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SHORT COMMUNICATION

Sperm competition and maternal effects differentially influence testis and sperm size in *Callosobruchus maculatus*

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Abstract

The evolutionary factors affecting testis size are well documented, with sperm competition being of major importance. However, the factors affecting sperm length are not well understood; there are no clear theoretical predictions and the empirical evidence is inconsistent. Recently, maternal effects have been implicated in sperm length variation, a finding that may offer insights into its evolution. We investigated potential proximate and microevolutionary factors influencing testis and sperm size in the bruchid beetle *Callosobruchus maculatus* using a combined approach of an artificial evolution experiment over 90 generations and an environmental effects study. We found that while polyandry seems to select for larger testes, it had no detectable effect on sperm length. Furthermore, population density, a proximate indicator of sperm competition risk, was not significantly associated with sperm length or testis size variation. However, there were strong maternal effects influencing sperm length.

Introduction

A wide variety of behavioural, physiological and anatomical adaptations can be attributed to selection from sperm competition (Birkhead & Møller, 1998). Theory is especially consistent in its prediction regarding sperm allocation when sperm competition proceeds under the raffle principle: males are predicted to increase the number of sperm allocated to an ejaculate based on the average sperm competition risk they face (Parker, 1998; and see Engqvist & Reinhold, 2005 for a discussion). Furthermore, because sperm production is positively associated with testis mass within species (Olar *et al.*, 1983; Moller, 1989; Gage, 1995; Bjork *et al.*, 2007; Scharer *et al.*, 2008), testis size should also increase with sperm competition risk.

Consistent with theory, testis size is positively associated with sperm competition risk across a range of taxa (Harcourt *et al.*, 1981; Gage, 1994; Hosken, 1997; Katvala *et al.*, 2008), and two experimental evolution studies found that testis size increases with higher sperm competition risk (Hosken & Ward, 2001; Hosken *et al.*, 2001; Pitnick *et al.*, 2001). This is also true at the intraspecific level. For example, in Salmon (*Salmo salar*), sperm competition is always higher for the small parr than for the large anadromous males, and consistent with predictions, parr have relatively larger testes (Gage *et al.*, 1995). Similar results were found in dung beetles *Onthophagus binodis* (Simmons *et al.*, 1999). There is also evidence that males can strategically adjust their investment in spermatogenesis. For example, in two moth species (*Plodia interpunctella*, Gage, 1995 and *Pseudaletia separata*, He & Tsubaki, 1992) and in the dung fly (*Scathophaga stercoraria*, Stockley & Seal, 2001), males increase their investment in testis mass when population density is high and there is greater risk of sperm competition (reviewed in Wedell & Hosken, 2009).

In contrast to testis size, variation in sperm size is less well understood (Pitnick *et al.*, 2009). Theory predicts that sperm competition should select for maximal sperm numbers, and hence sperm size should be reduced to some minimum except under special conditions (Parker, 1993, 1998), and there is evidence that short sperm are

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favoured during sperm competition in the cricket *Gryllus bimaculatus* (Gage & Morrow, 2003).

However, longer sperm may generate more power and could swim faster (Katz & Drobnis, 1990; Gomendio & Roldan, 1991), or deceive females about their sperm load, which could confer an advantage in sperm competition. In agreement with that, in the freshwater snail (*Viviparus ater*) males producing longer (apyrene) sperm had greatest success in sperm competition (Oppliger *et al.*, 2003), whilst in *Drosophila melanogaster*, males producing long sperm tend to outperform those producing short sperm, especially when competing within females with longer seminal receptacles (Miller & Pitnick, 2002). Furthermore, comparative studies report contrasting results, with some finding a positive association between sperm length and sperm competition risk (Gomendio & Roldan, 1991; Gage, 1994; Breed & Taylor, 2000; Morrow & Gage, 2000), whilst other have found a negative relationship (Stockley *et al.*, 1997) or no relationship (Hosken, 1997; Anderson & Dixson, 2002; Gage & Freckleton, 2003). In addition, there was no response of sperm length to varying levels of polyandry during experimental evolution studies in two species of fly, *S. stercoraria* and *D. melanogaster* (Hosken & Ward, 2001; Pitnick *et al.*, 2001). Nevertheless, there is some evidence that environmental factors influence sperm length. Sperm length increases with temperature in dung flies (Hellriegel & Blanckenhorn, 2002; but see Gage & Cook, 1994), with males's age in the rove beetle *Alleocharo bilineata* (Green, 2003) and decreases with larval density in *D. melanogaster* (Morrow *et al.*, 2008).

