Costs and benefits of giant sperm and sperm storage organs in *Drosophila melanogaster*

Susanne Zajitschek[1,2*], Felix Zajitschek[1,2*], Sarah Josway[1], Reem Al Shabeeb[1], Halli Weiner[1] & Mollie K. Manier[1]

[1] George Washington University, Department of Biological Sciences, Science and Engineering Hall, 800 22nd St. NW, Suite 6000, Washington, DC 20052, United States

[2] School of Biological, Earth and Environmental Sciences, University of New South Wales, Kensington Campus, Sydney, NSW 2052, Australia

* Joint first authors

Correspondence: Mollie Manier, manier@gwu.edu, +1.202.994.0126 (phone), +1.202.994.6100 (fax)

ABSTRACT

In *Drosophila*, long sperm are favoured in sperm competition based on the length of the female's primary sperm storage organ, the seminal receptacle (SR). This sperm-SR interaction, together with a genetic correlation between the traits, suggests that the coevolution of exaggerated sperm and SR lengths may be driven by Fisherian runaway selection. Here, we explore the costs and benefits of long sperm and SR genotypes, both in the sex that carries them and in the sex that does not. We measured male and female fitness in inbred lines of *D. melanogaster* derived from four populations previously selected for long sperm, short sperm, long SRs, or short SRs. We specifically asked: what are the costs and benefits of long sperm in males and long SRs in females? Furthermore, do genotypes that generate long sperm in males or long SRs in females impose a fitness cost on the opposite sex? Answers to these questions will address whether long sperm are an honest indicator of male fitness, if male post-copulatory success is associated with male pre-copulatory success, if female choice benefits females or is costly, and whether intra-genomic conflict could influence evolution of these traits. We found that both sexes have increased longevity in long sperm and long SR genotypes. Males, but not females, from long SR lines had higher fecundity. Our results suggest that sperm-SR coevolution is facilitated by both increased viability and indirect benefits of long sperm and SRs in both sexes.

Keywords: fecundity; Fisherian runaway selection; good genes; intralocus sexual conflict; longevity; mating success; pre-copulatory sexual selection; post-copulatory sexual selection; sperm; sperm storage organ

INTRODUCTION

Foundational questions in sexual selection ask how female preferences for elaborate male ornaments can evolve. That is, how do females benefit from these preferences, and what are the associated costs? There is ample evidence that, as predicted by theory (Zahavi, 1975), ornaments are costly to produce and thus serve as signals of genetic quality (e.g., Godin and McDonough, 2003; Kotiaho, 2000; Manica et al., 2016; Mobley et al., 2018; Zuk et al., 1995). Females will gain indirect benefits from mating with high-condition males by having high-condition offspring (good genes; Fisher, 1958; Zahavi, 1977), if condition is heritable. If ornament phenotype is also heritable, females will additionally benefit by producing sexy sons, and if female preference is heritable, a choosy female will have choosy daughters, who will also gain these indirect benefits. Likewise, males would benefit by mating with females exhibiting preference through also having choosy daughters.

In addition, intralocus conflict for either the trait that is exaggerated in males or its female preference (Lande, 1980; Rice, 1984) will constrain the evolutionary benefit of advantageous ornament or preference genotypes in males or females, respectively, by incurring fitness costs when those genotypes are expressed in the other sex (Bonduriansky and Chenoweth, 2009; Chippindale et al., 2001; Cox and Calsbeek, 2009; Pischedda and Chippindale, 2006). Thus, the benefit of being a successful male may be limited by any costs of also having unfit daughters (Foerster et al., 2007), and any benefit of choosy daughters may be limited by low fitness of a female preference genotype in males.

Principles of female preference and male ornament evolution can apply to traits under post-copulatory sexual selection, which is mediated by sperm competition (Parker, 1970) on the one hand and cryptic female choice (Eberhard, 1996; Firman et al., 2017) on the other. These two processes occur after mating in an analogous fashion to male-male competition and female choice, which comprise pre-copulatory selection that acts before mating. Male traits under pre-copulatory sexual selection often take the form of elaborate visual, audible, tactile, and/or chemical displays, and female preferences for them are based on sensory perception that leads to behavioral decisions (Candolin, 2003; Jennions and Petrie, 1997). In contrast, female preference under post-copulatory sexual selection occurs when female-mediated behavioral, morphological, or physiological processes bias paternity in favor of certain males (Pitnick and Brown, 2000), based on pre-copulatory (Pilastro et al., 2004; Sbilordo and Martin, 2014) or post-copulatory male traits (Wojcieszek and Simmons, 2012). Whether acting before or after copulation, female preference evolution follows similar expectations predicted under runaway selection (Fisher, 1958; Kirkpatrick, 1982), good genes (Iwasa and Pomiankowski, 1991; Zahavi, 1975), or sexy son (Pomiankowski et al., 1991)/sexy sperm (Keller and Reeve, 1995) hypotheses.

In *Drosophila*, the correlated evolution of sperm and sperm storage organs has become a model system in which to study the evolution of traits under post-copulatory sexual selection. In this lineage, sperm reach extraordinary lengths (Pitnick et al., 1995), driven by length of the female's primary sperm storage organ, the seminal receptacle (SR), which can be even longer (Pitnick et al., 1999). Long sperm have a competitive fertilization advantage against shorter sperm (Lüpold et al. 2012; 2016; Miller and Pitnick, 2002), but primarily within long SRs (Miller and Pitnick, 2002). This long sperm advantage occurs through as yet undescribed fluid dynamic processes during the displacement stage of sperm competition (Manier et al., 2010; 2013). Thus, variation in SR length is a proxy for the strength of cryptic female choice for sperm length, with longer SRs being more selective, or "choosier", based on the size of the post-copulatory male ornament, sperm length.

Male ornaments are typically considered to evolve under pre-copulatory sexual selection, because females are assumed to be agnostic to their mate's sperm traits, and it is difficult to imagine a sperm phenotype being "preferred" by that female. If *Drosophila* sperm length can be considered to be a male ornament, a number of patterns would be expected. (1) If this exaggerated trait has evolved under runaway selection, the male ornament and female preference should coevolve and be genetically correlated. (2) If long sperm carry indirect benefits consistent with a good genes model of ornament evolution, they should also be costly and condition-dependent (Zahavi, 1977), and possibly trade off with other male traits (reviewed in Manica et al., 2016). Finally (3), we could expect long sperm to display strong positive allometry (disproportionally longer for a given body size; Bonduriansky, 2007; Kodric-Brown et al., 2006; Voje, 2016), particularly if sperm length could be considered a "weapon" rather than a "display" (Eberhard et al., 2018).

In support of these predictions, (1) sperm length and SR length are coevolving both among species (Pitnick et al., 1999) and among populations within *D. mojavensis* (Pitnick et al., 2003), and there is a significant genetic correlation between the two traits (Lüpold et al., 2016). (2) Long sperm are also costly in terms of time required to reach reproductive maturity (Miller and Pitnick, 2002; Pitnick et al., 2003; Pitnick et al., 1995), and sperm length trades off with sperm number across species (Pitnick, 1996). Moreover, condition-dependence of sperm length increases in species with longer sperm (Lüpold et al., 2016), and (3) as expected for certain male ornaments, sperm length has the strongest positive allometry with body size ever measured for a sexually selected trait (Lüpold et al., 2016).

