cavity (Gamarra-Luques et al., 2006) or perhaps the last rejection resource of the females is to close the gonopore.

Presumably after the contact of the penis sheath with the female gonopore, the penis is introduced in the feminine duct. According to Bahl (1928), only the penis tip penetrates in the Indian apple snail *Pila globosa* and the presence of a heavily ciliated sperm groove in *P. canaliculata* (Catalán et al., 2002) suggests that the penis does not go too far in this case either. The sperm transference begins at least one hour after the intromission of the penis sheath (unpub. results), and no further negative reaction was observed thereafter. During this phase, the male's outer sheath gland releases a mucus secretion that is eaten by the female, a behavior that has been interpreted as a nuptial feeding to entice the female to remain in copula (Burela & Martín, 2007). After several hours and without any externally observable reason, the males withdraw the penis sheath and dismount; whether the male quits due to sperm depletion or because the female closes it gonopore to interrupt the transference cannot be determined for now.

Yusa (2004, 2007) reported that double paternity occurs in a single egg mass of *P. canaliculata*, suggesting that sperm mixing occurs within the female seminal receptacle and that sperm competition could be an important sexual selection mechanism in this snail. The generally brief and infrequent postcopula phase indicates that, if males display a mate guarding behavior to prevent a subsequent mating, it would be during the copula phase (*in copula* guarding *sensu* Sillén-Tullberg, 1981) or triggered only by the presence of prowler males.

On the basis of differential copula frequencies in experiments with multiple partners of both sexes and two sizes, Estebenet & Martín (2002) concluded that *P. canaliculata* shows size-assortative mating. However, the experimental design did not allow discerning the size preferences of each sex. The mate rejection behaviors displayed by females in the present study are interpreted as evidence that females are able to perceive a male's intention to mate. The knowledge of these behaviors will allow the study of sexual preferences (Jennions & Petrie, 1997), a necessary step to understand mate choice patterns in this snail.

ACKNOWLEDGEMENTS

This work was funded with grants by Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 6150/05) and Universidad Nacional del Sur (PGI 24/B108 and PGI 24B/144). SB and PRM are a postdoctoral fellow and a researcher in CONICET, respectively.

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Revised ms. accepted 9 December 2008