In addition to other environmental factors, maternal effects could affect male reproductive traits. In particular, mother's age could influence offspring's development because egg size decreases with maternal age in *Callosobruchus maculatus* (Fox, 1993). Recently, Dowling *et al.* (2007) investigated the effects of cytoplasmic and nuclear genes, and their interaction, on sperm viability and sperm length in the bruchid beetle *C. maculatus*. They found significant cytoplasmic and nuclear genetic effects on both sperm viability and sperm length. In addition, and unexpectedly, they found that mothers collected late in the culture regime ('old' treatment) produced sons with longer sperm than those of mothers that eclosed early in the culture regime. This maternal effect could be due to the adult age of the mothers (i.e. age from eclosion), variation in larval development time (affected by host size; Credland *et al.*, 1986) or larval competition (e.g. Bellows, 1982,) or level of larval competition within the beans, upon which *C. maculatus* develop. Here, we investigated testis and sperm size variation in the beetle *C. maculatus*. Specifically we tested whether polyandry drives the evolution of male investment in sperm and testis size, at both the phenotypic (reaction norm) and microevolutionary levels by (i) manipulating larval density (perceived risk of sperm competition; Gage, 1995) and (ii) subjecting populations to monogamy or polyandry for 90 generations. In addition, we investigated the plastic response of males to another environmental factor, mother's age, to test Dowling's *et al.* hypothesis of a maternal effect on sperm length [specifically that older mothers produce sons with longer sperm than younger mothers (Dowling *et al.*, 2007)].

Methods

Study species

Callosobruchus maculatus is a widely distributed pest of legumes. Females attach their eggs to the surface of beans, whereupon larvae hatch and burrow into the seed where they complete their larval development and eclosion. The beetles used in this experiment were derived from Niamey, Niger, and have been in culture on black-eyed beans (*Vigna unguiculata*) for over 90 generations at 27° C, 32% relative humidity and 16L : 8D photoperiod at the University of Lincoln. This population was maintained under polyandry, with approximately 60 males and 60 females housed together on 400 beans for their entire lifespan. To retain a constant population size and ratio of resources to beetles, we sieved and weighed the newly emerging adults at each generation and placed another 60 males and females (numbers estimated by mass) on 400 new beans. Prior to our experiments, this population size was increased to 2500 individuals on 120 g of black-eyed beans.

Maternal effects and larval density

We manipulated both larval density and mother's age to study their effects on the reaction norms of sperm and testis size. Males emerging into high density populations may face a higher risk of sperm competition and thus could use population density during larval development as an indication of the level of sperm competition expected as adults and adjust their investment in sperm accordingly (Gage, 1995).

Prior to beetle emergence, we isolated 400 beans in 'virgin chambers' (48-well cell culture plates; VWR International Ltd, Lutterworth, UK). Beans were checked every 24 h for emerging adults. We isolated the virgin adults, found by themselves in a cell, or only with their own sex. We formed two groups of females of different age: old females were collected just after emergence (< 24 h) and kept isolated until they were 4–5 days old (about a third of their lifespan) and young females which were between 0 and 24 h old at mating. These two age classes mimic the approximate age differences of mothers in the Dowling *et al.* (2007) study (day 24 vs. day 30 since laying date). The virgin (young or old) females were then allocated to one of two larval density treatments. For the high larval density treatment, virgin females (25 young or 25 old) were transferred with 25 virgin males (aged between 24 and 48 h from eclosion) to a container with limited resources for oviposition (50 beans). This quantity of beans per female was anticipated to yield an egg density of three to four eggs per bean. For the low larval density treatment, virgin females (25 young or 25 old) were transferred with virgin males (as above) to a container with abundant oviposition resource (800 beans). This quantity of beans per female was anticipated to yield approximately one egg per bean. All females were allowed to mate and oviposit on beans for 24 h at 27° C.

