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ADAPTATION TO EXPERIMENTAL ALTERATIONS OF THE OPERATIONAL SEX RATIO IN POPULATIONS OF *DROSOPHILA MELANOGASTER*

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Theory predicts that males adapt to sperm competition by increasing their investment in testis mass to transfer larger ejaculates. Experimental and comparative data support this prediction. Nevertheless, the relative importance of sperm competition in testis size evolution remains elusive, because experiments vary only sperm competition whereas comparative approaches confound it with other variables, in particular male mating rate. We addressed the relative importance of sperm competition and male mating rate by taking an experimental evolution approach. We subjected populations of *Drosophila melanogaster* to sex ratios of 1:1, 4:1, and 10:1 (female:male). Female bias decreased sperm competition but increased male mating rate and sperm depletion. After 28 generations of evolution, males from the 10:1 treatment had larger testes than males from other treatments. Thus, testis size evolved in response to mating rate and sperm depletion, not sperm competition. Furthermore, our experiment demonstrated that drift associated with sex ratio distortion limits adaptation; testis size only evolved in populations in which the effect of sex ratio bias on the effective population size had been compensated by increasing the numerical size. We discuss these results with respect to reproductive evolution, genetic drift in natural and experimental populations, and consequences of natural sex ratio distortion.

KEY WORDS: **Genetic drift, meiotic drive, sex ratio distortion, sexual conflict, sperm competition, *Wolbachia*.**

In species in which females mate multiply, the reproductive success of a male depends not only on his capacity to acquire matings, but also on his success in the subsequent competition among sperm for fertilization. Theory predicts that higher levels of sperm competition select for increased ejaculate size (Parker 1990; Williams et al. 2005), because the transfer of larger amounts of sperm increases fertilization success by either diluting or displacing

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competing ejaculates. Accordingly, males are expected to have larger testes relative to their body size when sperm competition risk is high. This prediction is supported by two sets of experimental data, one obtained from the fruitfly *Drosophila melanogaster* (Holland and Rice 1999), the other from the dung fly *Scathophaga stercoraria* (Hosken et al. 2001; Hosken and Ward 2001). In these experiments, the effect of sperm competition on testis size was assessed by allowing populations to evolve under polyandry (several males mating with each female) or under monogamy (a single male mating with each female). Both experiments showed that, as predicted by theory, males evolving under monogamy had smaller testes relative to their body size than males evolving under polyandry (Hosken et al. 2001; Hosken and Ward 2001; Pitnick et al. 2001).

The relationship between testis size and sperm competition has also been investigated in comparative studies. These revealed that, as predicted, relative testis size correlated positively with the presumed intensity of sperm competition across species of primates (Harcourt et al. 1981), micro- and megachiroptera (Hosken 1997, 1998), fish (Stockley et al. 1997), birds (Birkhead and Møller 1992), and butterflies (Gage 1994). However, due to their correlative nature, comparative studies are unable to differentiate between sperm competition per se and any variable with which it might be correlated. One potentially confounding factor is male mating frequency (Blanckenhorn et al. 2004). Because a mating involves both a male and a female, an increase in female mating rate will entail a correlated increase in male mating rate. As a consequence, the conditions under which sperm competition is intense are often also those that select for a male's capacity to perform large numbers of matings. Because both require a high rate of sperm production, it can be difficult to pinpoint the precise factor(s) selecting for increased testis size in species subjected to sperm competition.

In some cases, the effect of male mating rate can clearly be ruled out. For example, Birkhead and Møller (1992) found that testes are small in lekking species of birds, where female remating is virtually absent, but in which a very small number of males perform most matings. The same is true in primate species in which some males monopolize harems of several females. Here again, reproducing males have small testes despite their high rate of mating (Harcourt et al. 1981). Other studies, however, cannot avoid the confounding effect of male mating rate because they assess the level of sperm competition through the number of times that females mate (Gage 1994) or the size of the social groups in which matings take place (Hosken 1997, 1998).

