

# Sexual conflict selects for male and female reproductive characters

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**Background:** Strict genetic monogamy leads to sexual harmony because any trait that decreases the fitness of one sex also decreases the fitness of the other. Any deviation from monogamy increases the potential for sexual conflict. Conflict is further enhanced by sperm competition, and given the ubiquity of this phenomenon, sexual conflict is rife. In support of theory, experimentally enforced monogamy leads to the evolution of sexual benevolence. In contrast, with multiple mating, males evolve traits causing massive female fitness reductions when female evolution is restrained. Theory also predicts increased investment in spermatogenesis when sperm competition risk is high. While this supposition has correlational support, cause and effect has yet to be firmly established.

**Results:** By enforcing monogamy or polyandry in yellow-dung-fly lines, we have shown experimentally that males from polyandrous treatments evolved larger testes. Furthermore, females from this treatment evolved larger accessory sex glands. These glands produce a spermicidal secretion, so larger glands could increase female ability to influence paternity. Using molecular techniques, we have shown that, consistent with this idea, males' success as second mates is reduced in females from the polyandrous treatment. Nevertheless, males from polyandrous lines achieve higher paternity during sperm competition, and this finding further supports the testis evolution patterns.

**Conclusions:** These results provide direct experimental support for macroevolutionary patterns of testis size evolution. Furthermore, we have shown that sperm competition selects for traits likely to be important in sexual conflicts over paternity, a result only previously demonstrated in *Drosophila melanogaster*.

## Background

Strict genetic monogamy leads to sexual harmony because any trait decreasing the fitness of one sex also decreases it for the other. However, any deviation from monogamy increases sexual conflict because individuals' lifetime reproductive interests will not coincide [1]. Therefore, sexual conflict should increase with multiple mating [1], as does the potential for sperm competition [2]. As a result, sperm competition should enhance sexual conflict and thus lead to the evolution of characters that increase reproductive success in one sex even when they are costly to the other. Experimental confirmation was found when female *Drosophila melanogaster* flies were prevented from coevolving with males that evolved traits that caused massive female fitness reductions [3]. In contrast, in the same taxon experimentally enforced monogamy selects for sexual benevolence [4]. Therefore, in at least one species, traits evolving under multiple mating and sperm competition fit theoretical predictions.

Theory also predicts that when sperm competition is fun-

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damentally analogous to a raffle, the relative numbers of sperm in competition will be the primary determinant of success [2, 5, 6]. This should select for increased investment in spermatogenesis since larger testes typically produce ejaculates containing more sperm [7, 8, but see 9] and correlational evidence for positive associations exists [10–14]. Theory is less clear regarding the effects of sperm competition on sperm size [15] and indicates that increased sperm size will only be selectively favored under certain restrictive conditions [15 and see 9, 16]. Nevertheless, correlational evidence for positive associations between sperm competition risk and sperm size exists [11, 17, 18], although not in all taxa [12]. Furthermore, increased sperm size often results from sexual conflict over storage, with sperm size tracking changes in female morphology [18–21, 33]. However, studies of evolutionary influences of sperm competition on testis size and sperm length are typically correlational and do not establish cause and effect. Nevertheless, direct selection on testis size can lead to rapid size divergence as well as cause correlated responses in other characteristics, such as sperm size [22]. However, the only published experimental

study of microevolutionary influences of sperm competition did not exclude inbreeding effects [23].

We conducted an experimental study of the effects of sperm competition (ten generations of polyandrous [P] or monogamous [M] matings) on reproductive characters in male and female yellow dung flies. We also performed a sperm competition experiment that pitted P males against M males and compared the proportion of offspring that each fathered. The yellow dung fly, *Scathophaga stercoraria* (*Scatophaga*), is a model system for sperm competition studies [24]. Males that copulate second typically fertilize about 80% of the subsequent clutch, and fertilization success also depends on copula duration, which correlates negatively with body size [25–28]. Females may also influence paternity [27], and there is some evidence that sperm size, a highly heritable trait [29], influences sperm storage [28].