Response to selection: experimental evolution in monandrous and polyandrous lines

Two replicate monandrous and two replicate polyandrous populations were established from the ancestral population (Niamey, Niger). For each generation of the monandrous populations, we isolated beans carrying eggs in 'virgin chambers' before offspring emergence and collected virgin males and females every generation. Virgin females and males (all < 24 h post eclosion) were then paired and each pair was placed in a 40-mm Petri dish and observed until copulation had ceased. Finally, 60 singly mated females were transferred to 400 beans for oviposition. For each generation of the polyandrous populations, we sieved and weighed the newly emerging adults at each generation and placed 60 males and 60 females (estimated by mass) on 400 new beans. The males and females were housed together for their entire lifespan. When oviposition resources are available, about 80% of females *C. maculatus* remate within 24 h after the initial copulation (Eady *et al.*, 2004). Such a high remating rate ensures that females in our polyandrous treatment are actually polyandrous. Both treatments thus had equal census population sizes. We cannot exclude a difference in effective population size, and polyandrous populations are more likely to be smaller, because the probability that every male contributes offspring to the next generation is higher in monandrous lines where every male achieves copulation. Lines were maintained under these regimes for 90 generations before being used in the assays described in this study. In a side experiment, we tested for maternal effects by manipulating the level of larval competition and the mating rate of mothers (high/low larval competition and monandry/polyandry, in total four treatments) and measured offspring's lifetime reproductive success ($n = 214$). The results provided no evidence for a maternal effect of larval competition or mating rate. We nevertheless standardized selection prior to the assay for the four populations (two treatments × two replicates) by housing beetles individually under standardized conditions of mating number (single mating) and larval competition (one egg per bean) for one generation, to control for possible environmental effects.

Measurements on offspring

The following generation, for both the maternal effects/larval competition experiment ($n = 80$) and the experimental evolution assay ($n = 80$), we dissected male genitalia in insect saline under an Olympus SZH10 binocular microscope and measured the cross-sectional area of the two paired testes (each testis, one on either side of the gut, appears as a constricted structure made up of a large and small element, with a narrow 'waist' separating the two elements; Gill *et al.*, 1971) using Moticam image plus. Because testes in this species are approximately spherical, our 2-D measurement is likely to reflect 3-D measures. Following measurement, the testes were then ruptured and 10 randomly selected sperm located and photographed under phase-contrast lighting using a Nikon light microscope at 400× magnification. Photographs of sperm were then measured using Moticam image plus. Both sperm length and testis volume are highly repeatable in *C. maculatus*, $r_i = 0.88$ and 0.70 respectively (Rugman-Jones & Eady, 2008). Male body size was estimated by measuring elytra length, which is highly correlated to body mass (Wilson & Hill, 1989).

Statistical analyses

Due to difficulties of dissections, some of the testis measurements were incomplete (loss or damage of one or part of one of the testes in 15 cases). Because we wanted to use the total cross-sectional area of the testes, we used least-squares regression to predict the missing data. All regressions were performed on the complete dataset, regardless of the treatment group. Most of the missing data were for the smaller element of the paired testes. Thus, we regressed total testis area (sum of the four testes) on area of the two larger testes and one of the smaller testis ($R^2 = 0.98$) and used the regression equation to predict the missing data. We calculated the mean sperm length per male sampled (mean of 10 sperms). We used a linear model with elytra length as a surrogate for body size, and tested for the effect of mother's age (old or young) and larval density on testis area and sperm length.

In the experimental evolution dataset, we used line means as our unit of replication (Holland & Rice, 1999). Because our design only had four degrees of freedom (two replicates per treatment only), we only tested for the main effect of treatment (monandrous or polyandrous) with body size as a covariate – we could not include the interaction between mating treatment and body size in the model. To investigate this interaction indirectly, we performed a regression of testis volume or sperm length on body size for each population, and compared the intercepts and slopes between treatments. We found no significant difference in either slope (testes: $F_{1,3} = 4.23$, $P = 0.176$; sperm: $F_{1,3} = 0.02$, $P = 0.897$) or intercept (testes: $F_{1,3} = 5.73$, $P = 0.139$; sperm: $F_{1,3} = 0.002$, $P = 0.966$), indicating an absence of an interaction between body size and treatment.