To obtain an understanding of how the mating system shapes a species' reproductive biology, we need to assess the importance of mating frequency relative to that of sperm competition. One way to do so experimentally is to manipulate the population sex ratio. This approach was taken by Wigby and Chapman (2004), who derived selection lines of *D. melanogaster* under sex ratios of 3:1 male bias, 3:1 female bias, and an even sex ratio. By manually altering the population sex ratio, Wigby and Chapman increased the level of multiple mating in females (and hence sperm competition) in the male-biased lines and decreased it in the female-biased lines, compared to populations with an even sex ratio. In parallel to these changes, the number of mating opportunities for males decreased (male bias) or increased (female bias) relative to the control. If sperm competition were the prime agent behind the evolution of testis size, we would expect males from male-biased populations to have the largest testes. If, on the other hand, male mating rate is the important factor, the largest testes should be found in males from the female-biased treatment. Wigby and Chapman's (2004) experiment did not provide evidence to distinguish between these two hypotheses. Although the experimental manipulation resulted in differences in female mating rate between regimes, no change in male reproductive morphology was observed after 32 or 86 generations of selection (Wigby and Chapman 2004). Thus, males from different selection regimes differed neither in the size of their testes nor in the size of their accessory glands. These latter structures are the paired organs that produce proteins and peptides transferred along with sperm during mating (Chen 1984) and which may be involved in an antagonistic manipulation of the female's reproductive schedule (Eberhard and Cordero 1995; Wolfner 1997, 2002; Chapman 2001). Given this lack of response to selection, the impact of mating opportunities on the evolution testis size remains to be demonstrated.

Here, we present the results of a new evolutionary experiment in *D. melanogaster*. Our experimental design was based on Wigby and Chapman's (2004) approach of manipulating the mating system of populations through changes in the sex ratio. However, it deviated from that of the previous study in several ways. First, we concentrated on female bias, enabling us to cover a wider range of conditions and selection pressures. Thus, we subjected populations to sex ratios of 1:1, 4:1, and 10:1 (females: males). Second, our experimental treatments incorporated the fact that sex ratio bias entails a reduction in the effective population size (N_e). To address variations in N_e, we adapted the numerical population size of biased populations to maintain a roughly constant N_e across treatments, but included a 10:1 treatment without correction (with $N_e \approx 30$) as a contrast.

Measurements of male reproductive morphology as well as experimental data on the treatment effects in our setup demonstrate that testes size evolves in response to selection on male fertilization capacity rather than the intensity of sperm competition. After 28 generations of evolution, males from the most female-biased treatment (10:1) had larger testes than males from other treatments. Moreover, the evolutionary response was restricted to the 10:1 treatment with high effective population size, showing that the increase in genetic drift associated with sex ratio distortion

can impede adaptive responses to an altered mating system. We discuss these findings with respect to previous results on the evolution of male reproductive morphology, the role of genetic drift in adaptation, and the evolutionary impact of natural sex ratio distorters on their hosts.

Materials and Methods

MAINTENANCE OF SELECTION LINES

The *D. melanogaster* used in this experiment were derived from the Dahomey wild-type stock. The Dahomey stock has been maintained for over 30 years under laboratory conditions (large population size with overlapping generations, cf. Wigby and Chapman 2004). In preparation for this experiment, a population of Dahomey flies was maintained at 21°C (and otherwise identical rearing conditions) for a period of six months.

Selection lines were established under four different selection regimes (hereafter referred to as "treatments"). Three treatments consisted of applying alterations to the population sex ratio, with female bias of 1:1, 4:1, and to 10:1. Given that a bias in the population sex ratio leads to a decrease in the effective population size, the numerical population size was increased in parallel with female bias, to keep the effective population size roughly constant across these three treatments. The correction was based on the inbreeding effective population size with separate sexes, $N_e = 4N_mN_f / (N_m + N_f)$ (Crow and Kimura 1970, p. 350). Populations thus consisted of 50 females and 50 males (1:1), 125 females and 31 males (4:1), and 275 females and 28 males (10:1 high N_e). In a fourth treatment (10:1 low N_e), flies were maintained under a 10:1 female bias without correcting for the decrease in effective population sizes. These populations consisted of 91 females and nine males. Each treatment was replicated in four independently evolving lines (hereafter referred to as "lines").