## Results

Body size did not differ between males from crossed monogamous lines ([MX], in which crossing controlled for inbreeding differences between monogamous [M] and polyandrous [P] lines), and P males (mean  $\pm$  SE hind-tibia length: P =  $3.3 \pm 0.04$  mm, MX =  $3.5 \pm 0.07$  mm). Body size comparison: P v. MX;  $F_{1,9} = 3.26$ ;  $p = 0.10$ ), but P males had significantly larger testes than did MX males (mean  $\pm$  SE:  $0.954 \pm 0.016$  mm<sup>2</sup> v.  $0.920 \pm 0.046$  mm<sup>2</sup>. Testis area: Treatment [P v. MX]  $F_{1,8} = 10.05$ ;  $p = 0.013$ ; HTL  $F_{1,8} = 20.82$ ;  $p = 0.002$ ; the interaction was not significant [ $F_{1,7} = 1.88$ ;  $p = 0.21$ ] and was removed). To see how testis size had diverged and if differences between MX and P lines were due to selection via sperm depletion, we compared P, MX, and W (wild-type) flies. P males had larger testes than did MX or W males, which did not statistically differ (mean residual testis area  $\pm$  SE: P =  $0.042 \pm 0.012$  mm<sup>2</sup>, MX =  $-0.046 \pm 0.031$  mm<sup>2</sup>, W =  $-0.124 \pm 0.055$  mm<sup>2</sup>. ANOVA of relative testis area was calculated as residuals from a linear regression of testis area on body size of P, MX, and W males with males as the replicate and was significant:  $F_{1,47} = 12.3$ ,  $P < 0.0001$ . Fisher's PLSD: P > MX,  $p = 0.027$ ; P > W,  $p < 0.001$ ; MX = W,  $p = 0.10$ ). These results indicate that inbreeding differences did not cause divergence in testis size and hence confirm conclusions that sperm competition selects for testis size [23].

Sperm length did not differ across treatments (mean  $\pm$  SE sperm length: P =  $210.4 \pm 0.8$   $\mu$ m v. M =  $210.8 \pm 1.2$   $\mu$ m), and it was not associated with body size, testis size, or residual-testis size (ANCOVA or ANOVA of sperm size with factor, treatment (P, M), and covariate, HTL, with HTL alone or with either testis size measure: all  $F < 2.64$ , all  $p > 0.18$ ).

In the sperm competition experiment, copula duration

**Table 1**

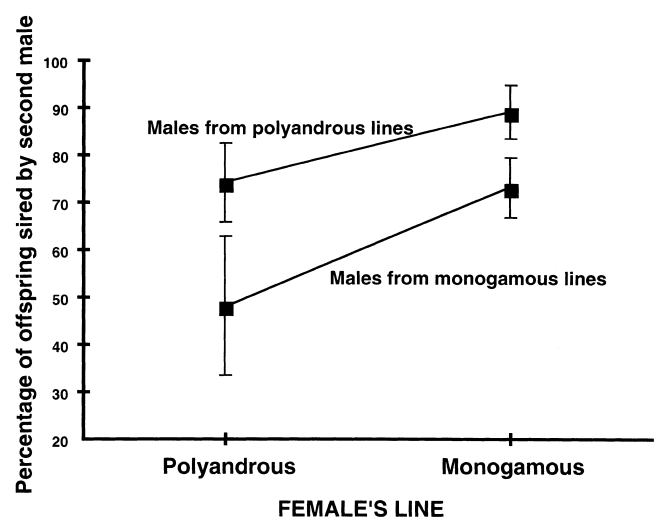
**Comparison of arcsine square root-transformed proportion of offspring fathered by the second male to copulate with a female.**

Source	DF	MS	F	P
Female	1	0.60	6.7	0.018
Male 2	1	0.47	5.3	0.033
Copula difference	1	0.28	3.1	0.095
Error	18	0.09		

The female (from M or P lines) and the second male to copulate (from M or P lines) were factors, and the difference in copula duration between first and second copulations was entered as a covariate (copula duration influences paternity). Results do not differ when copula durations are covariates. Shown is the simplest model obtained by backward elimination of all nonsignificant higher-order effects containing the covariate (NB the male-by-female interaction was not significant:  $F_{1,17} = 0.002$ ;  $p = 0.96$ ). Male body size did not differ between treatments (ANOVA:  $F_{1,6} = 0.062$ ;  $p = 0.82$ ).

did not significantly differ between P and M flies (males, P =  $38.6 \pm 1.8$  min v. M =  $36.7 \pm 1.9$  min; females, P =  $37.1 \pm 2.5$  min v. M =  $37.8 \pm 2.4$  min), nor were any interactions significant (ANOVA: all  $F < 1.11$ ; all  $p > 0.3$ ). A pairwise comparison also found no significant differences between a female's first or second copulation (d.f. = 24; paired  $t = -0.417$ ;  $p = 0.68$ ). However, treatment significantly influenced fertilization success (Table 1; Figure 1). Males from polyandrous lines were more successful during sperm competition, but male size did not differ between treatments. The proportion of sperm alive in the testes of three-week-old M and P males was also not

**Figure 1**



Interaction plot of P2 data (mean  $\pm$  SE) percentage of offspring sired by the second male to copulate) when males from polyandrous lines competed against males from monogamous lines in females from both lines. Both male and female effects are significant. Transformed data were used in the analysis; raw data are shown here for visual purposes only.