In order to better understand how male ornaments and female preferences coevolve, we need to elucidate the fitness consequences of genotypes controlling these traits for both the sex in which they are expressed and the sex in which they are not. Previous research on the fitness consequences of long sperm and long SRs have shown that production of long sperm incurs costs in delayed male reproductive maturity (Miller and Pitnick, 2002; Pitnick et al., 2003; Pitnick et al., 1995) while also conferring a fertilization advantage during sperm competition (Lüpold et al., 2012; 2016; in review; Miller and Pitnick, 2002). Similarly, long SRs are associated with extended development times and decreased longevity but increased fecundity (Miller and Pitnick, 2003, 2002). Moreover, females that experience low-quality environments as larvae have shorter SRs (Amitin & Pitnick, 2007), suggesting that the production of long SRs is metabolically costly and requires adequate resources.

Despite these advances, much remains unknown. Do males with long sperm also have higher pre-copulatory success (increased attractiveness, mating success), fecundity, or viability? Sperm size could be correlated with these traits due to genetic linkage with viability alleles (Gilbert and Uetz, 2016; Head et al., 2005; Svobodová et al., 2018), or there may be significant trade-offs (Ball and Parker, 1996; Dines et al., 2015; Foo et al., 2018) or even no relationship (Travers et al., 2016), depending on a range of ecological factors (Evans and Garcia-Gonzalez, 2016; Lüpold et al., 2014; Parker et al., 2013; Simmons et al., 2017). In addition, do females benefit from bearing SRs that select for longer sperm, or does cryptic female choice carry a cost? In order for female preference for a male trait to evolve, it must be strong enough to outweigh any costs associated with that preference, even if the male trait is not at first linked to viability (Chandler et al., 2013; Mead and Arnold, 2004). Therefore, quantifying the costs of female preference is critical for understanding preference-trait coevolution. Finally, is there evidence for intragenomic conflict at loci controlling sperm length and SR length, or do both sexes benefit from exaggerated reproductive traits? The strength and direction of female-male coevolution may be affected by fitness consequences incurred by trait genotypes in the sex not expressing the trait (Chenoweth et al., 2008; Chippindale et al., 2001; Cox and Calsbeek, 2009, Pischedda and Chippindale, 2006).

Here, we investigate the costs and benefits of long sperm and long SRs that may influence how they coevolve. This system has a unique advantage in that the female "preference" (SR length) is an easily and consistently quantifiable morphological trait, rather than a behavioral or cognitive process that may be more difficult to measure and is potentially affected by social learning (Danchin et al. 2018; Dion et al., 2019; Monier et al., 2019). We measured male mating success, male and female fertility, and male and female longevity in isofemale lines derived from populations that had been experimentally evolved to have long sperm, short sperm, long SRs, or short SRs. This experimental design allowed us to examine costs and benefits of long sperm and long SR genotypes both in the sex expressing the trait and in the opposite sex. Fitness consequences of exaggerated trait genotypes manifested in either sex could influence the dynamic of sperm-SR coevolution, either by reinforcing selection in the same direction on both sexes or imposing an antagonistic relationship between selection on males and females.

METHODS

Experimental populations

To determine fitness effects of sperm length or SR length, we quantified mating success, fecundity, and longevity in inbred isofemale lines derived from four *D. melanogaster* populations that had been previously selected for long sperm, short sperm, long SRs, or short SRs (initially reported in Miller and Pitnick, 2002; 2003). Briefly, these populations underwent 17 generations of selection for sperm length, and at least 30 generations of selection for SR length. For each generation of sperm length selection, males were dissected, and sperm length was measured after breeding with virgin females. Progeny of sires with the longest or shortest sperm contributed to the next generation. For each generation of SR length selection, females were transferred to individual vials after group mating, where they laid eggs before dissection and SR measurement. Progeny of dams with the longest or shortest SRs contributed to the next generation (for details see Miller and Pitnick, 2002; 2003).

Approximately 300 generations after the initial selection experiments described above, the long and short SR lines underwent eight additional generations of selection in order to re-establish highly significant divergence in SR length. For each generation of this second selection regime, 75 virgin pairs were housed in individual food vials (standard cornmeal-yeast-agar-molasses diet). After laying eggs for several days, females were dissected, and SR length measured. We selected the highest 5 or lowest 5 families for the next generation. All 5 families contributed equally (15 males and 15 females), and we avoided pairing siblings by mating Family 1 females with Family 2 males, Family 2 females with Family 3 males, etc. Eight generations of selection yielded SR lengths with non-overlapping distributions between the high line (mean = 2946 µm; min-max = 2552-3574 µm) and low line (mean = 2150 µm; min-max = 1841-2507 µm).

We then proceeded to generate panels of isolines for sperm and SR length selection regimes through 10 generations of full-sibling inbreeding for sperm lines and 15 generations for SR lines. Inbreeding of the SR lines began immediately after the second round of selection, and approximately 330 generations following initial sperm length selection. For each panel, the four most extreme isolines were identified and used for this experiment. For each isoline, a minimum of five female SRs and, on average, five sperm cells (range: 2-11 sperm) from each of at least four males (range 4-8, average: 5.56) were measured. To measure sperm, seminal vesicles from mature virgin males (5 days post-eclosion) were dissected into a large droplet of 1X phosphate buffered saline (PBS) on a glass slide, ruptured, and dragged several times to release the live sperm. The droplet was dried down at 55 °C, and sperm were fixed in 3:1 methanol:acetic acid, mounted in glycerol, and the slide sealed with nail polish. Sperm were visualized on a Nikon Ni-U upright light microscope at 200X magnification under darkfield. Images were captured with an Andor Zyla 4.2 camera and measured using the segmented line tool in ImageJ (https://https://imagej.nih.gov/ij/).

SRs were measured from mature virgin females (5-7d post-eclosion) that were stored frozen (−20°C) until dissection. Female reproductive tracts were dissected into 1X PBS, the SR gently unraveled with a fine insect pin, and the sample mounted under a coverslip, such that the SR was two-dimensional but not over-compressed. SRs were visualized at 100X magnification under phase contrast, and images were captured and measured as outlined above.

From across three experimental blocks conducted at different time points, a total of N = 1151 males and N = 1298 females were included in the final analyses. All stocks were maintained at ambient room temperature and light regime in polyethylene fly vials with cornmeal agar yeast molasses medium supplemented with live yeast. Experimental flies were reared by pairing 2-3 day old female and male flies from the same isoline for 48 hours, and emerging offspring were collected as virgins. All reproductive and behavioral assays were performed at the same time of day to reduce circadian rhythm effects. All individuals were collected as virgins under light CO_2 anesthesia, maintained in same-sex vials with densities of 10 females or 20 males, and were 2-5 days old when first mated.

