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# Strategic adjustment of copulatory plug size in a nematode

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## Abstract

Copulatory plugs (CP) are substances produced during copulation that block the genital openings of the female. In several species of Nematoda, males produce CP that are thought to impede female remating and thus sperm competition. The relatively large size of the CP in several nematodes, and its evolutionary loss in self-fertilizing populations of *Caenorhabditis elegans*, suggests that CP are costly to produce. If CP production is costly, the application of basic concepts of strategic ejaculation theory suggests a modulated allocation of CP in response to sperm competition risk. This hypothesis led us to predict that males perceiving a higher risk of sperm competition will produce larger CP. We tested these ideas with the entomopathogenic, gonochoristic nematode *Rhabditis regina*. Our first experiment provides evidence suggesting that production of CP is costly, because the size of CP is negatively affected by stressful conditions (high population density, small male adult size, and suboptimal food type). The results of our second experiment support the prediction that males adjust the size of CP to sperm competition risk: the average size of CP increased as the number of males competing for one female increased. Overall, our study supports the idea that in *R. regina* the production of CP is costly for males and that the size of the CP produced is influenced by sperm competition risk.

**Key words**: copulatory plug, sexual selection, sperm competition, strategic ejaculation, nematodes

During copulation, males of many species produce substances that block the genital opening(s) of the female, known as copulatory plugs (CP hereafter). These substances are produced by species from several unrelated taxa (e.g., Mattoni and Peretti 2004 on scorpions; Dunham and Rudolf 2009 on primates; Uhl et al. 2010 on spiders; Friesen et al. 2013 on snakes; Cutter 2015 on *Caenorhabditis* species; Carvalho et al. 2017 on butterflies), suggesting that they have evolved independently several times. Most authors consider that sperm competition is the selective pressure responsible for the evolution of CP (Parker 1970; Simmons 2001; Palopoli et al. 2015), which could act as physical barriers to further mating or decrease the attractiveness or sexual receptivity of the females (Timmermeyer et al. 2010). However, there are alternative functional explanations that have been less studied (see below).

In nematodes, the subject of this study, CP are present in several species. Although some experimental studies show that CP prevent insemination by other males (Barker 1994; Palopoli et al. 2008; Lan et al. 2017), other studies have failed to demonstrate this function (Hodgkin and Doniach 1997; Timmermeyer et al. 2010; Dong et al. 2014). This discrepancy could be explained by the ability of males to remove the CP of previous males (Barker 1994; García et al. 2007), since this behavior has not been observed in all cases (Lan

**Table 1.** Hypotheses on male fitness benefits provided by CP in nematodes

| Fitness benefit for the males producing the CP | Empirical evidence |
|---|---|
| H1. The CP impede insemination by subsequent males | *Caenorhabditis elegans*: (+) Barker (1994), (+) Palopoli et al. (2008), (–) Hodgkin and Doniach (1997)<br>*C. remanei*: (+) Palopoli et al. (2008), (–) Timmermeyer *et al.* (2010) |
| H1.1 The CP is a physical barrier against further copulations | — |
| H1.2 The CP contains "anti-aphrodisiacs" that render the female unattractive to (or repel) subsequent males | *Strelkovimermis spiculatus*: (+) Lan et al. (2017) |
| H1.3 The CP contains substances that inhibit or reduce female sexual receptivity | — |
| H2. The CP permits or improves the attachment of the mating pair during copulation | *Pelodera strongyloides*: (+) Wagner and Seitz (1983) |
| H3. The CP helps increase sperm transfer | *C. elegans:* (–) Hodgkin and Doniach (1997) |
| H4. The CP prevents or reduces, either passive or female-induced sperm loss | *C. elegans:* (–) Hodgkin and Doniach (1997)<br>*C. remanei*: (–) Timmermeyer *et al.* (2010)<br>*Strelkovimermis spiculatus*: (+) Lan et al. (2017) |
| H5. The CP is a physical barrier against the entry of pathogens | — |
| H6. The CP contains substances that protect against infections or provide nutrients for the female | *Pelodera strongyloides*: (+) Wagner and Seitz (1983)<br>*Strelkovimermis spiculatus*: (–) Lan et al. (2017) |
| H7. The CP contains hormone-like substances that stimulate ovulation and oviposition | *C. remanei*: (+) Timmermeyer *et al.* (2010), (+) Leighton and Sternberg (2016) |
| H8. The CP provides mechanical stimulation that increases ovulation and oviposition | — |