**Table 2****Results of the female-accessory-sex-gland size comparison.**

Source	DF	MS	F	P
Treatment (P or M)	1	0.0028	11.4	0.020
HTL	1	0.0054	21.8	0.006
Error	5	0.0002		

Treatment and body size significantly influenced gland size, with P females having larger glands than M females. The interaction between treatment and hind-tibia length was not significant ( $F_{1,4} = 0.03$ ;  $p = 0.86$ ) and was removed from the model.

significantly different (mean  $\pm$  SE%: M =  $79.5 \pm 3.3$ , P =  $76.1 \pm 23$ . Mann-Whitney U = 108.5;  $p = 0.32$ ).

Female treatment also significantly influenced paternity, with the low P2 from P females indicating sexual conflict over paternity (Table 1). To investigate potential mechanisms to explain the P2 data, we measured spermathecal and accessory reproductive gland (AG) size in females from each treatment. Neither female body size nor total or mean spermathecal volume differed significantly between the groups, and there were no significant interactions between female body size and spermathecal volume (female body size:  $F_{1,6} = 0.004$ ,  $p = 0.95$ ; two measures of spermathecal size: all  $F_{1,4} < 1.51$ ,  $p > 0.28$ ; the interactions:  $F_{1,4} < 1.45$ ,  $p > 0.29$ ). However, AG size was significantly larger in P females (residual means  $\pm$  SE: P =  $0.014 \pm 0.009$  mm<sup>2</sup> v. M =  $-0.014 \pm 0.011$  mm<sup>2</sup>; Table 2).

## Discussion

Testis size comparison of polyandrous (P) and monogamous crossed (MX) lines indicates that differences in inbreeding did not lead to the divergence in testis size previously reported and hence confirms conclusions that increased (relaxed) sperm competition leads to increased (reduced) testis size [23]. Furthermore, the divergence in testis size appears to be largely driven by an increase in testis size in P males since these, but not MX males, differed from wild-type (W) flies. Therefore, these results provide direct experimental evidence that sperm competition selects for larger testis size, and they strongly support models of numerical sperm competition [2, 5, 6] while providing microevolutionary verification of macroevolutionary patterns found across many taxa [10–14]. Similarly, in *Drosophila melanogaster* testis size decreases with relaxed sperm competition [44].

The lack of testis size difference between monogamous (M) and W males is surprising since in the field males essentially always experience sperm competition [25]. The most parsimonious explanation is simply that testis size in M males was not selected for and did not change. Alternatively, field males may be selected to invest more in somatic growth since large size is a critical component of mating success in nature. Since M males were mating

with larger females (due to the benign laboratory conditions), they may have maintained larger testes because of dilution effects (i.e., larger females require larger ejaculates to fill their sperm stores). The increased testis size of P males is certainly due to the increased sperm competition they faced compared with free-living males as females are extremely unlikely to copulate with three males per clutch in nature [25].

Unlike testis size, sperm length did not evolve in response to our treatments in spite of its high heritability [29]. This indicates that sperm length is not an important determinant of fertilization success in this species, as may have been expected based on the high  $h^2$  [30]. This result is also consistent with theory that predicts that sperm size should evolve independently of sperm competition [15]. However, we found no evidence for sperm size/number trade-offs. In contrast, direct selection on testis size in another fly caused correlated responses in sperm size indicative of pleiotropy between the two traits [22], and many studies have found positive associations between sperm size and sperm competition success or risk [17, 18]. However, decreased sperm competition did not consistently alter sperm length in *Drosophila* [44], and in yellow dung flies it appears that sperm length is maintained at some (quasi) stable optima (seemingly) independently of sperm number and sperm competition, perhaps by sperm/egg interactions [31, 32] or sexual conflict over optimal sperm size [29, 33].