Mating success

We observed male mating behavior to assess attractiveness (latency to mate), copulation duration, and the proportion of successful matings for males from each of the four selection regimes: long sperm, short sperm, long SR, and short SR. A subset of ten randomly selected males from each of three replicate group vials from each of the 16 isolines ($N = 460$) were tested for five consecutive hours (or until successful mating) each week over a period of six weeks. Individual males were transferred without anesthesia into a mating arena consisting of a polyethylene vial with a foam plug in the bottom to enhance visibility, containing a single 5 day old wild type (LHm) virgin female. For each mating arena, the cotton plug was pushed halfway down the vial, leaving approximately 2.5 cm of vertical space, to stimulate male-female interactions. For each successful mating, latency to mate and mating duration were recorded, after which males were returned to their original group vial. Males were transferred to new food vials three times a week, and dead males were removed without replacement, with date of death recorded for longevity analyses.

Fecundity

To measure female fecundity, experimental virgin females 2-3 days post-eclosion were paired individually with a wild type LHm male (5 to 7 days old) for 48 hours, after which the male was removed ($N = 160$). Each week, we subsampled progeny produced within a 24 hr period for each female over the course of her life (see Longevity, below).

Specifically, we allowed the eggs that had been laid within the specified weekly 24 hr period to develop, and counted the number of eclosed and uneclosed pupae, four days after the flies in a given vial had started hatching. All weekly counts from each female were summed to approximate lifetime reproductive output.

For male fecundity, we counted progeny produced by up to two randomly selected successfully mated males from each replicate group vial (N max/week = 96) for each week of the mating success assays (see above). Specifically, LHm females were separated from the males directly after mating, and transferred to a new individual food vial, where they were allowed to deposit eggs for 48 hours before being discarded. Adult offspring were counted as a proxy for male fecundity. In contrast to females, male offspring data were not measured on the same individuals over time, as the individual identity of males within a given vial was unknown. Any measure of fecundity is subject to both male and female effects, but by using standard wild type females we aim to distribute female effects in an unbiased way across male treatments and factors (selection regime, block, line).

Longevity

Males were kept in cohorts of initially 20 same-sex flies per vial (three replicate vials per isoline, populated one day post-eclosion, $N = 48$ vials). We checked for survival every two days, when flies were transferred to a new food vial. We tested how selection regime affected survival using Cox proportional hazard models (function *coxph*; Therneau, 2015), separately for each sex and each selected trait. Females were maintained individually to assess female reproductive success (10 replicates each, conducted in blocks 1 & 2).

Statistical Analyses

To analyse male fecundity and mating behavior, we used general linear mixed models (Bates and Maechler, 2009) and *lmertest* (Kuznetsova et al., 2017) to calculate *p*-values, tested with line fitted as a random effect. Degrees of freedom were based on the Satterthwaite approximation. In some cases, the response variable was square root transformed to satisfy model assumptions. Binomial data (mating success) were checked for overdispersion, and *p*-values were calculated in *afex* (Singmann et al., 2016). All analyses were performed using R (v 3.4.0, R Core Team, 2017).

RESULTS

Sperm and SR length

Long sperm lines had significantly longer sperm than short sperm lines (long mean ± SE: 1934.00 ± 10.78, n = 86 sperm cells; short: 1673.97 ± 10.46, n = 115 sperm cells; $t_{192.94}$ = 17.313, P = 2.2e-16). Likewise, long SR lines had significantly longer SRs than short SR lines (long: 2504.72 ± 58.74 µm, n = 22 SRs; short: 1640.56 ± 29.83 µm, n = 31 SRs; $t_{31.85}$ = 13.17, P = 1.98e-14). However, sperm lengths were not significantly different between SR selection regimes (long: 1840.02 ± 6.83 µm, n = 119 sperm measurements; short: 1855.07 ±

6.76 μm, n = 117 sperm cells; t_{234} = -1.567, P = 0.1185). Similarly, SR length in sperm selection treatments did not differ (long: 2138.50 ± 94.97μm, n = 6; short: 2237.69 ± 51.78 μm, n = 10; $t_{8.02}$ = -0.917, P = 0.39).

Fitness

In the sperm selection lines, long sperm males had lower mating success (χ^2 = 4.35, df = 1, P = 0.037; Fig 1a), suggesting that there is a pre-copulatory cost to the post-copulatory long sperm advantage found in previous studies (Miller and Pitnick, 2002). However, there were no differences in male attractiveness (mating latency; $F_{1,211}$ = 2.270, P = 0.133; Fig 1c) or copulation duration ($F_{1,192}$ = 0.553, P = 0.458; Fig 1e). Both males and females from long sperm lines trended toward higher fecundity, though this pattern was not statistically significant (males: $F_{1,5.8}$ = 3.997, P = 0.094; Fig 2a; females: $F_{1,6}$ = 3.560, P = 0.108; Fig 2c). We standardized fecundity within sex and selected trait (sperm or SR) by subtracting the mean and dividing the difference by the standard deviation, to directly compare fitness for both males and females (see Fig. 3). Standardized fitness did not differ between males and females for short sperm ($F_{1,54.9}$ = 0.119, P = 0.731) or long sperm lines ($F_{1,53.6}$ < 0.001, P = 0.988; Fig. 3a). We did find a longevity advantage to long sperm genotypes in both sexes (males: χ^2 = 32.50, df = 1, p = 0.001; sperm selected, females: χ^2 = 9.13, df = 1, P = 0.003; Fig 4a, c). Higher survival specifically occurred for older females (Fig 4c) and at all ages for males (Fig 4a).

In the SR selection lines, short SR males were more attractive (shorter mating latency; $F_{1,569}$ = 8.727, P = 0.003; Fig 1d) and copulated for longer ($F_{1,536}$ = 91.261, P < 0.0001;

Fig 1f), but long SR males ultimately had higher mating success (χ^2 = 5.82, df = 1, P = 0.0158; Fig 1b) and higher fecundity ($F_{1,5.8}$ = 6.118, P = 0.049, see Fig 2b). Females had higher relative fitness than males in short SR lines ($F_{1,52.4}$ = 10.419, P = 0.002; Fig. 3b) and males had higher relative fitness than females in long SR lines ($F_{1,55.2}$ = 7.485, P = 0.008; Fig. 3b). Interestingly, long SR females did not produce more offspring ($F_{1,6}$ = 0.413, P = 0.544; Fig 2d), but they did live longer (χ^2 = 4.64, df = 1, p = 0.031; Fig 4d), primarily at intermediate ages (Fig 4d). Male longevity was marginally longer between short and long selection regimes in SR selection lines (χ^2 = 2.88, df = 1, P = 0.090; Fig 4b).