*Note*: Females could benefit or not from receiving CP.
References with evidence in favor (+) or against (–) the proposed benefits are included.

et al. 2017). In fact, there are studies with opposite results even within the same species (Table 1). Thus, alternative functions for the CP have been proposed (Timmermeyer et al. 2010; Decraemer 2012) and at least some of them have empirical support (Table 1). The hypotheses list in Table 1 is not exhaustive and in particular cases other functions are possible. For example, in species of the genus *Trichodorus*, CP have a "central canal of variable diameter" which led Decraemer (2012) to suggest that the CP "might function as a kind of copulatory tube facilitating copulation."

The evolution of all the potential functions described in Table 1 could result from some type of sexual selection, although in some cases other kinds of selection pressures, alone or in combination with sexual selection could be important. For example, some hypotheses propose that reduction of sperm competition (intrasexual selection) is responsible for the evolution of CP by preventing female remating (H1) or displacement by other males during copulation (H2), or by increasing the amount of inseminated sperm (H3) or stimulating egg production while the sperm of the male producing the CP is predominant (H7 and H8). On the contrary, natural selection alone could explain the evolution of CP that prevent the entry of pathogens (H5). Furthermore, the hypotheses proposing that CP are "nuptial gifts" (H6 and H7) probably evolved by female choice, although in the case of H6 the importance of natural selection via natural enemies is evident. On the contrary, in the case of H4, although natural selection for prevention of sperm loss could be the main responsible for the evolution of CP, sexual conflict could be involved if females actively discard sperm as a way of post-copula mate choice ("sperm-dumping"). It is important to notice that the functional hypotheses in Table 1 only consider the fitness benefits for male nematodes producing CP. The fitness consequences for the females receiving CP could coincide with those of the male (e.g. if the CP are nuptial gifts) or be negative (e.g., if females obtain benefits from mating with multiple males and CP prevent remating). However, the female interests are beyond the scope of the research reported here.

Although we were not able to find studies measuring the fitness costs paid by male nematodes due to the production of CP (in fact, this type of study is rare in most animals producing CP; e.g., see Uhl et al. 2010 on a spider and Friesen et al. 2013 on a garter snake), it seems likely that these are substantial as judged by its relatively large size in several species (Sarr et al. 1987; Barker 1994; Hodgkin and Doniach 1997; Palopoli et al. 2008; Timmermeyer et al. 2010; Decraemer 2012; Dong et al. 2014; Lan et al. 2017; present study) and by its evolutionary loss in populations of *Caenorhabditis elegans* that evolved self-fertilizing hermaphroditism and, thus, experience reduced sperm competition (Palopoli et al. 2008). Considering these potential costs, if sperm competition is the main selective pressure responsible for the evolution of CP (Table 1: H1 and some versions of H2, H3, H7, and H8) and if the optimal size of CP is directly proportional to the risk of sperm competition, we expect that males perceiving a higher risk of sperm competition will produce larger CP. This hypothesis is an application of basic principles of strategic ejaculation theory (Wedell et al. 2002; DelBarco-Trillo 2011; Kelly and Jennions 2011) to the production of CP (Ramm and Stockley 2007).

