

EVOLUTIONARY AND FUNCTIONAL SIGNIFICANCE OF LENGTHY COPULATIONS IN A PROMISCUOUS APPLE SNAIL, *POMACEA CANALICULATA* (CAENOGASTROPODA: AMPULLARIIDAE)

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ABSTRACT

The peculiar adaptations of the dioecious freshwater snails belonging to the family Ampullariidae have recently been recognized as important, albeit neglected, models for evolutionary ecology studies. A review of the literature, mostly reports from unsystematic observations under laboratory conditions, revealed considerable variation (38 min to 20 h) in the duration of copulation in this family, both at inter- and intraspecific levels. The aims of our study were to investigate if the lengthy copulations of *Pomacea canaliculata* occur naturally in the wild and if a significant part of the copulation duration was to accomplish genital connection or to impede the access of other males, rather than being necessary solely for sperm transfer. The effect of single *vs* repeated copulations in terms of female reproductive output, and the effect of some proximate factors (male size, mating status, time of the day and prowler males) on the duration of copulation were also evaluated. Our field results indicate that lengthy copulations previously reported for *P. canaliculata* were not an artefact. The laboratory evidence obtained indicates that the completion of the genital connection or a preinsemination mate guarding can account for only 20% of the copulatory period and that there is no postinsemination guarding. Most of the copulation time is apparently necessary to transfer an amount of sperm that will ensure the fertilization of the thousands of ova produced during the entire lifetime of females. Our laboratory experiments also showed that there was no effect of male size, the time at which copulation began, the mating status of snails or of the presence of prowler males on the duration of copulation.

INTRODUCTION

The peculiar suite of morphological, physiological and behavioural adaptations of Ampullariids, or apple snails, constitutes an excellent, though relatively unexplored, model for addressing important questions in evolutionary ecology (Hayes *et al.*, 2009). *Pomacea canaliculata* (Lamarck 1822) is one of the best known species of this family of dioecious freshwater snails; both males and females mate frequently with different partners, at least under laboratory conditions, and females lay aerial egg masses composed of hundreds of complex cleidoic eggs with calcareous shells (Andrews, 1964; Albrecht, Carreño & Castro-Vázquez, 1996; Burela & Martín, 2007, 2009; Heras *et al.*, 2007). During copulation the penis sheath of the male, an ancillary copulatory organ, produces a mucous secretion that is ingested by the female (Burela & Martín, 2007), a behaviour interpreted as nuptial feeding (*sensu* Vahed, 1998, 2007) or an oral nuptial gift (*sensu* Gwynne, 2008).

Several authors have observed the ampullariid mating behaviour (Bahl, 1928; Nono & Mane, 1931; Demian & Ibrahim, 1971; Guimaraes, 1981a, b; Berthold, 1989, 1991; Heiler *et al.*, 2007). All the descriptions agree on the basic pattern: matings are subaquatic 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. However, the details of the genital connection and insemination are not readily observable and remain obscure.

Notwithstanding this overall uniformity in the mating behaviour, our review of the few reports available on the

duration of the copulation (i.e. the period with genital contact; Burela & Martín, 2009) depicted a great inter- and intraspecific variation in this trait (Fig. 1), ranging from 38 min in *Lanistes ovum* to 12.80 h on average in *P. canaliculata*. Copulations of up to 12 h have been reported for several genera (*Pomacea*, *Marisa* and *Lanistes*), but by far the longest copulations were those recorded for *P. canaliculata* (up to 19.78 h). Knowledge on mating behaviour and copulation duration of almost all these apple snails comes from unsystematic observations carried out under laboratory conditions.

Pomacea canaliculata is located in the upper extreme of the continuum of copulation durations not only within the Ampullariidae, but also within the Caenogastropoda, a highly diverse clade of mostly aquatic, dioecious and internally fertilizing snails (Barker, 2001; Strong, 2003), in which the copulation duration is highly variable (from 5 min to 15 h; Baur, 1998). Several nonmutually exclusive hypotheses can be proposed to explain the lengthy copulations in *Pomacea canaliculata*. The most basic is that they are an artefact provoked by laboratory conditions. The optimum conditions given in laboratory studies in general (*ad libitum* food, no predators, no interference of other snails in some cases, constant temperatures etc.) could have artificially extended the copulation durations.

Another plausible explanation could be the anatomical complexity of the male and female reproductive systems in *Pomacea*, which are much more complex than those of other apple snails (Berthold, 1991; Catalán, Fernández & Winik, 2002; Gamarra-Luques *et al.*, 2006). The copulatory male apparatus collects the sperm from the sperm pit located next to the male

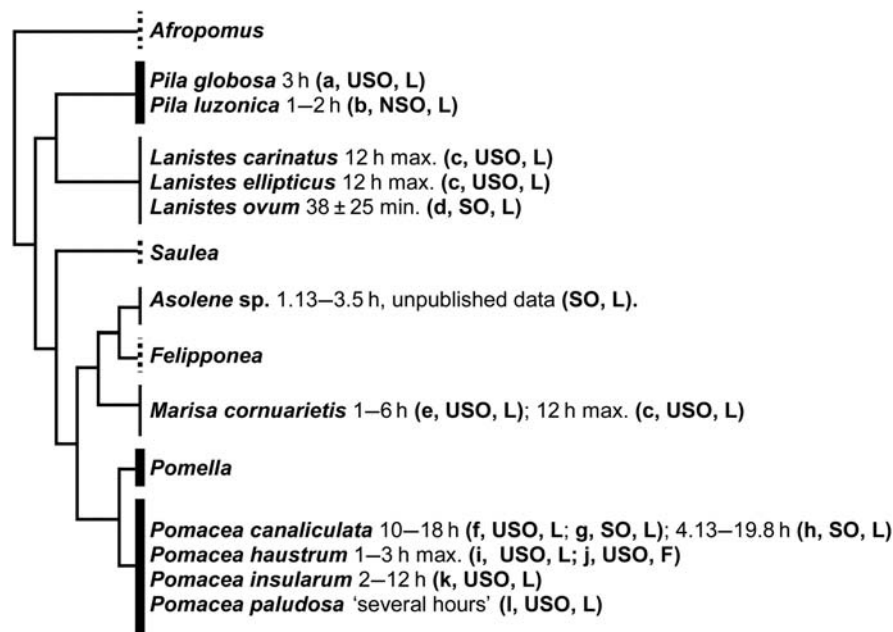


Figure 1. Mapping of selected reproductive traits on a molecular phylogeny of the Ampullariidae at generic level (after Hayes *et al.*, 2009: fig. 1b). Copulation durations taken from the following references: (a) Bahl (1928); (b) Nono & Mane (1931); (c) Berthold (1991); (d) Heiler *et al.* (2007); (e) Demian & Ibrahim (1971); (f) Andrews (1964); (g) Albrecht *et al.* (1996); (h) Burela & Martín (2007); (i) Guimarães (1981a); (j) Guimarães (1981b); (k) J. Bernatis (personal communication); and (l) Hanning (1979). Thick, thin and dashed bars indicate calcareous, gelatinous or unknown egg capsules, respectively. Copulation observations: USO, unsystematic; SO, systematic; L, in laboratory; F, in field.