DISCUSSION

In our study, genotypes producing long sperm or SRs confer multiple fitness benefits and few costs for both sexes (Table 1), suggesting that higher genetic quality is required to produce these traits. In particular, long selection lines for both sperm and SR phenotypes had increased longevity in males and females. By selecting for longer sperm, long SRs might also select for higher fitness genotypes in sons and daughters. Thus, the evolution of long sperm and long SRs may be driven by both viability selection (e.g., increased longevity) and indirect benefits (long SRs select for longer sperm, which confer fitness benefits to both sons and daughters). Together with a genetic correlation between the traits (Lüpold et al., 2016), these fitness benefits may aid in fueling a Fisherian runaway process. An alternate explanation for our results is that the selection and inbreeding history of the populations used in this experiment has led to the capture of genes conferring increased longevity in long sperm and long SR lines. It is important to note that increased longevity in both males and females is not necessarily indicative of increased lifetime reproductive success, which was not quantified here. Evaluation of fitness in unrelated populations with known sperm and SR phenotypes will be required to determine if sperm length and SR length are actually linked to "good genes".

We unexpectedly found that long SR genotypes in females confer increased longevity with no fecundity benefit, in contrast to previous work that showed that females with long SRs have higher reproductive output but at a cost to survival (Miller and Pitnick, 2003). These previous results may be due to increased storage capacity of both sperm and detrimental male ejaculate proteins (Chapman et al., 1995). In that study, long SRs were 40% longer than those reported here (3.5 mm vs 2.5 mm) and unlikely to occur naturally, perhaps because of these costs. Our more moderate SR lengths are comparable to those found in local wild *D. melanogaster* in the Washington, D.C. area (mean 2.5 mm, unpubl. data), and also on par with SR phenotypes shown to select for longer sperm (Miller and Pitnick, 2002). These moderately long SRs come with a longevity benefit, while also mediating sperm choice for longer sperm, perhaps reaping viability benefits for both sons and daughters. We thus find that both long sperm and long SRs may be honest signals of genetic condition.

Our results identified a tradeoff in males between long sperm and mating success, suggesting evolutionary modularity for traits under pre-copulatory versus post-copulatory sexual selection. In other words, long sperm confer only a post-copulatory advantage through sperm competitive outcome, with no premating benefits with regards to mating success. For males with long SR genotypes, reproductive success was mixed, with decreased attractiveness and copulation duration but increased mating success. This outcome may be due to more persistent courtship by long SR males, despite lower attractiveness, though we did not quantify courtship effort. At the same time, females mated to less attractive long SR males produced more progeny, suggesting a disconnect between male attractiveness and male fecundity. Higher fecundity in long SR males also further supports the hypothesis that genotypes associated with long SRs are of higher quality.

Most studies that examine the relationship between pre-copulatory and post-copulatory processes ask if mating success and attractiveness (pre-copulatory) is correlated with paternity outcome (post-copulatory). This study flips that question by starting with traits associated with paternity success (sperm and SR length) and looking for an association with premating outcome. We would not necessarily expect to find a difference between comparisons of pre-copulatory success with post-copulatory outcome, as opposed to associating post-copulatory outcome with pre-copulatory success. However, most studies in other species have found that pre-copulatory success is a good predictor of post-copulatory outcome (Evans et al., 2003; Hosken et al., 2008; Lewis and Austad, 1994; Polak and Simmons, 2009; Sbilordo & Martin, 2014; McDonald et al., 2017), though it matters which traits are considered (Ala-Honkola and Manier, 2016). Here, however, we did not find an association between sperm length and premating success, in concordance with Droge-Young et al. (2012) and Travers et al. (2016). It is possible that pre-copulatory and post-copulatory effort trade off in *D.*

melanogaster (Filice and Dukas, 2019), and that both are so costly that males may invest in only one or the other.

In conclusion, sperm length and SR length in this system do not appear to have fitness costs in the opposite sex. Rather, both long sperm and long SR phenotypes seem to confer fitness advantages to both males and females (with few costs). Long SR females and long sperm males lived longer (viability benefits), and by selecting for long sperm, long SRs in females may provide indirect benefits through increased longevity in both sons and daughters. These results suggest not only that long sperm are indeed an honest signal of good genes, but that female preference can also be an indicator of female quality. The costs and benefits incurred by female preferences have received less empirical attention than selection on male traits, primarily because female preferences (and concomitant costs and benefits) are more difficult to measure. Our work here suggests that selection driving male-female coevolution is not always antagonistic and can actually align to benefit both sexes.

ACKNOWLEDGEMENTS

We are grateful to Zachary Boor, Ayesha Monga Kravetz, and Michael West for experimental assistance, and to Bob Cox for helpful discussion. Kirstin Berben conducted the last round of SR length selection. The manuscript markedly benefited from comments from two anonymous reviewers. (Financial disclosure statement removed for blinding purposes). We declare no conflicts of interest.

LITERATURE CITED

Ala-Honkola, O., Manier, M.K., 2016. Multiple mechanisms of cryptic female choice act on intraspecific male variation in *Drosophila simulans*. Behav. Ecol. Sociobiol. 70, 519–532. https://doi.org/10.1007/s00265-016-2069-3

Ball, M.A., Parker, G.A., 1996. Sperm competition games: External fertilization and "adapative" infertility. J. Theor. Biol. 180, 141–150.

Bates, D., Maechler, M., 2009. lme4: Linear mixed-effects models using S4 classes.

Bonduriansky, R., 2007. Sexual selection and allometry: a critical reappraisal of the evidence and ideas. Evol. Int. J. Org. Evol. 61, 838–849.

Bonduriansky, R., Chenoweth, S.F., 2009. Intralocus sexual conflict. Trends Ecol. Evol. 24, 280–288. https://doi.org/10.1016/j.tree.2008.12.005

Candolin, U., 2003. The use of multiple cues in mate choice. Biol. Rev. Camb. Philos. Soc. 78, 575–595.

Chandler, C.H., Ofria, C., Dworkin, I., 2013. Runaway sexual selection leads to good genes. Evolution 67, 110–119. https://doi.org/10.1111/j.1558-5646.2012.01750.x

Chapman, T., Liddle, L.F., Kalb, J.M., Wolfner, M.F., Partridge, L., 1995. Cost of mating in *Drosophila melanogaster* is mediated by male accessory gland products. Nature 373, 241–244.

Chenoweth, S.F., Rundle, H.D., Blows, M.W., 2008. Genetic constraints and the evolution of display trait sexual dimorphism by natural and sexual selection. Am. Nat. 171, 22–34.

Chippindale, A.K., Gibson, J.R., Rice, W.R., 2001. Negative genetic correlation for adult fitness between sexes reveals ontogenetic conflict in *Drosophila.* Proc. Natl. Acad. Sci. 98, 1671–1675.

Cox, R.M., Calsbeek, R., 2009. Sexually antagonistic selection, sexual dimorphism, and the resolution of intralocus sexual conflict. Am. Nat. 173, 176–187.

Dines, J.P., Mesnick, S.L., Ralls, K., May-Collado, L., Agnarsson, I., Dean, M.D., 2015. A trade-off between precopulatory and postcopulatory trait investment in male cetaceans. Evolution 69, 1560–1572. https://doi.org/10.1111/evo.12676

Droge-Young, E.M., Manier, M.K., Lüpold, S., Belote, J.M., Pitnick, S., 2012. Covariance among premating, post-copulatory and viability fitness components in *Drosophila melanogaster* and their influence on paternity measurement. J. Evol. Biol. 25, 1555–1563. https://doi.org/10.1111/j.1420-9101.2012.02540.x

Eberhard, W.G., 1996. Cryptic female choice. Princeton University Press, Princeton, N.J.