In this article, we first looked for evidence of costs in the production of CP by manipulating 2 factors that could affect adult quality in the entomopathogenic, gonochoristic nematode *Rhabditis regina* Schulte and Poinar (1991): adult density, that negatively affects adult size (Canales-Lazcano et al., unpublished experiments), and diet, which could affect other aspects of individual quality. We reasoned that if production of CP is costly, smaller (i.e., grown at high density [HD]) and lower quality (i.e., fed with low-quality diet) males will produce smaller CP. Then, in a second experiment, we tested the hypothesis that sperm competition risk affects the size of CP by experimentally manipulating the social environment of *R. regina* in a way that simulated different degrees of sperm competition risk. Specifically, we tested if the CP produced by males are larger when they copulate in presence of competitor males than when copulating in their absence.

## Materials and Methods

### Species studied, laboratory culture, and general experimental procedures

*Rhabditis regina* is a gonochoristic (i.e., only males and females exist) entomopathogenic nematode. According to Schulte and Poinar (1991), the larvae mature sexually in 24 h and adults live about 4 days. As in most nematode species (von Lieven et al. 2005), males have a pair of copulatory organs called spicules, that are inserted into the vulva previous to sperm transfer (von Lieven et al. 2005; García et al. 2007). Males deposit CP that block completely the genital opening and invade part of the uterus after insemination (Figure 1), and then immediately after they separate from the females. When females give birth (to L1 larvae) the CP are lost and they can mate again (J. Canales Lazcano, personal observation). There is male competition for mates as judged from the common finding of multiple males with their tails coiled around the same female, even if she is already copulating or plugged (Canales Lazcano J, personal observation). Despite detailed observations of several mating couples approached by males, which even coiled their tails around the female besides the copulating male, no cases of displacement of the mating male have been observed (Canales Lazcano J, personal observation). As mentioned above, males attempt to mate with plugged females but they are unable to curl their tails around the area were the vulva is located, probably because of the presence of CP (Canales Lazcano J, personal observation). For the last reason, we consider unlikely that males deposit CP on top of CP produced by previous males.

The nematodes used in our experiments came from a culture founded with wild nematodes collected parasitizing third instar larvae of *Phyllophaga* spp. beetles in corn fields in the town of Jerécuaro, state of Guanajuato (Mexico), on 2 different dates (August 2012 and August 2014; Jiménez-Cortés et al. 2016). The laboratory colony is divided in 2 groups, one fed with pupae of *Tenebrio molitor* beetles and the other with raw beef. The *T. molitor* diet is considered a diet of higher quality than that of raw beef since *R. regina* is an entomopathogen. The nematodes are maintained in 500 mL plastic containers with food (either *T. molitor* pupae or raw beef) and 2 mm of 2% agar. The containers are kept at room temperature (25 ± 2°C) with 75% humidity and reseeded every week. The nematodes are suspended in the water of each medium with a Pasteur pipette. The same pipette is used to move the nematodes immersed in the medium to conical tubes to wash them 3 times with sterile distilled water. Once free of organic material, they are deposited again in the culture medium. Once a container with fresh culture medium is "seeded" with nematodes, they start to feed and reproduce and thus the number of individuals' increases with time, rapidly in the first days, until reaching a maximum density that depends on the amount of culture medium.

### Looking for costs in the production of CP

We looked for evidence of costs in the production of CP by manipulating the density of nematodes (that has a negative effect on adult size; Canales Lazcano J et al., unpublished experiments) and diet. We hypothesized that if CP are costly to produce, smaller (i.e., grown at HD) and lower quality (i.e., fed with low-quality diet) males will produce smaller CP. In this experiment, we used 150 mm diameter and 20 mm height Petri dishes with 30 mL of 2% agar and either 5 mg of raw beef or 20 pupae of *T. molitor* (whose total weight is also about 5 mg). Each of these dishes was seeded with 3 mL of adult nematodes suspended in distilled water and coming