genital pore, then the penis uncoils from the penis sac and extends along the penis sheath channel to emerge by a T-shaped sulcus before reaching the female genital pore (Gamarrá-Luques *et al.*, 2006). It has been assumed that the penis must reach a specific location in the complex oviduct of *P. canaliculata* before insemination can begin. The identity of this location is still disputed. Andrews (1964) proposed that the penis must reach the noncoiled portion of the shell gland, whereas Gamarrá-Luques (2006) argued it must reach the bursa copulatrix. The preparation of both genital systems and their complete connection could probably account for an important part of the presumed copulation duration. Three genera of apple snails produce eggs with calcareous shells (*Pomacea*, *Pomella* and *Pila*; Hayes *et al.*, 2009; Fig. 1). In *Pomacea*, in which the egg masses are aerial and cleidoic (Pizani, Estebenet & Martín, 2005), the oviduct attains the greatest complexity relative to the genera that lay gelatinous egg masses underwater, with a coiled portion or capsule gland related to the deposition of the calcareous shell around the egg capsule (Catalán *et al.*, 2002). Hence, a longer time for the penis to reach the place of sperm release would be expected in this genus and probably also in the others that lay calcareous eggs.

The biparental egg masses observed in *P. canaliculata* constitute strong evidence of sperm mixing in the female genital tract and, therefore, of sperm competition for fertilization of ova (Yusa, 2004, 2007). Therefore, an alternative explanation for the lengthy copulations is that males perform mate guarding to impede the access of other males, prolonging the genital contact more than the strictly necessary to fertilize all of the ova (Alcock, 1994). On the basis of the infrequent discovery of sperm in the genital duct of copulating males, Gamarrá-Luques *et al.* (2006) suggested that sperm transference occupies only a small part of the copulation duration. Mate guarding is a frequent and taxonomically widespread male strategy to increase the paternity rate when the risk of sperm competition is high (Stockley, 1997).

The hypothesis that each egg mass is preceded by a single copulation in *P. canaliculata* has been rejected by Albrecht *et al.* (1996), although the reproductive significance of each of the lengthy copulations is not clearly understood yet. Females of *P. canaliculata* maintained with a single male during all their lifetime produce from 1,316 to 10,869 eggs (mean 4,506) (Estebenet & Martín, 2002) and under such conditions they copulate more frequently (2.9 times/week) than they lay egg masses (1.4 times/week; Albrecht *et al.*, 1996), suggesting that a continuous replenishment of sperm is needed to avoid sperm depletion or to replace old sperm with decreased vigour or quality. Paradoxically, isolated females are able to store sperm in the receptaculum seminis for up to 140 days and to produce 3,000 viable eggs without further copulation (Estebenet & Martín, 2002).

Several proximate factors presumably affect copulation duration in *P. canaliculata*. For instance, females crawl above the waterline to lay their egg masses, usually during the night, a process that can last up to 5 h (Estebenet & Martín, 2002). Albrecht *et al.* (1996) reported that four out of nine females that were observed in copulation in the evening and during the next morning laid an egg mass in between, indicating that the copulation was interrupted for egg laying. Hence, the nocturnal egg laying behaviour could limit the duration of the copulations that began during the afternoon.

Another possible factor influencing copulation duration is male size. The testis begins to differentiate early in male development (at *c.* 10 mm; Gamarrá-Luques, 2006; Tamburi & Martín, 2009) but, notwithstanding, testis mass increases with body mass even among mature males (bigger than 30 mm; Albrecht, 1998). Big males are probably able to produce and transfer a higher amount of sperm than small ones and hence, assuming other factors to be equal, their copulations should last longer.

In the context of sperm competition, female mating status could modify the time invested in a given copulation by males (Stockley, 1997). Being the first in inseminating a virgin female

assures absolute paternity until the next copulation and under low mate encounter rates it would be very advantageous to inseminate the female with a quantity of sperm sufficient for its entire reproductive life (reproductive assurance strategy). However, when mating with a nonvirgin female, the sperm competition risk would require an even higher time investment to overcome the sperm of precedent males (Wedell, Gage & Parker, 2002). This strategy of 'sperm loading' (Dickinson, 1986; García-González & Gomendio, 2004) has been proposed as an explanation for the lengthy copulations of other invertebrates. In the presence of prowler males crawling around the mating couple and seeking to gain access to the female, the length of copulations is expected to increase under both the sperm-loading and mate-guarding hypotheses. A co-occurring effect could be the lack of copulatory experience in males, which are the sex that have the active role in copulation (Burela & Martín, 2009).

The aims of our study were to investigate if the lengthy copulations of *Pomacea canaliculata* occur naturally in the wild and if a significant part of the copulation duration is not devoted to sperm transference but to accomplish genital connection or to impede the access of other males. Additionally, we evaluated the significance in this promiscuous snail, in terms of lifetime reproductive output, of a single *vs* repeated copulations, and also the effect of some proximate factors that could explain the great variation of copulation duration in this species.