All three traits (testis volume, sperm length and elytra length) were normally distributed in both datasets (Kolmogorov-Smirnov test, all $P > 0.05$). Residuals from all analyses were checked and did not deviate from normality (Kolmogorov-Smirnov test, all $P > 0.05$). Analyses were performed in R. We initially fitted the maximal model, removing nonsignificant effects (e.g. body size) in a stepwise manner, starting with the highest order interactions (Crawley, 2007). Results from the minimal models are reported.

Results

Maternal age and larval competition

Increasing larval density significantly reduced male size, as measured by elytra length ($F_{1,78} = 21.08$, $P < 0.0001$; high density 1.89 mm ± 0.02, low density 2.02 mm ± 0.01). Both testis cross-sectional area and sperm length were positively related to male elytra length (testes: $F_{1,76} = 11.07$, $P = 0.001$; sperm: $F_{1,75} = 3.98$, $P = 0.050$). Thus, male size was included as an explanatory variable in all models. For both testis area and sperm length, there was no significant interaction between elytra length and either of the two treatments, larval density (testes: $F_{1,74} = 0.01$, $P = 0.923$; sperm: $F_{1,73} = 1.24$, $P = 0.268$) and maternal age (testes: $F_{1,76} = 2.51$, $P = 0.118$; sperm: $F_{1,75} = 1.33$, $P = 0.253$). Cross-sectional testis area was not affected by either mother's age or larval density (mother's age: $F_{1,76} = 1.31$, $P = 0.256$; larval density: $F_{1,76} = 0.95$, $P = 0.333$, Fig. 1b). In contrast, we found a significant effect of mother's age on the sperm length of the sons: older mothers produced offspring with longer sperm (old mother: 0.167 μm ± 0.002; young mother: 0.164 μm ± 0.002, $F_{1,75} = 4.05$, $P = 0.048$, Fig. 1a). To verify this result, we reiterated the analysis on a random subset of the data and found a similar trend. Larval density did not affect sperm length ($F_{1,75} = 0.71$, $P = 0.402$, Fig. 1a).

Experimental evolution

Body size was included as a covariate but had no significant effect on either testis area ($F_{1,1} = 0.55$, $P = 0.59$) or sperm length ($F_{1,1} = 21.03$, $P = 0.14$). We found a borderline effect of selection regime on testis area ($F_{1,2} = 18.31$, $P = 0.050$, Fig. 2a): in the monandrous lines testes were smaller (mean ± standard error of the lines: 0.45 μm^2 ± 0.019) than in the polyandrous lines (0.64 μm^2 ± 0.024). The trend was in the same direction for both replicates. Sperm length was not affected by selection regime ($F_{1,1} = 0.53$, $P = 0.543$, Fig. 2b). Animals from monandrous lines were also smaller than those from polyandrous lines ($F_{1,2} = 19.35$, $P = 0.048$; elytra length = 1.87 mm ± 0.013 and 2.04 mm ± 0.014 respectively, Fig. 2c).

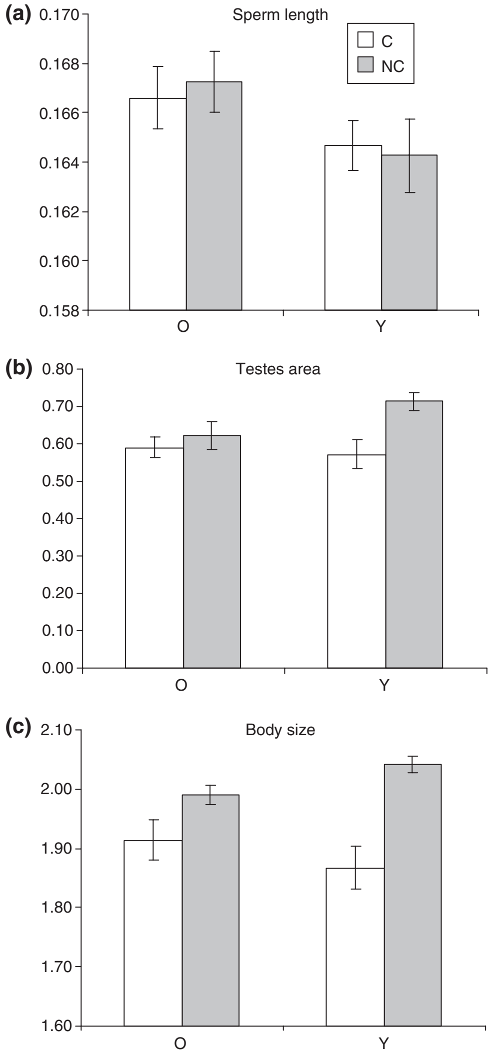

Fig. 1 Effects of larval density (C, competition; NC, no competition) and mother's age (O, old mother; Y, young mother) on (a) sperm length (μm), (b) testis size (μm^2) and (c) elytra length (mm). Error bars indicate standard errors.