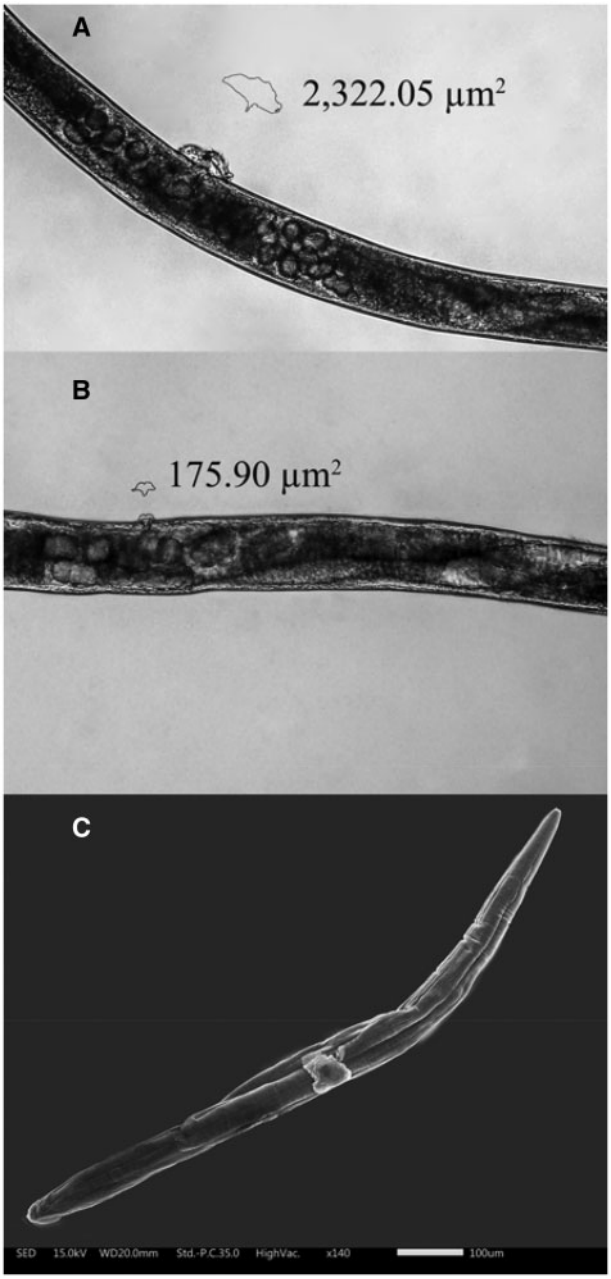


**Figure 1.** CP deposited in the female during copulation by males of *Rhabditis regina* and its measurement. (**A**) Mated female with a relatively large CP. (**B**) Mated female with a relatively small CP. In A and B, the contour of the CP whose area was used as a proxy of its size is shown slightly displaced from the CP. (**C**) Scanning Electron Microscope (model JSM-IT300) photography of a female with a relatively large CP, the longitudinal folding in the middle part of the female body is an artifact of sample preparation (microphotography courtesy of Drs Orlando Hernández and Jorge Contreras).

either from the raw beef or the *T. molitor* pupae culture containers. Although we did not measured if the amount of nematodes were the same in the 3 mL of solution coming from the raw beef or from *T. molitor* pupae, we think it was similar because of the way in which the 3 mL of nematodes seeded were obtained: The collection of nematodes from the culture was made with distilled water injected with disposable 3 mL Pasteur pipettes in the culture containers. The pressure of the water helped to suspend most

nematodes from the medium, which were then captured by retrieving the water with the same pipette. The nematodes from several culture containers (seeded at different times but with the same type of food, i.e., raw beef or *T. molitor* pupae) were mixed in a 50 mL glass tube and concentrated by decantation of the excess water until a "saturated" nematode suspension reaching the 40 mL mark of the glass tube was reached. From these tubes, we took the 3 mL of nematodes for seeding each Petri dish. As mentioned above, the number of nematodes increases rapidly after seeding and pilot observations indicate that after 8 days they reach their maximum density. So, we tested the effect of nematode density by comparing CP size in plugged females collected 4 ("low" density, LD) or 8 days ("high" density, HD) after seeding the Petri dishes. A limitation of our experimental design is that if mating rates are higher in the HD treatment (which we do not know) and CP are smaller in this treatment, we will not be able to distinguish if this result is due to the smaller size of males or to their higher mating rate or to both (see "Discussion" section). However, possible differences between diets within density treatments would not have this problem.