MATERIAL AND METHODS

The snail

Pomacea canaliculata is a freshwater snail whose natural range extends from the La Plata river basin southwards to the Encadenadas del Oeste basin in Southern Pampas, Argentina (Martín, Estebenet & Cazzaniga, 2001), but that has been dispersed worldwide by man and has been listed among the "100 of the world's worst invasive alien species" (Lowe *et al.*, 2000). Our study was centred on *P. canaliculata* populations from the Curamalal stream (37°14'31"S, 62°08'04"W), a short permanent watercourse located near the species' southern natural limit of distribution, and that has been the subject of previous studies (Martín & Estebenet, 2002; Estebenet & Martín, 2003). The maximum size in this area is around 80 mm and in most populations the females attain larger sizes than males (Estebenet, Martín & Burela, 2006); the degree of dimorphism in laboratory cohorts is quite variable since size at maturity is relatively constant in females and very plastic in males (Tamburi & Martín, 2009).

Copulation duration of P. canaliculata in the field

Copulation duration was recorded in April 2006 for naturally occurring mating couples in a ditch that runs along Provincial Route 85 (37°15.86'S, 062°06.38'W) and drains to the Curamalal Grande stream. A pond-like section of the ditch was selected due to its very low turbidity, narrow shape (43.5 m long and 2.3–4.3 m wide) and shallowness (maximum depth of 0.35 m), which greatly facilitated the continuous observation from the shore without disturbing the snails. The only aquatic macrophyte present was the pennywort *Ludwigia* sp., forming small and sparse mats.

The observation period lasted 26 h and a complete inspection from the shore was performed each hour from around the pond to detect mating activity; water temperature was recorded at the start of each inspection. Since it was not always possible to observe the penis sheath inserted in the mantle cavity of the female (Burela & Martín, 2009), a couple was

considered in copulation if the male was mounted and motionless on the right (sutural) side of the body whorl of the female's shell. The couples that were already engaged in copulation at the beginning of the observation period, and every new copulating couple detected, were marked with a numbered stake.

General rearing and experimental conditions

Unless otherwise stated the individuals used in the experiments were sexually mature snails (measuring >30 mm in shell length) obtained from laboratory stocks originated from egg masses collected at different times in the Curamalal Grande stream. They were maintained in a rearing room at 25°C in 20-l aquaria filled with CaCO₃-saturated water, under a 14 h light/day photoperiod and fed with lettuce *ad libitum*. The water of the aquaria was changed once a week and the shell length measured. As early as possible (at a shell length of almost 15 mm) snails were sexed according to the aspect of the gonad visible through the translucent shell (Takeda, 1999) and maintained separately thereafter.

The copulation duration trials were performed in 3-l glass aquaria with tap water at 25°C and without food. A single pair of randomly selected males and females was placed in each aquarium. Series of 10–20 couples were continuously observed and the start (intromission of the penis sheath into the pallial cavity) and the end (withdrawal of the penis sheath) of copulations were recorded (Burela & Martín, 2009). Most trials were started at 8.00 a.m. and performed under continuous artificial illumination, except for the time-of-day trials (see below). Couples that did not copulate after a fixed period of time (4–12 h from the moment of pairing to the beginning of copulation) were separated.

Duration of sperm transfer

Virgin females (shell length 35–40 mm) were randomly coupled with virgin males (shell length 30–35 mm), and randomly assigned to the seven different levels of copulation duration. To estimate the portion of the copulation during which sperm is transferred, the duration of the copulation was controlled by gently pulling apart the partners after periods of 1, 2, 4, 6, 8, 10 or 12 h from the start; the emergence of the penis through the T-shaped sulcus of the penis sheath was recorded. During the trials several copulations ended spontaneously and their duration was recorded. Once mated, the females from both spontaneous and controlled duration copulations were maintained isolated in 3-l aquaria under the same general rearing conditions and the egg masses recorded for each female until a period of 6 weeks without new ovipositions had passed. After each oviposition the couple was relocated to another aquarium and the egg mass was incubated at room temperature until hatching ceased, when the numbers of hatchlings, dead embryos and eggs without a developed embryo were recorded. The total number of eggs and egg masses per female, the duration of the oviposition period (weeks), the oviposition rate (egg masses per week) and the percentage of eggs with embryos were calculated.

Effect of single vs repeated copulations and male size on female reproductive output

To evaluate the relative reproductive significance of a single copulation with males of different size we maintained females during all their reproductive lifetime under two artificial mating regimes. Virgin females (36 females, shell length 41–49 mm) and virgin males of the same age (18 small,

31–36 mm and 18 big, 40–47 mm) were randomly paired in 3-l glass aquaria with tap water and were observed at hourly intervals up to the completion of the first copulation (most of them completed the copulation within the first 2 days of the experiment).

Half of the females in each male size group were maintained with the same male throughout the experiment to allow repeated copulations. To obtain a group of once-mated females, without the confounding effect of leaving the female alone in the aquarium (Estebenet & Martín, 2002), the males in the other half of each size group were replaced immediately after the first copulation by a second male whose penis sheath had been surgically severed (at the level of the mantle edge, 2 weeks previously). Thereafter the couples were maintained in the 3-l aquaria under the same general rearing conditions and the egg masses recorded daily until 6 weeks had passed without new ovipositions; copulations were recorded three times per day (8.00, 12.00 and 20.00), 5 days a week during the first month. After each egg mass was laid, the couple was relocated to another aquarium and the egg mass was incubated until hatching ended. The reproductive variables were recorded and calculated as in the previous experiment.

Effect of proximate factors on the duration of copulation

Male size. The females used in this experiment were collected in the Curamalal Grande stream (shell length 40–50 mm). The males were randomly chosen from two size categories: small and big (shell lengths 31–34 and 40–43 mm, respectively); all of them had shared aquaria with females to allow them to obtain some copulatory experience. The period of time permitted to begin copulation was 12 h.

Time of day. Virgin females (shell length <34 mm) were randomly coupled with males that had mating experience in the lab (shell length 34–45 mm); the pairs were randomly assigned to three different start times: 8.00, 12.00 and 16.00; daylight hours lasted from 7.00–8.00 to 18.00–19.00 during these trials. The period of time allowed for the beginning of copulation was 4 h. The same experiment was repeated with females from the same laboratory stock, but which had copulated at least once in other experiments.

Mating status of females and males. In a first series of trials virgin females (shell length 33–40 mm) were paired with virgin males (shell length 30–35 mm) during periods of 6 h until the beginning of the copulation. After 48 h the same procedure was repeated with a new virgin male for each of these once-mated females until a second copulation was achieved. In a second series, virgin males (shell length 35–38 mm) were paired with presumably nonvirgin females collected in the field (shell length 35–40 mm); for each male the same procedure as in the first series was used to obtain the first and second copulations with field-collected females.