The sperm competition experiment largely confirmed the testis evolution data; larger testes are advantageous during sperm competition. The fact that males did not differ in sperm quality or length further supports this conclusion. In contrast, no differences were found in the fertilization success of beetle morphs differing in testis size, even though sperm apparently compete numerically [34]. Likewise, sperm competition success in *Drosophila* lines did not covary with testis size and sperm number [44]. In yellow dung flies numerical sperm competition is well supported [25–28], and hence differences in competitive ability based on testis size were expected. Furthermore, males did not adjust copula duration relative to female mating status, males from both treatments mated for equal durations, and their body sizes did not differ. Therefore, P males must be more efficient at displacing rival sperm as their success during competition was greater. Additionally, since MX males did not differ from wild-type males, our result supports comparisons across species and suggests that testis size evolution is more strongly affected by sperm competition than by sperm depletion [35].

Males from polyandrous lines also had P2 values in the range of those typically reported for wild-type males [25]. However, the very low P2 values obtained in matings with P females appears indicative of sexual conflict over

paternity, at least between the female and last male, and of the strong nature of the sperm competition selection imposed on P flies. The selective benefit of lower P2 in the context of this experiment may come either from the “sexy-sperm” hypothesis, which suggests that females should provide increased opportunity for direct competition between males’ sperm [37], or from increased variation within a clutch. However, conditions favoring increased variance are probably restrictive [36]. The result certainly supports recent work showing P2 variation attributable to females [27], and this is one of a growing number of studies to show unequivocal male and female influences on paternity [38–41].

There was no difference between female sperm store size across our treatments. Similarly, female storage organs did not respond to direct selection on testis length in another fly [22]. This tentatively suggests that differences in female accessory gland size in our treatments were not due to pleiotropy, although this requires further investigation. Given the male results, we also do not think the difference is due to inbreeding variation. However, a larger accessory gland may be expected in P females since the secretion may be required for lubrication, protection of the female reproductive tract during copula, and/or production of a hostile insemination site [42]. The gland contents are certainly extruded during copula [42], and they have been shown to have spermicidal properties (Bernasconi et al., personal communication). This latter property potentially provides a mechanism for the P2 reduction observed for P females.

## Conclusions

Overall, our results provide direct experimental evidence that sperm competition selects for larger testis size, as predicted by theory, and support macroevolutionary patterns. Furthermore, male and female characters independently influence fertilization success, as previously shown in *Drosophila* [41]. Sperm competition and sexual conflict therefore select for characters in both sexes that are beneficial in the coevolutionary struggle over paternity.

## Materials and methods

Thirty-five female flies were collected at Fehraltorf, Switzerland in Autumn 1998 and brought to the laboratory. They were allowed to lay eggs, and the flies that developed were used as the parental generation of the experimental flies. Second-generation laboratory flies were divided into two treatments, polyandrous (P) and monogamous (M), and each replicated four times ( $n = 8$ ). P females copulated with three males in succession before laying one clutch of eggs, while M females copulated only once. From each clutch, three to four flies were collected at emergence, housed, and given food, sugar, and water as needed. A subset of these flies were used for starting the next generation. After ten generations, virgin males and non-virgin females were frozen for subsequent investigation (see [23] for further details). To eliminate inbreeding differences as a confounding factor, we crossed M lines (MX) and compared testes size of MX and P males. One male from each cross was measured ( $n = 7$ ), and these measurements were used as independent data points in the analysis, which compared them with mean P line values (lines were the replicate). In addition, relative testes size of P, MX, and wild-

type (W) males were compared. Males were the replicate in this analysis because of the asymmetry created by treating P lines as replicates but also treating MX and W males as replicates. W males were second-generation laboratory flies that originated from the same wild population as the lines. All measurements were made with a binocular microscope and OPTIMAS software. After testes measurement in the M and P males, sperm were released from the ejaculatory duct into a drop of insect ringer on a microscope slide. After air drying, slides were rinsed in distilled water, and the length of 30 sperm/slide (per male,  $n = 10$  males/line) were measured blindly. The percentage of sperm alive in the testes of P and M males, which is one aspect of sperm quality, was also assessed blindly. For females, the three sperm stores (spermathecae) and accessory reproductive glands (AG) were dissected out in a drop of insect ringer on a microscope slide ( $n = 5$  females/line). Spermathecae and AGs were gently placed beneath a coverslip, and the perimeter of each spermathecae was recorded and subsequently used for calculating spermathecal volume. For AGs, area was calculated as for testis area. Body size (hind tibia length, or HTL) was also measured for all flies included in the analysis. In the sperm competition experiment, males from P lines competed against M males, in both M and P females, and as both first and second mates. However, females never copulated with males from the same line as there may have been coevolution that advantaged/disadvantaged males with whom females had been evolving. Copulation duration was measured (blindly), and paternity was assigned (blindly) to a random sample of 12 offspring from each of 22 competitive crosses by the use of microsatellite loci [43].

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