The effect of adult density and type of diet on the size of CP was measured by comparing the size of the plug produced under 4 different conditions: 1) low density (LD) and *T. molitor* diet (i.e., low level of food competition and good diet), 2) a low-density population and raw beef diet (i.e., low level of food competition and poor diet), 3) high density (HD) and *T. molitor* diet (i.e., high level of food competition and good diet), and 4) HD and raw beef diet (i.e., high level of food competition and poor diet). Three Petri dishes for each condition were set-up. Thirty randomly chosen plugged females from each treatment (≈ 10 from each Petri dish) were fixed in a formalin-glycerin solution (composed by one part of glycerin, 10 parts of 40% formaldehyde and 89 parts of distilled water) at 55°C. Three photographs of each CP were taken under a microscope (Zeiss™, model Primo Star) at 10X magnification, each photograph was taken from a different angle trying to "correct" for the curvature of the nematode (we made an effort to take one picture exactly from above the CP, and 2 lateral pictures, one from each side of the female, that also allowed observing the whole CP). We measured the area ($\mu m^2$) of each CP in the 3 photographs with the AxioVision Rel. 4.7® software (see, e.g., Figure 1A and B), and used the average of the 3 photographs as a proxy for CP size. Since adult size is negatively affected by density (Canales-Lazcano J et al., unpublished experiments), we took 1 photograph of each female under the microscope at 4× magnification, and measured female area with the AxioVision Rel. 4.7® software. We considered female area as an estimate of female size, and used it as a covariable in the analyses. We performed an analysis of covariance, followed by Tukey's Honestly Significant Difference (HDS) post-hoc tests, and calculated partial eta squared to compare the effect sizes of the covariable (female size) and the treatment (Richardson 2011).

### Effect of number of competing males on CP size

The effect of sperm competition risk was manipulated by varying the number of virgin males competing for one virgin female. Larval nematodes (L2 stage) were collected with an entomological pin from the general culture containers (with raw beef as food). These larvae were raised individually in 25 mL plastic containers with 2 mm of 2% agar and a very small piece of pork meat (an unmeasured amount, collected with the tip of an entomological pin, which was enough for complete development into adult according to preliminary observations). After 2 days (which is the time taken by an L2 larva to molt into adult), the sex of the individuals was determined and 36 h later the experimental treatments were established. Every female was individually placed in the same recipient where it had developed in order to provide an environment with availability of food that they already inhabited. After 5 min, the 3 treatments were established by varying the number of virgin males introduced with single virgin females: 1) one male, 2) 2 males, and 3) 4 males. These groups were maintained together for 4 h to ensure copulation, and then the females were collected, fixed, and, later, their body area and the area of their CP measured as in the previous experiment. For logistical reasons, the experiment was made in 3 blocks, established at 2 weeks intervals. Each block consisted in 20 replicates of each treatment; 3 replicates of each treatment were excluded from the analyses because one of the experimental individuals was lost. We compared female size between treatments with Analysis of Variance (ANOVA). The effect of "sperm competition risk" (treatment) and block on CP size was first assessed by means of a nested ANOVA. Since only treatment had an effect, we calculated Tukey's HDS post-hoc tests between treatments. We performed all statistical tests with the software Statistica™ V. 10.

## Results

### Effect of adult density and diet quality on CP size

Our analysis of covariance showed that the size of the CP was affected by the environment in which the nematodes developed ($F_{3,\ 115} = 8.45$, $P = 0.00004$). The covariable female size had a positive effect on CP size ($P = 0.0023$): females grown at low densities were larger (Figure 2A) and received larger CP (Figure 2B) than females grown at high densities. However, female size had a smaller effect size (partial eta squared = 0.078) than that of the density/diet treatment (partial eta squared = 0.181). Males produced larger CP when growing at low-density than at high-density conditions (Tukey's HDS tests, *P*-values between 0.0115 and $< 0.0001$; Figure 2B). Diet affected the size of CP only at high-density conditions (Figure 2B): males fed with *T. molitor* pupae (that we consider a higher quality diet given that wild *R. regina* are entomophagous) produced larger CP than males fed with beef (Tukey's HDS test, $P = 0.017$), despite the fact that females fed with beef tended to be larger than those fed with *T. molitor* (Figure 2A). In contrast, in low-density conditions no difference in the size of CP was found between nematodes fed with the 2 different diets (Tukey's HDS test, $P = 0.99$).