In every generation, larvae were reared at a constant density of 300 individuals per 1/3 pint (190 mL) bottle containing 65 mL of sugar-yeast medium (SY, 10% w/v autolyzed yeast powder, 10% dextrose, 2% agar, 0.3% propionic acid, 3 g Nipagin per liter). Adults were collected as virgins within 8 h from eclosion under cold anesthesia and kept separated by sex in groups of 20 individuals in vials containing 7 mL of SY medium and live yeast. At the age of 1–2 days, adult flies were placed into cages in numbers corresponding to the treatment. The cages were sized in a way to maintain a constant volume per fly across treatments and contained a plate of grape juice medium and live yeast paste. The flies were allowed to interact and lay eggs in cages over a period of four days. Every day the cages were supplied with fresh grape juice medium and yeast paste ad libitum. At the time of food renewal, flies were briefly anaesthetized under CO_2 when the food plates were changed. Eggs laid over the final 24 h of the four-day interaction period were used to found the next generation. Eggs were incubated at 18°C for 48 h to allow larvae to hatch, after which first instar larvae were transferred to culture bottles at a constant density. With the exception of the egg incubation period, lines were maintained at 21°C throughout the selection process.

ASSESSMENT OF TREATMENT EFFECTS

We performed an experiment to assess the effect of our sex ratio treatments on male mating rate and the incidence of female multiple mating (which determines the risk of sperm competition experienced by males). The experiment was performed using non-selection line flies from the wild-type Dahomey stock, as well as a copy of this stock into which we had twice back-crossed the recessive eye-color mutant *scarlet* (*Dahomey scarlet*). For each treatment, we set up three replicate cages containing flies in numbers that matched the selection treatments, with all females being homozygous *D. scarlet* and half of the males being homozygous *D. scarlet* and half being Dahomey wild-type. Flies were reared according to the maintenance scheme above during the two generations preceding the experiment.

Flies were left for four days in the cages with daily changes of food, replicating the conditions in the selection scheme. At the end of the mating period, all females were transferred singly to vials containing 7 mL of SY food and left to lay eggs for four days, after which they were discarded, and the offspring emerging from each of these vials were then scored. For each female, we recorded whether offspring were absent, purely wild-type (i.e., sired by at least one wild-type male), purely *scarlet* (sired by at least one *scarlet* male), or a mixture of wild-type and *scarlet* (sired by at least one each of a *scarlet* and a wild-type male). For vials with offspring, we recorded whether there were few ($\leq$10) or many ($>$10) offspring produced.

MORPHOLOGICAL MEASUREMENTS

Morphological measures were taken after 28 generations of selection. The flies to be measured were reared under conditions identical to those used during population maintenance. Upon eclosion, flies were separated by sex to maintain virginity and placed in groups of 10 individuals in vials containing 7 mL of SY medium and live yeast. Flies were stored for a week before being measured.

Wing area

We measured wing area as a proxy for body size in males and females of all selection lines, following the protocol developed by Gilchrist and Partridge (2001). Both wings of an individual were mounted on a glass microscope slide. A digital picture of the slide was taken at 100× magnification under a compound microscope and the area of the wing was determined by measuring the distance between six landmarks defined in Gilchrist and Partridge (2001) using the software ImageJ (http://rsb.info.nih.gov/ij). Wherever possible, both wings of an individual were measured and their sizes averaged. Measures were transformed into units of millimeter square using a standard slide.