Discussion

We found evidence for a microevolutionary influence of sperm competition risk on testis size with males from monandrous lines tending to have smaller testes than males from polyandrous lines. The observation of smaller testes when sexual selection and sexual conflicts are removed (monandry) suggests a cost for producing large testes, balanced by the benefit of large testes under

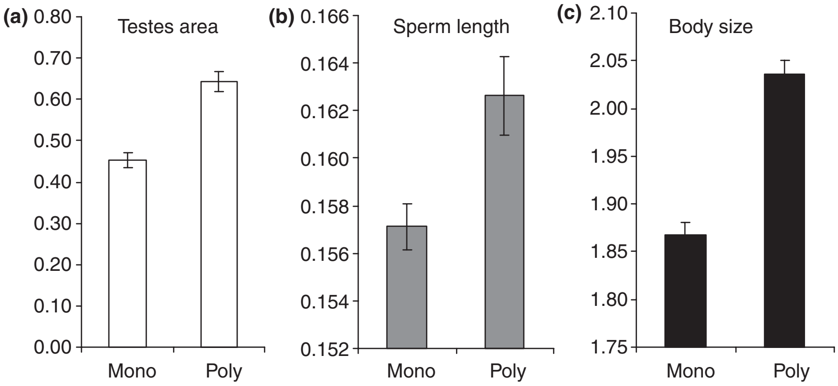

Fig. 2 Effects of mating treatment (Mono, monandrous; Poly, polyandrous) on (a) testis area in μm^2, (b) sperm length in μm and (c) elytra length in mm. Error bars indicate standard errors.

polyandry. This is in agreement with previous experimental evolution studies in other species (Hosken & Ward, 2001; Hosken *et al.*, 2001; Pitnick *et al.*, 2001) and with the ample correlational evidence for increased spermatogenic investment in response to elevated sperm competition level (Harcourt *et al.*, 1981; Gage, 1994; Hosken, 1997), particularly across the bruchid beetles where Katvala *et al.* (2008) have shown a positive correlation between female remating rate and male testis size. This suggests that bigger testes provide a fitness benefit to male *C. maculatus* under sperm competition. Larger testes are typically associated with higher sperm production (Olar *et al.*, 1983; Moller, 1989; Gage, 1995; Bjork *et al.*, 2007; Scharer *et al.*, 2008). Sperm number influences sperm competition success in *C. maculatus* (Eady, 1995), although sperm competition success was elevated only after the number of sperm transferred was reduced by approximately 80% (i.e. sperm number may be a minor factor in determining the level of sperm precedence in this species). Nevertheless, when males inseminate many sperm, females are less likely to remate (Eady, 1995), although this may be due to ejaculate components other than sperm (Edvardsson, 2007). Thus, the evolution of larger testes might be a pleiotropic effect of selection for larger ejaculates, as predicted by theory (Parker, 1998).

Consistent with previous findings (Hosken & Ward, 2001; Pitnick *et al.*, 2001), we found no effect of polyandry on sperm length in the lines exposed to experimental evolution. As outlined above, we had no clear prediction about how sperm competition would affect sperm length because theory (Parker, 1998) and empirical data (Gage, 1994; Hosken, 1997; Stockley *et al.*, 1997; Tomkins & Simmons, 2000; Morrow & Gage, 2001; Miller & Pitnick, 2002; Gage & Morrow, 2003; Oppliger *et al.*, 2003) do not produce an unambiguous picture. The absence of a microevolutionary response could result from a low genetic variation for sperm length, as found in dung beetles (Simmons & Kotiaho, 2002), or a low evolvability (coefficient of additive genetic variation), as found in *D. melanogaster* (Morrow *et al.*, 2008), but it seems more likely that sperm length plays little role in sperm competition in this species (Rugman-Jones & Eady, 2008). We should also note however, that the degree of replication in our study means that our statistical power to detect significant sperm length evolution is limited, although the power was high enough to detect testis size evolution.