Density of prowler males. Laboratory females (33–41 mm) and males (32–38 mm), both with proven copulatory experience, were used. One female and three males were randomly selected and put together in an aquarium and they had a period of time of 6 h to begin copulation. After one male initiated copulation, one or two of the remaining males were carefully removed from the aquarium in order to obtain the density levels of 1 and 0 prowler males, respectively; for the two-prowler males treatment, one of the noncopulating males was retrieved and immediately returned to the aquarium, to ensure that all treatments received the same disturbance.

Statistical analyses

Data were analysed by nonparametric tests. One-way Kruskal–Wallis tests were performed to test for the effect of male size, time of day and male density on copulation duration, and also to test for the effect of the controlled copulation

duration on number of eggs and egg masses per female, duration of the oviposition period, oviposition rate and percentage of eggs with embryos. Wilcoxon's tests for paired samples were used for the duration of the first and second copulations of males and females. Two-way Kruskal–Wallis tests for equal group sizes (Zar, 1984) were used for the reproductive output variables in the experiment of single *vs* repeated copulations with males of different size.

RESULTS

Copulation duration in the field

From a total of 47 copulatory events observed during the observation period in the field, 31 were observed for their full duration (complete) and the others were either already initiated or were not finished at the end of the observation period (incomplete). The start and ending of copulations showed no clear pattern during the observation period (Fig. 2). The number of couples engaged in copulation increased after dusk, but showed no clear relation with water temperature or with sunlight. The duration of complete copulations showed a bimodal frequency distribution (Fig. 3), with a group of short copulations (<6 h) and a smaller group (25.8%) of lengthy copulations (between 8 and 19 h); the incomplete copulations showed a similar frequency distribution.

Controlled duration of copulation

Out of the 31 copulations in this experiment, 12 ended spontaneously before the completion of the duration assigned *a priori*. The distribution of these uncontrolled durations was clearly bimodal, with a group of short (mean = 1.31 h, range = 0.77–2.52 h, $n = 7$) and a group of lengthy copulations (mean = 9.96 h, range = 7.23–11.50 h, $n = 5$). None of the females that experienced short spontaneous copulations deposited egg masses.

The emergence of the penis through the T-shaped sulcus of the sheath was observed in all but two of the interrupted copulations (one in the 1-h and one in the 10-h level). Neither of the two females which had engaged in the 1-h copulations deposited egg masses, but only one out of three engaged in the 2-h ones failed to do so. The total number of eggs and egg masses per female showed significant differences among controlled levels of copulation duration ($X^2_5 = 12.874$, $P = 0.025$ and $X^2_5 = 11.336$, $P = 0.045$, respectively). The duration of the oviposition period, the laying rate of egg masses and the percentage of eggs with embryos were not different among duration levels in which at least one female laid egg masses ($X^2_4 = 6.155$, $P = 0.188$, $X^2_4 = 4.497$, $P = 0.343$ and $X^2_4 = 3.674$, $P = 0.452$, respectively). The grand mean value for the oviposition rate was 5.14 weeks (± 4.25), 1.82 week⁻¹ (± 1.12) for the egg mass laying rate and 56.19 % (± 27.35) for the percentage of eggs with embryos. No differences were detected between lengthy spontaneous copulations and the 10-h level of controlled copulations in any of the above mentioned variables ($X^2_1 < 0.747$ and $P > 0.387$ in all cases).

The curves of accumulated number of eggs per female *vs* days since copulation showed large differences between treatments where different durations of copulation were permitted (Fig. 4). At first, the oviposition rate was constant and quite similar for the different treatments, but it decreased earlier and more noticeably in those with shorter copulation duration; the temporal oviposition pattern in females from lengthy spontaneous copulations was similar to that of the 10-h females. For the whole set of spontaneous and controlled copulations the duration was positively correlated with the total number of

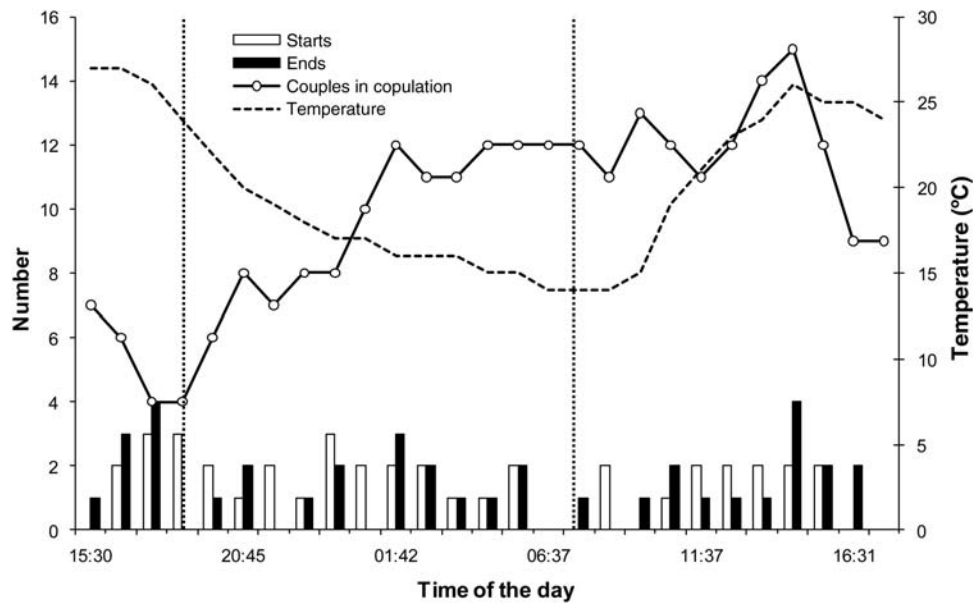


Figure 2. Number of copulating pairs and copulations starting and ending during field observations. Hours between dotted lines correspond to night.