Eberhard, W.G., Rodríguez, R.L., Huber, B.A., Speck, B., Miller, H., Buzatto, B.A., Machado, G., 2018. Sexual selection and static allometry: The importance of function. Q. Rev. Biol. 93, 207–250. https://doi.org/10.1086/699410

Evans, J.P., Garcia-Gonzalez, F., 2016. The total opportunity for sexual selection and the integration of pre- and post-mating episodes of sexual selection in a complex world. J. Evol. Biol. 29, 2338–2361. https://doi.org/10.1111/jeb.12960

Evans, J.P., Zane, L., Francescato, S., Pilastro, A., 2003. Directional postcopulatory sexual selection revealed by artificial insemination. Nature 421, 360–363.

Filice, D.C., Dukas, R., 2019. Winners have higher pre-copulatory mating success but losers have better post-copulatory outcomes. Proc. R. Soc. B 286, 20182838.

Firman, R.C., Gasparini, C., Manier, M.K., Pizzari, T., 2017. Postmating female control: 20 years of cryptic female choice. Trends Ecol. Evol. 32, 368–382. https://doi.org/10.1016/j.tree.2017.02.010

Fisher, R.A., 1958. The genetical theory of natural selection, [2d rev. ed.]. ed. Dover Publications, New York.

Fisher, R.A., 1915. The evolution of sexual preference. Eugen. Rev. 7, 184.

Foerster, K., Coulson, T., Sheldon, B.C., Pemberton, J.M., Clutton-Brock, T.H., Kruuk, L.E., 2007. Sexually antagonistic genetic variation for fitness in red deer. Nature 447, 1107.

Gilbert, R., Uetz, G.W., 2016. Courtship and male ornaments as honest indicators of immune function. Anim. Behav. 117, 97–103. https://doi.org/10.1016/j.anbehav.2016.04.013

Godin, J.G.J., McDonough, H.E., 2003. Predator preference for brightly colored males in the guppy: a viability cost for a sexually-selected trait. Behav. Ecol. in press.

Head, M.L., Hunt, J., Jennions, M.D., Brooks, R., 2005. The indirect benefits of mating with attractive males outweigh the direct costs. Plos Biol. 3, 289–294. https://doi.org/Artn E33 Doi 10.1371/Journal.Pbio.0030033

Hosken, D.J., Taylor, M.L., Hoyle, K., Higgins, S., Wedell, N., 2008. Attractive males have greater success in sperm competition. Curr. Biol. 18, R553–R554. https://doi.org/10.1016/j.cub.2008.04.028

Iwasa, Y., Pomiankowski, A., 1991. The evolution of costly mate preferences II. The "handicap" principle. Evolution 45, 1431–1442.

Jennions, M.D., Petrie, M., 1997. Variation in mate choice and mating preferences: A review of causes and consequences. Biol. Rev. Camb. Philos. Soc. 72, 283–327.

Keller, L., Reeve, H.K., 1995. in: Advances in the Study of Behavior, Vol 24. Why Do Females Mate with Multiple Males - the Sexually Selected Sperm Hypothesis. pp. 291–315.

Kirkpatrick, M., 1982. Sexual selection and the evolution of female choice. Evolution 36, 1–12.

Kodric-Brown, A., Sibly, R.M., Brown, J.H., 2006. The allometry of ornaments and weapons. Proc. Natl. Acad. Sci. 103, 8733–8738.

Kotiaho, J.S., 2000. Testing the assumptions of conditional handicap theory: costs and condition dependence of a sexually selected trait. Behav. Ecol. Sociobiol. 48, 188–194.

Kuznetsova, A., Brockhoff, P., Christensen, R., 2017. lmerTest package: Tests in linear mixed effects models. J. Stat. Softw. 82, 1–26. https://doi.org/10.18637/jss.v082.i13

Lande, R., 1980. Sexual dimorphism, sexual selection, and adaptation in polygenic characters. Evolution 34, 292–305.

Lewis, S.M., Austad, S.N., 1994. Sexual selection in flour beetles: the relationship between sperm precedence and male olfactory attractiveness. Behav. Ecol. 5, 223–224.

Lüpold, S., Manier, M.K., Puniamoorthy, N., Schoff, C., Starmer, W.T., Luepold, S.H.B., Belote, J.M., Pitnick, S., 2016. How sexual selection can drive the evolution of costly sperm ornamentation. Nature 533, 535–538. https://doi.org/10.1038/nature18005

Lüpold, S., Tomkins, J.L., Simmons, L.W., Fitzpatrick, J.L., 2014. Female monopolization mediates the relationship between pre- and postcopulatory sexual traits. Nat. Commun. 5, 3184. https://doi.org/10.1038/ncomms4184

Manica, L.T., Macedo, R.H., Graves, J.A., Podos, J., 2016. Vigor and skill in the acrobatic mating displays of a Neotropical songbird. Behav. Ecol. arw143.

Manier, M.K., Belote, J.M., Berben, K.S., Lüpold, S., Ala-Honkola, O., Collins, W.F., Pitnick, S., 2013. Rapid diversification of sperm precedence traits and processes among three sibling *Drosophila* species. Evolution 67, 2348–2362. https://doi.org/10.1111/evo.12117

Manier, M.K., Belote, J.M., Berben, K.S., Novikov, D., Stuart, W.T., Pitnick, S., 2010. Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster.* Science 328, 354–357. https://doi.org/10.1126/science.1187096

Mead, L.S., Arnold, S.J., 2004. Quantitative genetic models of sexual selection. Trends Ecol. Evol. 19, 264–271. https://doi.org/10.1016/j.tree.2004.03.003

Miller, G.T., Pitnick, S., 2003. Functional significance of seminal receptacle length in *Drosophila melanogaster.* J. Evol. Biol. 16, 114–126. https://doi.org/10.1046/j.1420-9101.2003.00476.x

Miller, G.T., Pitnick, S., 2002. Sperm-female coevolution in *Drosophila*. Science 298, 1230–1233. https://doi.org/10.1126/science.1076968

Mobley, K.B., Morrongiello, J.R., Warr, M., Bray, D.J., Wong, B.B., 2018. Female ornamentation and the fecundity trade-off in a sex-role reversed pipefish. Ecol. Evol. 8, 9516–9525.

Parker, G.A., 1970. Sperm competition and its evolutionary consequences in insects. Biol. Rev. Camb. Philos. Soc. 45, 525-.

Parker, G.A., Lessells, C.M., Simmons, L.W., 2013. Sperm competition games: A general model for precopulatory male–male competition. Evolution 67, 95–109. https://doi.org/10.1111/j.1558-5646.2012.01741.x

Pilastro, A., Simonato, M., Bisazza, A., Evans, J.P., 2004. Cryptic female preference for colorful males in guppies. Evolution 58, 665–669.

Pischedda, A., Chippindale, A.K., 2006. Intralocus sexual conflict diminishes the benefits of sexual selection. PLoS Biol. 4, e356.