Testes and accessory gland size

In male flies, we also measured the size of testes and accessory glands. These measures were performed on the same individuals used for the body size dataset. Following the protocol of Bangham et al. (2002), testes and accessory glands were dissected in phosphate-buffered saline (PBS). The organs were placed in 200 μl of PBS on a glass microscope slide and testes were carefully uncurled. An image of the slide was captured at 100× magnification under a compound microscope. The area of testes and accessory glands was measured with the ImageJ software and transformed in units of millimeter square. Wherever possible, both testes and both accessory glands of an individual were measured and their sizes averaged.

MEASURES OF THE RATE OF FERTILIZATION

We conducted additional experiments to establish whether changes in reproductive morphology were associated with changes in the capacity of males to successfully fertilize multiple females. These assays were performed after 24 generations of selection. We concentrated on the 1:1 and 10:1 high N_e selection lines between which a maximum divergence in reproductive biology would be expected. In our assays, we subjected flies from the two regimes to a 10:1 sex ratio (females:males) and measured the rate of fertilization, defined here as the proportion of females fertilized after four days of interactions in a cage. Because virgin females are usually willing to mate, matedness in this assay is expected to reflect the males' capacity to perform multiple matings, rather than their capacity to persuade females to mate. Flies for the experiments were reared under the same conditions used during selection line maintenance. For each selection line assayed, we set up three replicate cages at a 275:28 (F:M) sex ratio and left flies to interact for four days. Cages were maintained at 21°C and supplied daily with fresh grape juice plates and live yeast paste. At the end of the interaction period, the live males in each cage were counted and then discarded. The females were isolated in individual vials containing 7 mL SY medium and live yeast and stored at 25°C. After about seven days, females were scored as fertilized if the vials in which they had been stored contained offspring (pupae and/or larvae).

STATISTICAL ANALYSIS

Assessment of treatment effects

The estimation of rates of male and female multiple mating using eye-color mutants is complicated by the fact that single matings cannot be distinguished from multiple matings with males of the same genotype (wild-type or *scarlet*). To overcome this problem, we used Bayesian inference to estimate mating parameters. This approach, detailed in the Appendix, provided us with an estimate and 95% credible intervals of the probability of a female mating at least once (nonvirginity) and the probability of a female mating twice (as opposed to once) for each treatment, all based on our experimental data. The expected rate of male mating in each treatment was calculated as the expected total number of matings (based on the number of females and the probabilities of female mating and double mating), divided by the number of males.

Morphological measurements

We analyzed the effect of treatment, line, and sex on wing area using a partially nested analysis of variance (ANOVA) including the factors "treatment," line nested within treatment ("line in treatment"), and "sex," as well as all interactions (i.e., those of "sex" with "treatment" and "line in treatment"). Measures of testes and accessory gland size were analyzed with a nested ANOVA including the factors "treatment" and "line nested within treatment," as well as covariates as detailed in the Results section.

Rate of fertilization

Measurements on the rate of fertilization consisted of the numbers of fertilized and virgin females in each replicate cage. Data on two replicate assays (from two different 1:1 lines) were discarded before analysis because of errors during cage setup. Due to their binomial nature, the data were analyzed using a Generalized Linear Model with logit link function. The model included the number of live males recovered from the cage as a covariate, as well as the factors "treatment" and "line in treatment." All statistical analysis were performed in R 2.3.1 (R Development Core Team 2006).