Our manipulation of larval density provided no evidence that males adjust their reproductive investment, either testis size or sperm length, in response to information on the reproductive environment. This contrasts with the plastic response of testis volume and sperm number to larval density observed in the moth *P. interpunctella* (Gage, 1995). Our high larval density treatment could be confounded with a higher mating rate for the mothers (males can find the 25 females more easily on 50 rather than 800 beans). This would further increase the competition between males, which is expected to drive the same effect on testes and sperm size as a high level of larval competition. Because we found no effect, our results are conservative. However, in our experiment, we cannot distinguish the effect of sperm competition risk from the effect of larval competition for resources because controlling resource availability to larvae is difficult when larvae develop exclusively inside the bean. The best approximation might be to limit the number of larvae per black-eyed bean to two or three, to limit the effect of competition on offspring size, especially amongst males (Giga & Smith, 1991). It might also be the case that larval or population density is a poor predictor of sperm competition risk in this species. For example, if there is sperm displacement, sperm competition is the same whether a male competes with one or more ejaculate, which obscures the relation between the level of sperm competition and population density (Stockley & Seal, 2001). Another possible explanation for the absence of a plastic response of the reproductive traits measured to larval density might be that dispersal rather than adaptation to sperm competition is used as a response to high density. In conditions of high larval density (Messina & Renwick, 1985), *C. maculatus* presents an imaginal polymorphism (Utida, 1954), with a flying form that enables dispersal of individuals away from areas of limiting food resources. The active form also appears to be in reproductive diapause: females show little ovarian activity (Zannou *et al.*, 2003), whilst males have reduced testis size and rudimentary accessory glands, devoid of secretory material (Gill *et al.*, 1971). The absence of an increased investment in testes in response to an increase in larval density (and thus risk of sperm competition; Gage, 1995) is arguably indicative of an evolutionary history of investment into dispersal characteristics when resources are limiting.

Sperm length was influenced by another environmental factor, mother's age, as the sons of older females produced longer sperm. A similar result was found by Dowling *et al.* (2007). In our experiment, the plastic response in sperm size was produced by females of old adult age (as determined by days since eclosion) rather than by individuals that had a longer period of larval development (but were newly eclosed) or by offspring of eggs laid later in the previous generation. Our experimental design also allowed us to disentangle the effects of larval competition and maternal age on sperm length. Maternal age (measured as time because of the mothers' eclosion) affected offspring sperm length, thus confirming Dowling *et al.*'s suggestion that older mothers produce sons with longer sperm.

The precise mechanism underlying the maternal effect is unclear. Wasserman & Asami (1985) and Fox & Dingle (1994) have reported a longer development time of offspring of older females, thus it is possible that longer sperm result from a longer period of sperm individualisation. While this is conjecture, Pitnick *et al.* (1995) have demonstrated there is a correlational link between development time and sperm size across *Drosophila*. Irrespective of the mechanism, the maternal effect observed here and in Dowling *et al.* (2007) illustrates a plastic response of sperm length to an environmental factor. Sperm length is not usually considered to be a plastic trait, despite evidence for an effect of male's age and body size in the rove beetle *A. bilineata* (Green, 2003), temperature in dung flies (Hellriegel & Blanckenhorn, 2002) and larval density in *D. melanogaster* (Morrow *et al.*, 2008). A maternal influence has also been reported for the dispersal polymorphism in *C. maculatus*; eggs laid by older females are more likely to develop into the active (dispersal) form, than eggs laid by young females (Sano-Fujii, 1979). Given that the dispersal polymorphism is associated with differences in development time, fecundity, delayed reproduction, lower water content and higher lipid content (Messina, 1990), it is possible that the maternal effects on sperm length and dispersal polymorphism are also connected in some way.

Overall, our results reinforce the conviction that testis size responds to selection from sperm competition over microevolutionary time-scales. However a plastic response to one potential proximate indicator of sperm competition risk, larval density, was not observed. In contrast, sperm length was unaffected by sperm competition at both the microevolutionary and proximate levels, but was influenced by a maternal effect, female age. This is consistent with previous findings in this species, but whether there is any advantage to producing longer sperm, or whether there are functional trade-offs involving sperm length, remain to be investigated.

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