Pitnick, S., 1996. Investment in testes and the cost of making long sperm in *Drosophila*. Am. Nat. 148, 57–80. https://doi.org/10.1086/285911

Pitnick, S., Brown, W.D., 2000. Criteria for demonstrating female sperm choice. Evolution 54, 1052–1056. https://doi.org/10.1111/j.0014-3820.2000.tb00107.x

Pitnick, S, Markow, T.A., Spicer, G.S., 1995. Delayed male maturity is a cost of producing large sperm in *Drosophila.* Proc. Natl. Acad. Sci. U. S. A. 92, 10614–10618.

Pitnick, S., Marrow, T., Spicer, G.S., 1999. Evolution of multiple kinds of female sperm-storage organs in *Drosophila*. Evolution 53, 1804–1822. https://doi.org/10.1111/j.1558-5646.1999.tb04564.x

Pitnick, S., Miller Gary T., Schneider Karin, Markow Therese A., 2003. Ejaculate-female coevolution in *Drosophila mojavensis.* Proc. R. Soc. Lond. B Biol. Sci. 270, 1507–1512. https://doi.org/10.1098/rspb.2003.2382

Pitnick, Scott, Spicer, G.S., Markow, T.A., 1995. How long is a giant sperm? Nature 375, 109. https://doi.org/10.1038/375109a0

Polak, M., Simmons, L.W., 2009. Secondary sexual trait size reveals competitive fertilization success in *Drosophila bipectinata* Duda. Behav. Ecol. 20, 753–760. https://doi.org/10.1093/beheco/arp056

Pomiankowski, A., Iwasa, Y., Nee, S., 1991. The evolution of costly mate preferences I. Fisher and biased mutation. Evolution 45, 1422–1430.

R Core Team, 2017. R: A language and environment for statistical computing., R Foundation for Statistical Computing. Vienna, Austria.

Rice, W.R., 1984. Sex chromosomes and the evolution of sexual dimorphism. Evolution 38, 735–742.

Sbilordo, S.H., Martin, O.Y., 2014. Pre- and postcopulatory sexual selection act in concert to determine male reproductive success in *Tribolium castaneum*. Biol. J. Linn. Soc. 112, 67–75. https://doi.org/10.1111/bij.12262

Simmons, L.W., Lüpold, S., Fitzpatrick, J.L., 2017. Evolutionary trade-off between secondary sexual traits and ejaculates. Trends Ecol. Evol. 32, 964–976. https://doi.org/10.1016/j.tree.2017.09.011

Singmann, H., Bolker, B., Westfall, J., Aust, F., 2016. afex: Analysis of factorial experiments.

Svobodová, J., Bauerová, P., Eliáš, J., Velová, H., Vinkler, M., Albrecht, T., 2018. Sperm variation in Great Tit males (*Parus major*) is linked to a haematological health-related trait, but not ornamentation. J. Ornithol. 159, 815–822. https://doi.org/10.1007/s10336-018-1559-7

Therneau, T., 2015. A package for survival analysis in S.

Travers, L.M., Garcia-Gonzalez, F., Simmons, L.W., 2016. Genetic variation but weak genetic covariation between pre- and post-copulatory episodes of sexual selection in *Drosophila melanogaster*. J. Evol. Biol. 29, 1535–1552. https://doi.org/10.1111/jeb.12890

Voje, K.L., 2016. Scaling of morphological characters across trait type, sex, and environment: a meta-analysis of static allometries. Am. Nat. 187, 89–98.

Wojcieszek, J.M., Simmons, L.W., 2012. Evidence for stabilizing selection and slow divergent evolution ofmale genitalia in a millipede (*Antichiropus variabilis*). Evolution 66, 1138–1153. https://doi.org/10.1111/j.1558-5646.2011.01509.x

Zahavi, A., 1977. The cost of honesty (further remarks on the handicap principle). J. Theor. Biol. 67, 603–605.

Zahavi, A., 1975. Mate selection- a selection for a handicap. J. Theor. Biol. 53, 205–214.

Zuk, M., Johnsen, T.S., Maclarty, T., 1995. Endocrine-immune interactions, ornaments and mate choice in red jungle fowl. Proc. Biol. Sci. 260, 205–210.

Tables

Table 1. Summary of results showing fitness benefits (+) or costs (-) of long phenotypes. Parentheses indicate a marginally insignificant trend, and NS indicates no significant difference between long and short phenotypes.

	Sperm		SR	
	♂	♀	♂	♀
Mating success	-		+	
Latency	NS		-	
Copulation duration	NS		-	
Fecundity	(+)	(+)	+	NS
Longevity	+	+	(+)	+