Results

TREATMENT EFFECTS ON MALE AND FEMALE MATING RATES

Altering the population sex ratio had profound effects on the mating system (Table 1). As expected, increasing female bias led to an increased proportion of females remaining unmated (Table 1, "rate of female mating"). Although almost all females were fertilized at an even sex ratio and moderate female bias (4:1), 25–30% of females remained unmated at the 10:1 female bias. In parallel, the probability of a female mating doubly, as opposed to singly, declined (Table 1, "rate of female double mating"). Although the estimated rate was 80% at 1:1 and 70% at 4:1, it dropped to around 15% at a sex ratio of 10:1. Furthermore, the credible intervals of these estimates overlap between 1:1 and 4:1 but not the 10:1 treatments. Thus, as far as female mating patterns are concerned, a quantitative shift occurs between the 4:1 and the 10:1 treatment, whereas 1:1 and 4:1 are similar. Because double mating translates directly into sperm competition risk (defined as the proportion of ejaculates that reside in doubly mated females), a significant drop in sperm competition risk occurs only with extreme female bias (Table 1, "sperm competition risk"). The pattern of female double

Table 1. Effects of sex ratio treatments on the mating system. The rate of female mating (probability of mating vs. nonmating) and double mating (mating twice vs. mating once) were estimated with the Bayesian approach detailed in the Appendix. The sperm competition risk (proportion of ejaculates residing in doubly mated females) and the rate of male mating (matings per male) were estimated from female mating rates. The degree of sperm limitation (proportion of mated females producing ≤10 offspring) was estimated from the experimental data. Credible intervals for the Bayesian estimates are given in parentheses.

Parameter	1:1	4:1	10:1 high N_e	10:1 low N_e
Rate of female mating	0.99	0.96	0.76	0.71
	(0.96,1.0)	(0.93,0.98)	(0.72,0.79)	(0.66,0.77)
Rate of female double mating	0.81	0.70	0.18	0.13
	(0.66,0.96)	(0.60,0.79)	(0.14,0.23)	(0.06,0.22)
Sperm competition risk	0.90	0.82	0.31	0.23
Rate of male mating	1.79	6.58	8.81	8.11
Sperm limitation	0.02	0.08	0.27	0.38

mating and sperm competition contrasts with rate of male mating, calculated on the basis of female mating rates (Table 1, "rate of male mating"). Here, mating rate is low in the 1:1 treatment compared to the 4:1 and 10:1 treatments, both of which are somewhat similar. Thus, in terms of male mating rate a quantitative shift occurs between the 1:1 and the 4:1 treatment.

Elevated male mating rates in female-biased population were associated with depletion in male mating resources (Table 1, "sperm limitation"). Accordingly, the proportion of females that produced few (≤10) offspring was higher in treatments with an altered sex ratio compared to those with an even sex ratio (contrast 1:1 vs. other treatments, linear coefficient $\alpha = -2.57$, $P < 0.0001$). Furthermore, the proportion of sperm-limited females was significantly higher in the 10:1 than in the 4:1 treatment ($\alpha = -1.69$, $P < 0.0001$) and was also higher in the 10:1 high-N_e as compared to the 10:1 low-N_e treatment ($\alpha = -0.26$, $P = 0.004$). Quantitatively, sperm limitation was substantial only in the 10:1 treatments, where roughly a third of mated females produced few offspring (compared to 2% and 8% in the 1:1 and 4:1 treatments, respectively; Tab 1., "sperm limitation").

MORPHOLOGICAL MEASUREMENTS

Wing area

We obtained measures of wing area for an average of 16.0 ± 1.4 (SD) males and 19.6 ± 0.6 females per line. Analysis of the data showed, unsurprisingly, that male and female body size differed significantly (Table 2, "sex"). However, we did not detect a significant difference in average male and female size between treatments (Table 2, "treatment"), nor an interaction between treatment and sex (Table 2, "treatment × sex"). These results were confirmed by separate ANOVAs for each sex, neither of which revealed a significant treatment effect (males: $F_{3,12} = 2.78$, $P = 0.09$; females: $F_{3,12} = 0.44$, $P = 0.72$). Excluding the 10:1L treatment (in which evolution might have been dominated by drift) gave the same result for females ($F_{2,9} = 1.02$, $P = 0.39$) whereas in males there was a tendency for greater wing area in the 1:1 treatment (treatment effect $F_{2,9} = 4.04$, $P = 0.056$; Tukey HSD test between treatment: 1:1 vs. 4:1 $P = 0.0003$; 1:1 vs. 10:1 high N_e $P < 0.0001$; 4:1 vs. 10:1 high N_e $P = 0.2$).