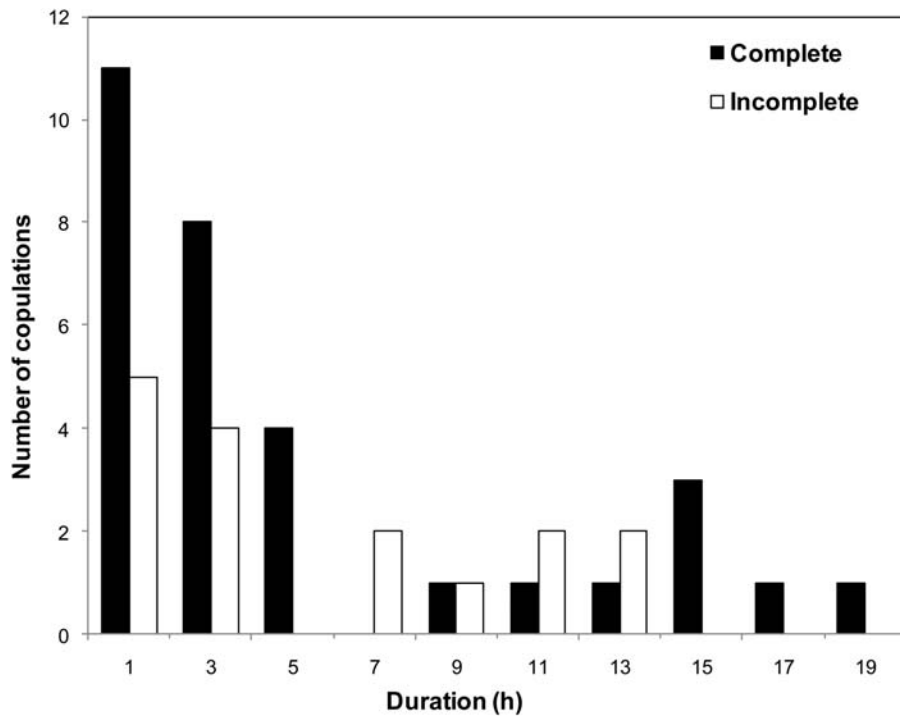


Figure 3. Frequency of durations for complete and incomplete field copulations.

eggs later laid by the female (Spearman's rank correlation: $r_s = 0.887$, $P < 0.0001$; Fig. 5) and egg masses ($r_s = 0.825$, $P < 0.0001$).

Single vs repeated copulations with males of different size

The oviposition period lasted 56.53 ± 26.26 days (mean \pm SD); the experiment was interrupted after 190 days at which time only 25% of the females were alive. Up to 10 copulations were recorded during the first month of the experiment

for females paired with the males with intact penis sheaths. The males without a penis sheath were often seen trying to mount a female, but at the end of the experiment it was confirmed that the penis sheath had not regenerated in any of them. No significant effect of male size or the frequency of repetition of copulations was detected on any of the reproductive variables (Table 1); the grand mean values for the total number of eggs and egg masses were $2,412.08 (\pm 994.87)$ and $13.11 (\pm 5.90)$, respectively, 8.07 weeks (± 3.75) for the oviposition period, $1.67 \text{ week}^{-1} (\pm 0.38)$ for egg-mass laying rate

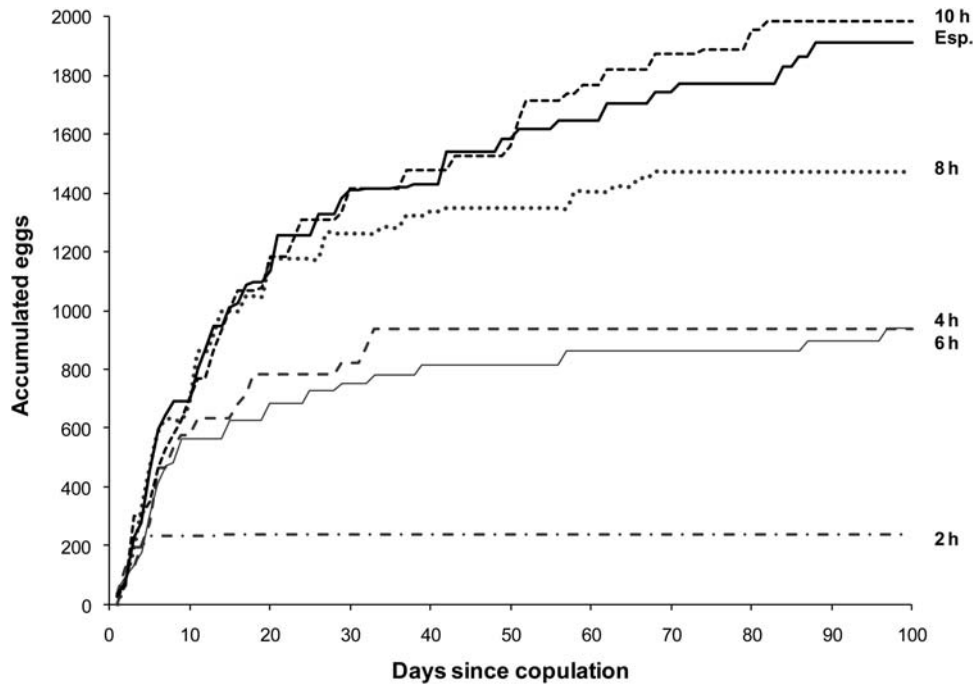


Figure 4. Number of accumulated eggs under the different treatments of controlled copulation durations.