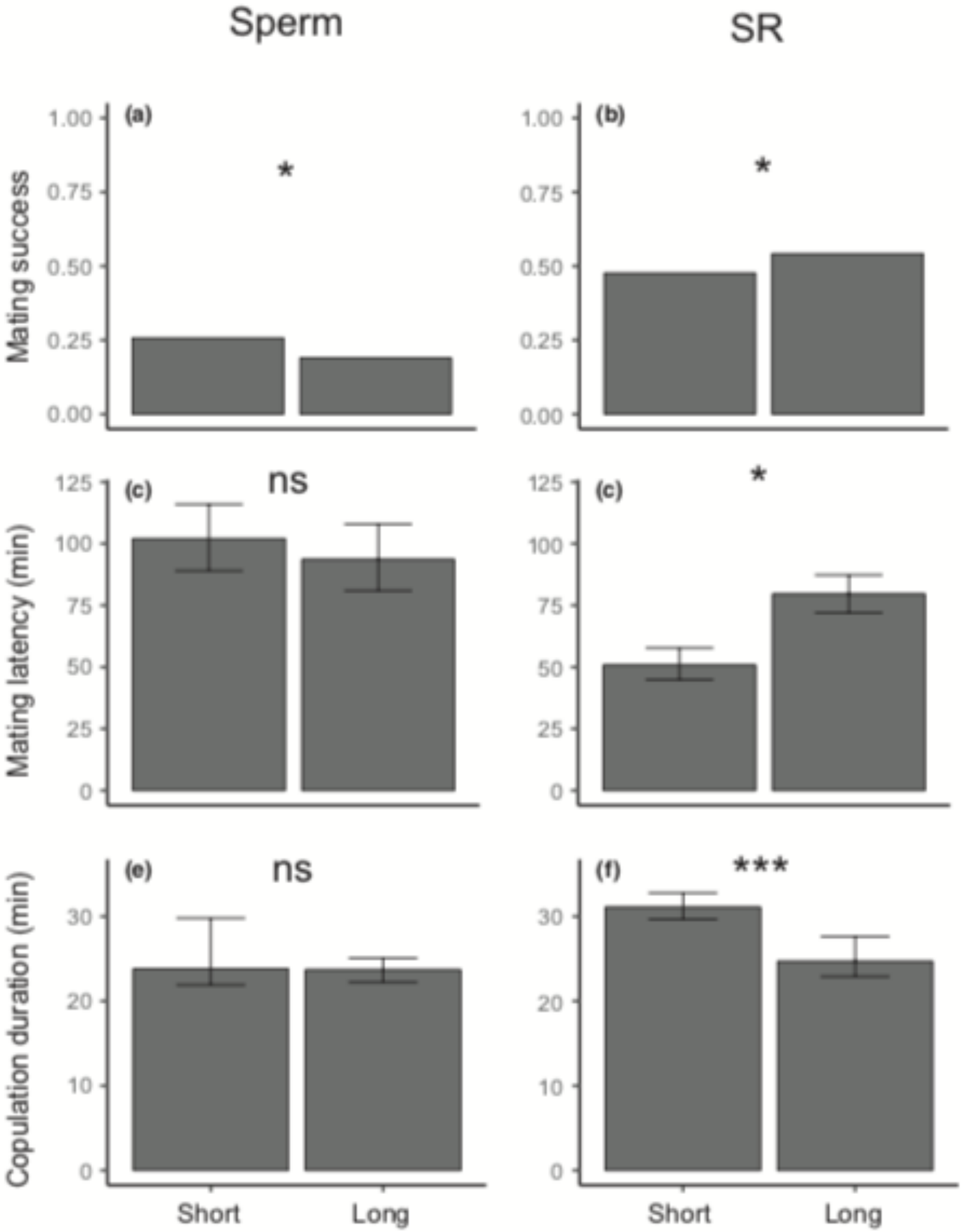
Figure captions

FIGURE 1. Mating success (A, B), mating latency (C, D), and copulation duration (E, F) in males from the sperm selected (A, C, E) and SR selected (B, D, F) lines.

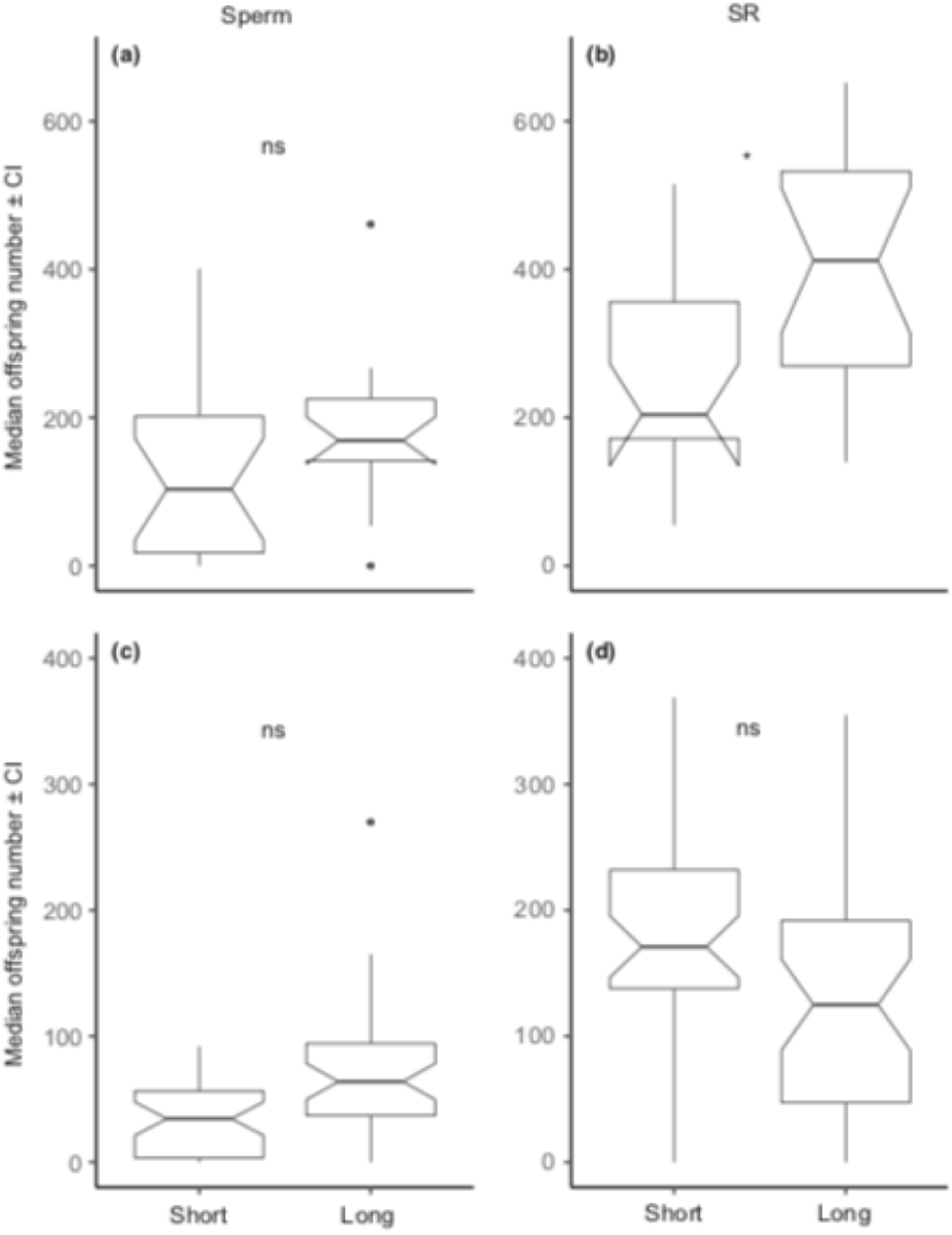
FIGURE 2: Number of offspring produced after mating trials by subsamples of males from long sperm (A), long SR (B), short sperm (C), and short SR (D) lines.