### The number of competing males is positively related to CP size

We found no significant differences in female size between-treatments ($F_{2,\ 168} = 0.038$, $P = 0.96$; Figure 3A). We performed a nested ANOVA and found no effect of block on CP size ($F_{2,\ 162} = 1.97$, $P = 0.143$). As we expected, the average size of the CP increased as the number of males competing for one female increased ($F_{2,\ 162} = 7.98$, $P < 0.000001$; Figure 3B), suggesting a graduated response of males to sperm competition risk (Tukey's HDS's: 1 Male *vs*. 2 Males: $P = 0.0008$; 1 Male *vs*. 4 Males: $P = 0.00002$; 2 Males *vs*. 4 Males: $P = 0.015$).

## Discussion

Sperm competition is considered the main selective pressure responsible for the evolution of CP (Parker 1970; Simmons 2001; Palopoli et al. 2015), although alternative explanations exist (Table 1).

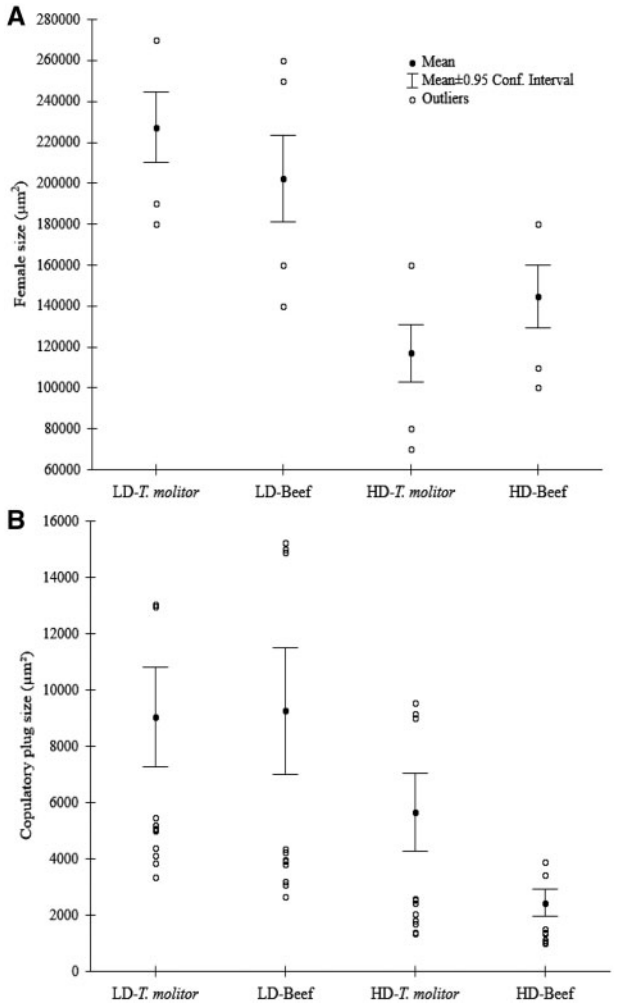


**Figure 2.** Effect of population density (which negatively affects body size) and diet quality on mean size ($\mu m^2$) of (**A**) the body of female *Rhabditis regina* nematodes and (**B**) the CP received by such females. We consider that *Tenebrio molitor* pupae are a better quality diet than raw beef given that *R. regina* is an entomopathogenic nematode that attacks beetle larvae. LD: low density; HD: high density.