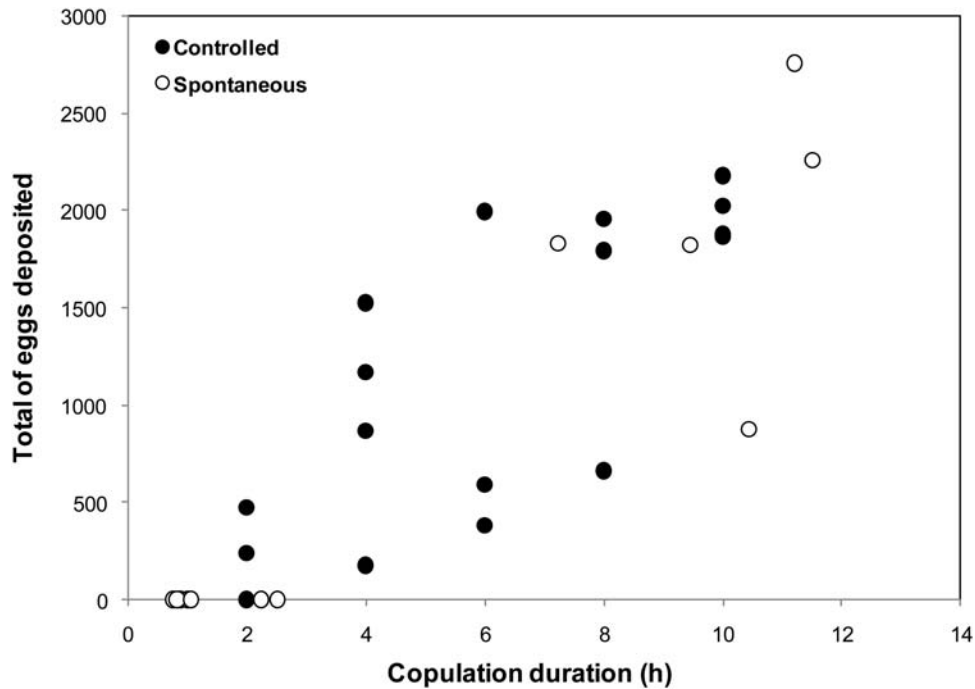


Figure 5. Total eggs deposited by each female in controlled and spontaneous copulations.

and 61.18% (± 21.21) for the percentage of eggs with embryos.

The number of accumulated eggs per female increased in a similar way among treatments (Fig. 6). During the first month the oviposition rate was high and quite constant but decreased steadily thereafter; the greater output in the 'big males-repeated copulations' treatment was mainly due to three females that survived longer than the others.

Effects of proximate factors on copulation duration

No significant effect of the proximal factors tested (male size, mating status, time of the day and density of prowler males) was found on the copulation duration ($P > 0.190$ in all cases; Table 2; Fig. 7). The duration of copulation in these five experiments ranged between 2.58 and 24.00 h, with a grand mean of 12.15 ± 3.97 .

DISCUSSION

Lengthy copulations of *Pomacea canaliculata* occur both in the field and under laboratory conditions. Sperm transfer makes up 80% of the copulation duration, suggesting that the contributions of mate guarding and of the process of genital connection are not important, and that most of the long duration can be explained by the high fecundity and sperm-storing capacities of the females. Our results showed that copulations in the field can last up to 19 h, although a high proportion of couples displayed mating behaviours that lasted <6 h. There was no clear relationship between copulation frequency and water temperature, or with daylight. The total number of eggs and egg masses produced during the reproductive life of a female that copulated only once was positively correlated with the duration of the copulation. Females permitted to copulate more than once did not show a higher lifetime reproductive output or success than the once-mated ones and in both cases there was no effect of male size. The size of the male partner, the time of the day at which the copulation began, the mating

Table 1. Two-way Kruskal–Wallis tests [$X^2_{1,3}$ (*P*-value)] for the reproductive variables in the experiment of single *vs* repeated copulations with males of different size.

Variables	Size	Copulations	Size × copulations
Total number of eggs	0.7848 (0.3758)	0.0641 (0.8002)	0.1692 (0.6808)
Total number of egg masses	0.1692 (0.6809)	0.6419 (0.2001)	0.0903 (0.7637)
Oviposition period	0.0563 (0.8124)	1.2615 (0.2614)	0.0250 (0.8743)
Egg mass laying rate	0.3614 (0.5478)	0.0090 (0.9244)	0.0360 (0.8494)
% embrionated eggs	0.0640 (0.8002)	0.0040 (0.9495)	0.4414 (0.5064)

status of snails (virgin or mated) and the number of prowler males in the aquarium showed no effect on the duration of copulation.

Our field results indicate that the lengthy copulation durations (4–20 h) previously reported in laboratory observations of *Pomacea canaliculata* (Andrews, 1964; Berthold, 1991; Albrecht *et al.*, 1996; Burela & Martín, 2007) were not entirely an artefact. However, only 25.8% of the field copulations lasted >8 h and the rest lasted <6 h. As it was not always possible to observe the penis sheath inserted in the mantle cavity of the female, probably many of these short copulations were in fact phases of the mating behaviour occurring before the start of insemination (i.e. courtship and the first part of copulation up to insemination posture; Burela & Martín, 2009). In the laboratory at 25°C these two phases can last at least 2.0 h (± 1.1) and 20 min, respectively. However, in the field, with lower water temperatures during most of the observation period (53.8% of the time below 20°C) it is possible that they could last longer. This group of short copulations observed in the field may be equivalent to those copulations of short duration (up to 2.52 h) that ended spontaneously without insemination (no egg laying was recorded) in the controlled-duration experiment.

In our field study there was no clear daily pattern of copulatory activity. In laboratory observations under 14 light/day and 23–27°C, Albrecht *et al.* (1996) recorded a higher frequency of copulations during the morning (07.00–08.00, 45%) than in the afternoon (13.30–14.30, 37%) or evening (20.00–21.00, 18%), but copulatory activity was not monitored during the night. Also under laboratory conditions (12 h light/day, 26°C), Heiler *et al.* (2008) recorded a higher level of general activity during the night, especially at the beginning of the dark period, suggesting that this could be an innate behaviour to reduce the risk of predation by visual predators. Wada & Yoshida (2000) also showed that a higher proportion of *P. canaliculata* snails were not buried in the soil of inundated paddy fields during night hours. Guimarães (1981b) reported