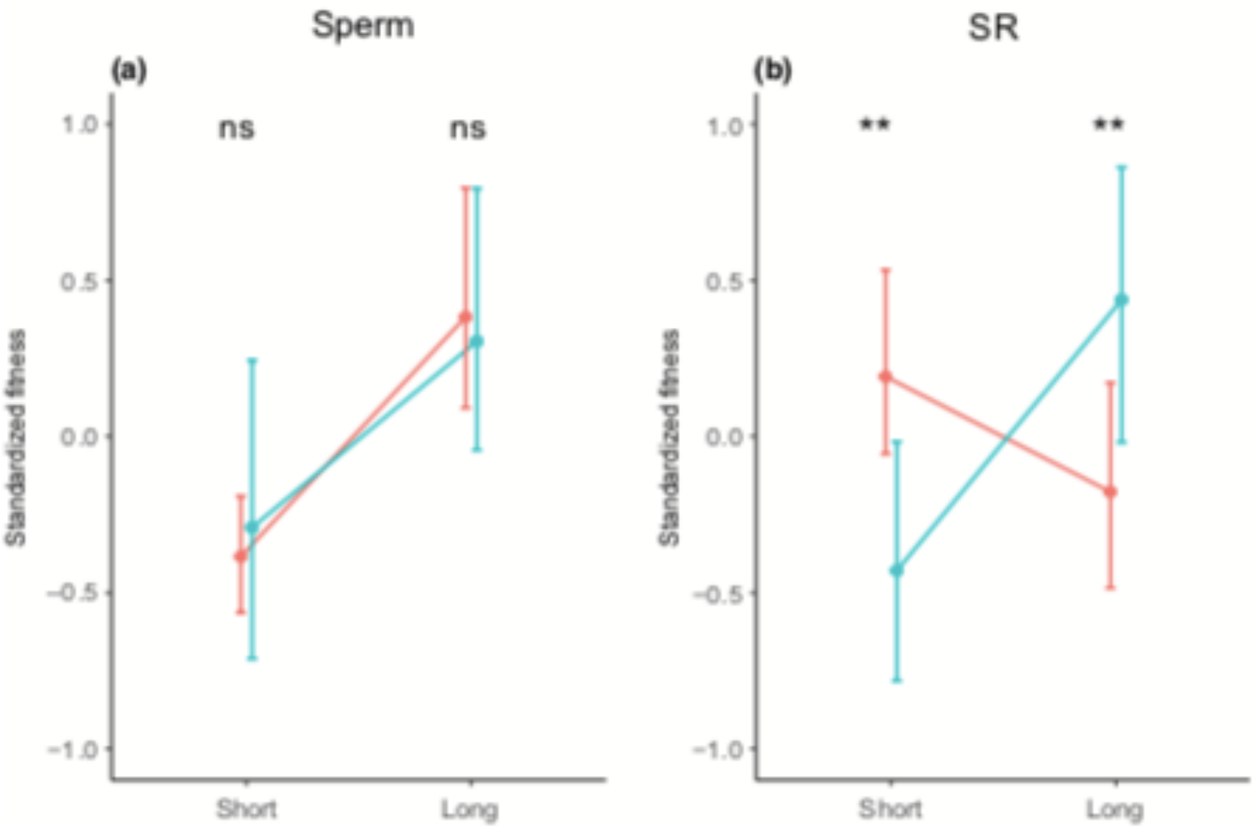
FIGURE 3: Standardized male and female fitness (mean ± bootstrapped 95% CI), for A) sperm and B) SR lines. Blue: male; red: female.

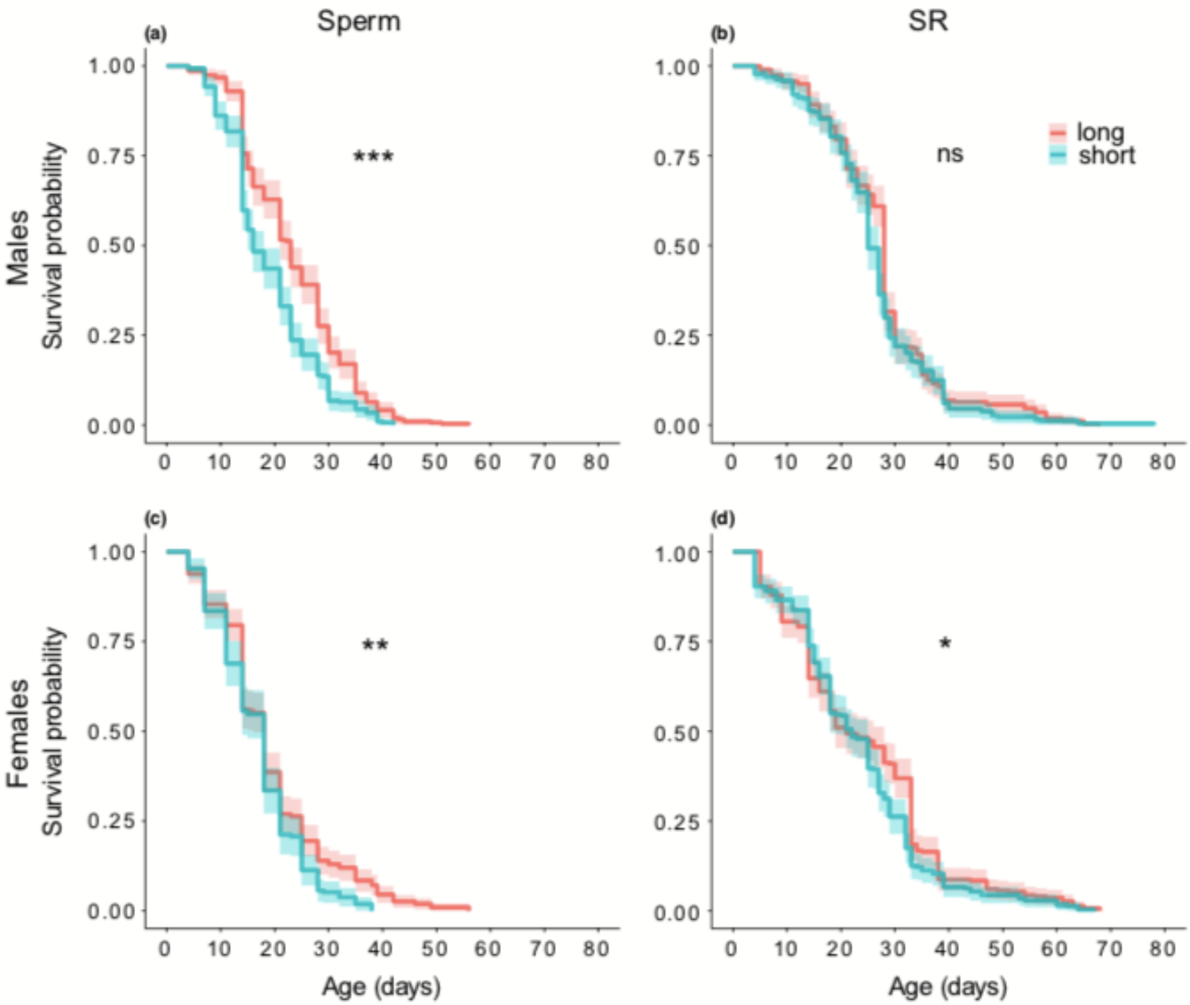
FIGURE 4. Survival curves of males from sperm selected lines (A) and of SR selected lines (B) and of females from sperm (C) and SR selected lines (D). Line colours represent selection regime (blue: short trait values; red: long trait values). Shaded areas represent 95% confidence intervals. Age refers to adult age


Sperm
SR
Mating success
1.00
0.75
0.50
0.25
0.00
(a)
*
(b)
*
Mating latency (min)
125
100
75
50
25
0
(c)
ns
(c)
*
Copulation duration (min)
30
20
10
0
(e)
ns
(f)
***
Short
Long
Short
Long

562


Sperm
SR
(a)
(b)
(c)
(d)
Median offspring number ± CI
Median offspring number ± CI
600
400
200
0
400
300
200
100
0
ns
ns
ns
Short
Long
Short
Long


Sperm
SR
(a)
(b)
ns
ns
**
**
1.0
0.5
0.0
−0.5
−1.0
Standardized fitness
Short
Long


(a)
Sperm
(b)
SR
Males
Survival probability
1.00
0.75
0.50
0.25
0.00
***
ns
long
short
0
10
20
30
40
50
60
70
80
(c)
(d)
Females
Survival probability
**
*
Age (days)
Age (days)

566