The global ANOVA also showed that sexual dimorphism had changed within individual lines, as indicated by the significant "line in treatment" by "sex" effect in Table 2. It appears that this last effect is mostly due to variance in sexual dimorphism between lines of the two 10:1 treatments (Fig. 1). However, there is no statistically significant difference between treatments in the among-line variance in dimorphism (expressed as mean male size/mean female size − 1, Lovich and Gibbons 1992) (Bartlett test, $K^2 = 5.1$, df = 3, $P = 0.17$).

Testes and accessory gland size

We obtained measures of both testes and accessory gland sizes for an average of 15 ± 1.6 (SD) males per replicate line (Fig. 2). The

Table 2. ANOVA results for male and female wing area for flies from the sex ratio lines. The table gives the number of degrees of freedom (df), the *F* ratio (*F*), the *P* value (*P*), and the error term used to calculate the *F* ratio for each term in the model.

Term	df	*F*	*P*	Error term
Treatment	3	1.33	0.31	Line in treatment
Sex	1	1930.34	<0.0001	Line in treatment×sex
Line in treatment	12	12.87	<0.0001	Residual
Treatment×sex	3	0.80	0.52	Line in treatment×sex
Line in treatment×sex	12	2.29	0.008	Residual

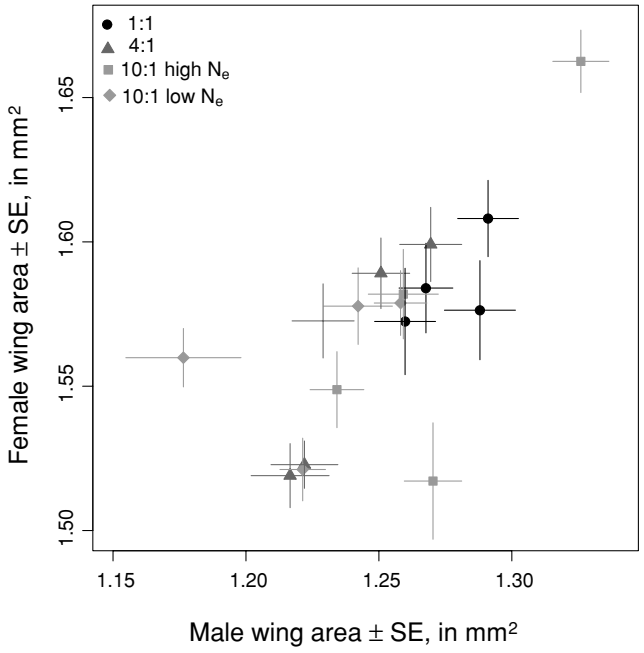

Figure 1. Male and female wing size in the sex ratio selection lines. The figure shows mean female wing size against mean male wing size for individual selection lines of all treatments. Error bars indicate the standard error of the means.