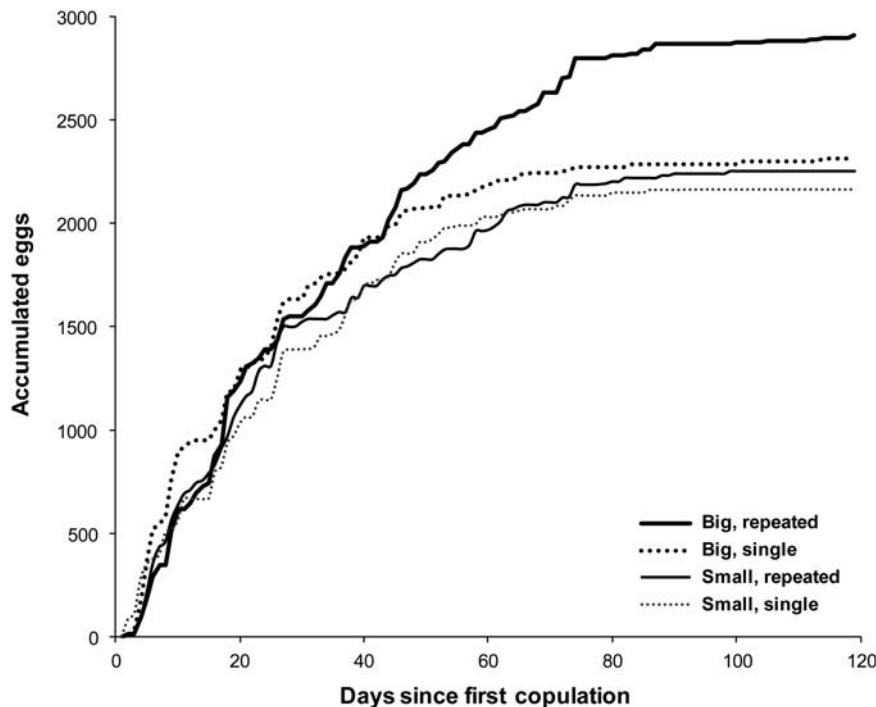


Figure 6. Number of accumulated eggs of females from each treatment in the experiment of single *vs* repeated copulations with males of different sizes.

for *Pomacea haustum* a higher frequency of copulations in the middle of the night in a tropical lake. In our field observations, the increase in the number of snails engaged in copulation during the night could be due to a prolongation of copulation duration because of the fall of temperature down to 14°C after sunset; an alternative explanation is that it may be related to the avoidance of specialized malacophagous birds that we have observed feeding in the area, like the snail kite, a visual and diurnal predator (Tanaka, Souza & Módena, 2006).

The hypothesis that the lengthy copulations are due to a lengthy process of genital connection that presumably must be completed before sperm transfer can start should be rejected, since sperm transference begins only 2 h after penis-sheath intromission (i.e. 20% of the mean copulation duration). Further evidence against it comes from the fact that in *Pila*, a genus with large calcareous-shelled eggs, copulations are short (1–3 h, Fig. 1) as also occurs in some species of *Pomacea* (1–3 h in *P. haustum*). Nevertheless, the genital connection in

P. canaliculata is almost as lengthy as the whole copulation of other apple snails such as *Lanistes ovum* and *Asolene* species.

In the sperm transfer experiment the lifetime reproductive output of the once-mated females was positively correlated with the duration of copulation, suggesting steady sperm transfer after the first 2 h and up to 10 h of the duration of copulation. On the other hand, the amount of sperm transferred was not different between the 10-h copulations and the copulations that ended spontaneously (9.96 h on average). This evidence indicates that males do not devote a significant part of the copulation duration solely to impeding the access of prowler males. If a preinsemination guarding exists (*sensu* Dickinson, 1986), it would represent <20% of total copulation time (the 2-h period during which no transfer of fertile sperm occurs). Postinsemination guarding was not observed in that experiment, although it would be more likely to observe it when other males are present (Alcock, 1994). However, the presence of the male mounted on the female shell after the copulation ended (postcopulation guarding; Dickinson, 1986) is infrequent and short in duration (<1 h), both in isolated couples (Burela & Martín, 2009) and in the presence of prowlers (this study). The lack of response in the duration of copulation to the presence of prowler males can be explained if only the rate of sperm transference is affected (García-González & Gomendio, 2004).

The fact that the females that copulated for <2 h did not lay eggs indicates that this period does not involve the transfer of fertile sperm, but it does not preclude that of seminal fluid with nonfertile material. In addition to fertile (eupyrene) sperm the testis of *P. canaliculata* produces two different types of nonfertile sperm or parasperm: apyrene and oligopyrene sperm (Winik, Catalán & Schlick, 2001; Winik *et al.*, 2009). At least the apyrene sperm reaches the bursa copulatrix, where it can be found mixed with eupyrene sperm (Andrews, 1964), neither of which apparently reach the receptaculum seminis (Winik

Table 2. Kruskal–Wallis tests for the proximate factors that could affect copulation duration.

	df	X ² value	P-value
Male size	1	1.410	0.235
Time of day			
Virgin females	2	0.622	0.733
Mated females	2	3.289	0.193
Mating status			
Females	1	1.427	0.232
Males	1	1.600	0.206
Prowler males	2	0.210	0.350

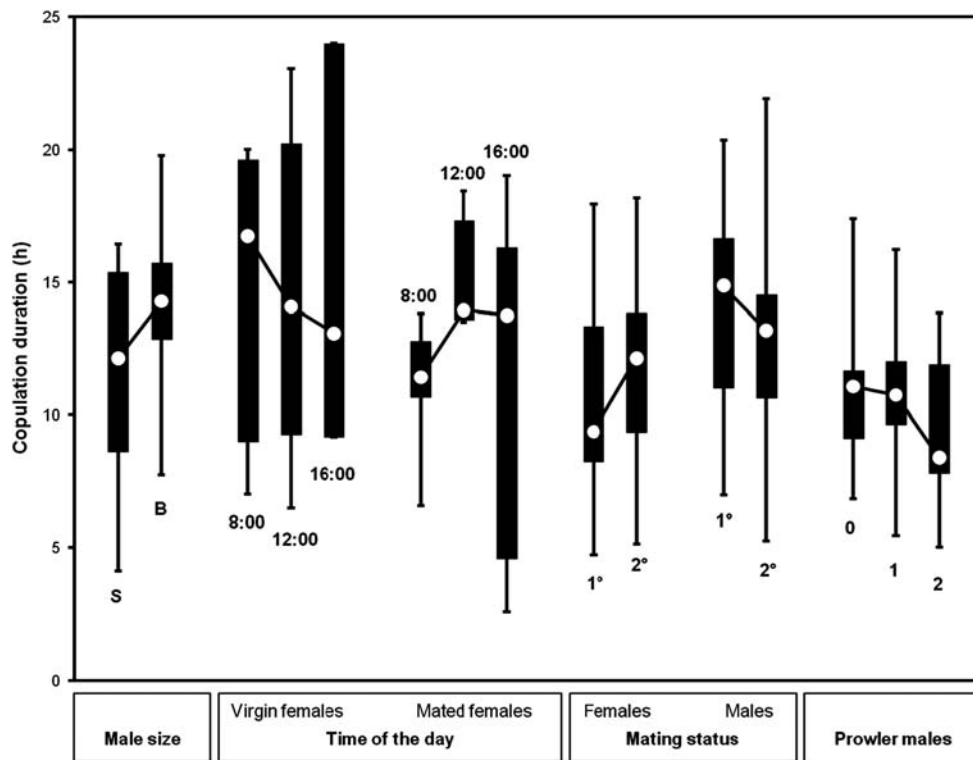


Figure 7. Copulation durations in the experiments to test the effects of proximate factors (circle, median; box, interquartile range; bar, minimum and maximum values). Male size: S, small; B, big. Time of day: copulations beginning at 8.00, 12.00 and 16.00. Mating status: 1°, first copulation; 2°, second copulation. Prowler males: 0, 1 or 2 prowler males with the mating pair.