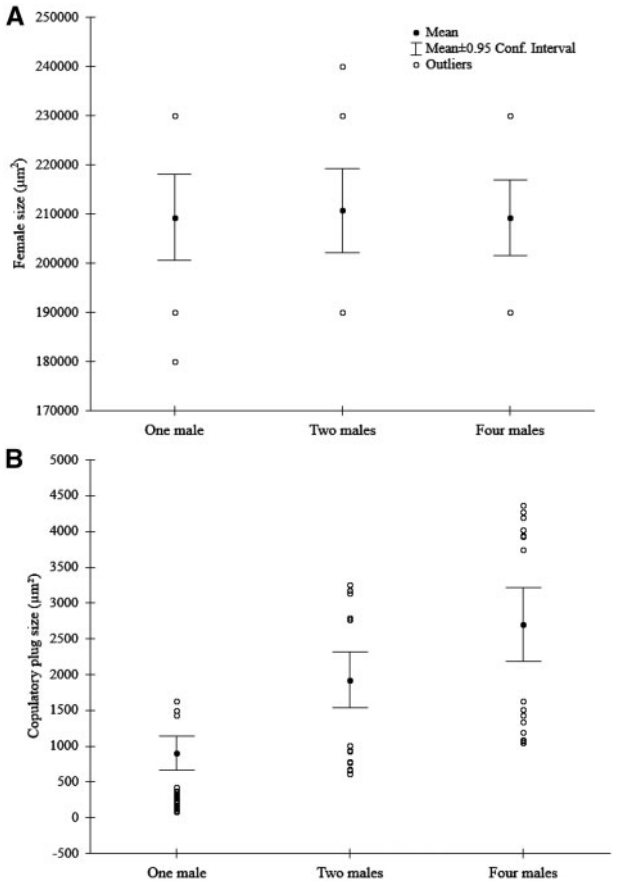


**Figure 3.** Sperm competition risk and the size ($\mu m^2$) of the CP deposited on female *Rhabditis regina* nematodes. (**A**) The size ($\mu m^2$) of virgin females experimentally exposed to different numbers of virgin males did not differ between treatments. (**B**) The size ($\mu m^2$) of the CP increased as the number of virgin males cohabiting with one virgin female increased.

If ejaculate production is costly, strategic ejaculation theory predicts strategic allocation of ejaculates in response to several factors including sperm competition risk (Wedell et al. 2002; DelBarco-Trillo 2011; Kelly and Jennions 2011). We applied these ideas to the production of CP (see also Ramm and Stockley 2007) and propose that the production of CP is costly for males of the nematode *R. regina* and that the size of the CP they produce should be directly proportional to the risk of sperm competition they perceive. Our experimental results are consistent with the idea that CP are costly to produce (Figure 2) and support the prediction that males adjust the size of CP to sperm competition risk (Figure 3B).

Previous experiments (Canales Lazcano J et al., unpublished results) with *R. regina* showed that male size is negatively affected by population density and, thus, we predicted that if CP production is costly males raised under HD conditions would produce smaller CP. As expected, in our first experiment, we found that at higher densities, when competition for resources is more intense and male size is smaller than at low densities, CP were significantly smaller than those produced by males raised at low densities. A potential problem with this result is that not only males but also females are of smaller size under high-density conditions and, thus, we cannot exclude the alternative explanation that males adjust the size of CP to female size. However, this hypothesis is also derived from strategic ejaculation theory and also assumes that CP production is costly (Wedell et al. 2002; DelBarco-Trillo 2011; Kelly and Jennions 2011).