et al., 2009). If the parasperm is transferred only during the first part of the copulation then its function would be to act as an antagonist of the sperm of previous mates, as suggested for other freshwater snails (Baur, 1998). Yusa (2004, 2007) showed that in *P. canaliculata* the sperm of the final mate replaces that of previous mates and the parasperm could play a role in this form of sperm competition (Winik *et al.*, 2009).

The reproductive output of females of 10-h level copulation duration was similar to that of females whose copulations ended spontaneously before the completion of the assigned duration (mean duration 9.96 h). From our results it seems feasible that at least the first 8 h of the first copulation of a virgin female are necessary for the transfer of sufficient sperm to ensure the fertilization of all the ova produced during its entire lifetime, without a new insemination. If so, an important part of the long copulation duration could be explained by the high fecundity of *P. canaliculata* females (average 4,500 eggs, range 1,316–10,869; Estebenet & Martín, 2002; Martín & Estebenet, 2002). The capacity of a female *P. canaliculata* to lay thousands of eggs after a single copulation has been considered a key factor in their ability to establish new populations (Keller, Drake & Lodge, 2007; Jerde, Bampfyld & Lewis, 2009). Our results indicate that the total amount of eggs laid did not differ between treatments in which one copulation was allowed and those in which several copulations were allowed, supporting the idea that the amount of sperm transferred in a single copulation is enough to fertilize all the ova a female can produce.

Given the steady transfer of sperm during copulation, it would be reasonable to expect a longer duration in bigger snails, although this was not observed in our experiment. Perhaps the female limits the duration of the sperm transfer, either by a physical limitation of the bursa copulatrix or a decision as to how much time to invest with each mating partner ('quantitative' female choice). However, as judged by their duration, the amount of sperm received in the first copulation of a virgin female does not limit the amount received in the second a few days later (see below). Perhaps the eupyrene sperm is soon transferred to, and stored in, the multiple pouches of the receptaculum seminis (Andrews, 1964). Male size did not affect the reproductive output of females fed *ad libitum* (single *vs* repeated copulations experiment), probably due to the fact that even a small male is able to transfer, in a single copulation, an amount of sperm which is more than sufficient to fertilize all the ova a big and well-nourished female can lay during its reproductive lifetime. Coincidentally, Tamburi & Martín (2009) reported that male size has no effect on the total number of eggs laid by a female and their viability.

In the time-of-day experiment there was no restriction in the duration of the copulations that began during the afternoon due to the nocturnal egg-laying behaviour of *P. canaliculata*. For virgin females a limiting effect of the time of day would only appear if the egg laying occurred during the same day of the copulation. However, only 56% of the virgin females laid egg masses in the same day of their initial copulation (18 females from the experiment of single *vs* repeated copulations and 7 from the experiment of sperm transfer); Albrecht *et al.* (1996) also reported that females do not always lay an egg mass within a few hours after copulation.

Males do not invest less time in sperm transfer with virgin females than with previously mated ones (mating status experiment), as expected in the sperm-competition risk models (Wedell *et al.*, 2002). A feasible explanation could be that the ending of copulation is not under direct male control; the male withdraws the penis sheath and dismounts from the female shell without any apparent reason, though a closure of the female genital pore cannot be ruled out (Burela & Martín, 2009).

Our experiments indicate that the amount of sperm transferred in one uninterrupted copulation is enough to fertilize all

the ova a female can produce in its lifetime. This raises the question of the advantage of multiple matings for the female in this species, for which copulations can be more frequent than oviposition episodes (Albrecht *et al.*, 1996). Females' energetic input is probably not seriously limited by the total time devoted to multiple matings since they can crawl and forage freely during copulation (Albrecht *et al.*, 1996; Burela & Martín, 2007). Possibly the female's interest is to obtain a higher genetic diversity (Jennions & Petrie, 2000) or at least to insure against the possibility of being inseminated uniquely by a sterile, defective or incompatible male (Birkhead & Pizarri, 2002). Oppliger *et al.* (2003) provided evidence of nongenetic or direct benefits of multiple copulations in the freshwater caenogastropod *Viviparus ater*.

Although it appears to be in the interest of females to copulate with different males, it would not be advantageous for them to spend a long time with each partner; on the other hand, it would be in the males' interest to stay a long time transferring sperm to each female, so as to prevail over the sperm of rival males (Wedell *et al.*, 2002). Thus, a sexual conflict arises due to these opposite interests. It has been suggested that the nuptial feeding behaviour (or oral nuptial gift) in *P. canaliculata* is a male strategy to entice the female to remain in copulation for longer than the female's optimum duration (Burela & Martín, 2007), a functional explanation well supported for nuptial gifts in insects (Vahed, 1998, 2007).

The evidence obtained in this study indicates that lengthy copulations are not entirely a laboratory artefact and that they cannot be explained by the complexity of the genital systems or by the occurrence of mate-guarding behaviour. A significant part of the duration could be explained by the high fecundity of females of *P. canaliculata* and by the advantages (to both female and male) of assuring a quantity of sperm sufficient for the rest of the female's reproductive life. However, the great variability in the duration of copulation remains largely unexplained. Possibly the duration of the copulation reflects a conflict of sexual interests that can vary both in magnitude and sign during lifetime (Chapman, 2006); for instance, the female's interest in genetic diversity is expected to decrease as successive matings are achieved and the male's interest to transfer a large amount of sperm could be related to the residual reproductive value of the female. The multiple pouches of the receptaculum seminis suggest that a cryptic female choice may be operating, as has been suggested in other snails with promiscuous mating systems (e.g. Haase & Baur, 1995).

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