In the first experiment, we also manipulated the type of diet by raising individuals either in beef or in pupae of *Tenebrio molitor* beetles, a diet we consider of better quality than the former since in the field *R. regina* is a entomopathogen of beetle larvae (*Phyllophaga*). It is interesting that, although our unpublished experiments did not show an effect of diet on adult size (Canales Lazcano J et al., unpublished results; see also Figure 2A), under high-density conditions males fed *T. molitor* produced larger CP than males fed the lower quality diet (raw beef) even though female size tended to be (not significantly) larger in the last treatment (Figure 2A). An additional point to consider in relation to the results of this experiment is that several studies on the costs of ejaculate production in different animal groups (Torres-Vila and Jennions 2005; Perry et al. 2013) consider a reduction in ejaculate size in consecutive copulations as evidence that ejaculates are costly to produce for the male. This idea also could be applied to the production of CP and explain, at least partially, our results. If mating rates are higher in the HD treatment (something we do not know), the smaller size of the CP observed could result from the inclusion of CP produced

by recently mated males. In conclusion, our results suggest that the production of CP might be costly for males at least under some circumstances.

We predicted that selection favors males that adjust the size of CP to sperm competition risk because fixed production of large CP, independently of the magnitude of sperm competition risk, would be selected against because males sometimes would produce unnecessarily large CP. On the contrary, fixed production of small CP would be selected against since males will sometimes lose in sperm competition if, for example, smaller CP are easier to dislodge by subsequent males (as observed in some butterflies [Matsumoto and Suzuki 1995] and spiders [Uhl et al. 2010]; see also Baker [1994] and García et al. [2007] on the nematode *C. elegans*). Our results agree with our prediction and, thus, provide support to the application of strategic ejaculation theory to CP production. Females received larger CP when housed with 4 males than when housed with only one male (Figure 3B). Furthermore, the CP transferred in the treatment with 2 males and one female were of an intermediate size, indicating that males adjusted the investment in CP to sperm competition risk in a more or less continuous way. Understanding the mechanisms employed by males to achieve this adjustment is a fascinating topic for future research.

Our application of strategic ejaculation theory is based in the untested assumption that the optimal size of CP is directly proportional to the risk of sperm competition. Although our results are consistent with this assumption, we do not know what is the advantage for the male of producing larger CP as the number of competing males increases. A number of possibilities come to mind depending on the function considered (Table 1). First, larger CP could resist more attempts of unplugging than smaller CP (Table 1: H1.1). In this respect, as mentioned in the methods section, detailed observations of several plugged females interacting with males showed no evidence of male behavior directed at dislodging CP (Canales Lazcano J, unpublished observations). Second, if CP contain "anti-aphrodisiacs" a larger amount of these could be necessary to repel several males (Table 1: H1.2). However, our unpublished observations indicate that males find plugged females sexually attractive, judging from the fact that they curl around the female and appear to try to insert their spicules on the female's body wall besides the CP. Another possibility is that CP contain substances that inhibit the sexual receptivity of females (Table 1: H1.3) and larger amounts could be necessary to face the stimuli provided by several males. Although a plugged female with a male curled around her presents an undulant and vigorous movement (not exhibited by unplugged females) that disturbs the male (Canales Lazcano J, unpublished observation), suggesting a female rejection response, it is not known if chemicals in the CP induce this female behavior. Finally, although it could be argued that a larger CP provides a better male-female attachment for resisting copula-interruption attempts by multiple males, and despite the fact that in *R. regina* we have observed males curling their tails besides mating males and moving in a way that could be interpreted as attempts to dislodge the copulating pair (although male displacement has not been observed), hypothesis H2 (Table 1) can be discarded in *R. regina* because males always separate from the female after finishing the production of CP, that is, CP do not appear to be used for attaching the mating couple.

Finally, it is important to mention that the results presented in Figure 3B could be produced by males depositing additional CP in already plugged females, besides or on top of previously produced CP. However, as mentioned in the "Methods" section, we consider this possibility unlikely because several non-systematic observations indicate that males are unable to curl their tails around the plugged vulva, apparently because of the presence of a previous plug (Canales-Lazcano J, personal observation). Although we frequently found multiple males with their tails coiled around copulating or plugged females, these males coiled their tails beside copulating males or beside previously deposited CP, which in both cases prevents access to the vulva, and we have never observed them depositing CP. However, continuous observations of mating and plugged females cohabiting with additional virgin males will provide a definitive answer.

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