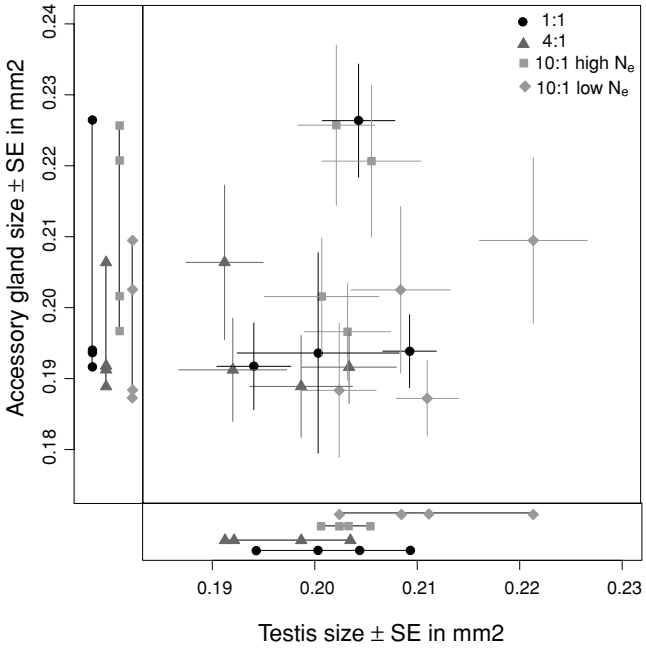

Figure 2. Testis and accessory gland size in the sex ratio selection lines. The figure shows mean accessory gland and testis size for individual selection lines of all treatments. To facilitate the visual comparison between treatments, the line means are also depicted outside the axes by the respective symbols. Error bars indicate the standard error of the means.

treatments did not differ in accessory gland size (nested ANOVA of log-transformed values, treatment effect: $F_{3,12} = 1.37$, $P = 0.30$). Using wing area as a covariate showed that larger males have larger accessory glands, but that residual accessory gland sizes do not differ between treatments (wing area effect $F_{1,227} = 4.69$, $P = 0.031$, treatment effect $F_{3,12} = 0.54$, $P = 0.7$).

In contrast to accessory gland size, testis size differed significantly between treatments (nested ANOVA of log-transformed values, treatment effect: $F_{3,12} = 3.39$, $P = 0.038$). Here, wing area had no significant effect when included as a covariate ($F_{1,228} = 3.47$, $P = 0.09$). However, including accessory gland size as a covariate had a highly significant effect (accessory gland size $F_{1,253} = 23.06$, $P < 0.0001$; treatment effect $F_{3,12} = 3.89$, $P = 0.037$), and the model including accessory gland size as a covariate fitted the data significantly better than a model without ($F_{1,253} = 22.50$, $P < 0.0001$). A post hoc analysis showed that the significant treatment effect was due to the fact that males from the 10:1 high-N_e treatment have significantly larger testes than males from other treatments, relative to their accessory gland size (Tukey HSD test on residuals of log-transformed testis size on accessory gland size; 10:1 high N_e vs. 1:1, $P = 0.020$; 10:1 high N_e vs. 4:1, $P = 0.0005$; 10:1 high N_e vs. 10:1 low N_e, $P = 0.029$; all others $P > 0.5$).

In summary, the above data show that, overall, accessory gland size was positively associated with testis size. However, the relative size of the two organs diverged between treatments, such that flies evolving under the 10:1 high-N_e regime had significantly larger testes relative to their accessory gland size than flies from the other treatments.

RATE OF FERTILIZATION

Although there was considerable heterogeneity among lines, the proportion of females fertilized after four days of mating was higher in the 10:1 high-N_e lines (64.5 ± 3.2%, mean ± SE of mean) than in the 1:1 lines (60.9 ± 2.7%). The difference between the treatments was statistically significant (Table 3).

Discussion

The results of the present study are relevant to three main areas, (1) adaptation to the mating system, (2) the limits to this adaptation

Table 3. GLM results on measures of fertilization rate. The table gives the number of degrees of freedom (df), the *P* value (*P*), and the percentage of deviance explained (% Dev. exp.) for each term in the model.

Term	df	*P*	% Dev. exp.
Number of males	1	0.78	–
Treatment	1	0.001	6.5
Line in treatment	6	<0.0001	38.1

imposed by genetic drift, and (3) the effect of natural sex ratio distorters on reproductive evolution of their hosts.

ADAPTATION TO THE MATING SYSTEM

We use female bias in the sex ratio as a means to manipulate the degree of multiple mating and sperm competition in populations of fruitflies. Female bias is predicted to lead to a decrease in the level of multiple mating by females, hence reducing sperm competition. Concomitantly, female bias should increase mating rates in males, due to reduced competition between males for access to mating partners. Our experimental data matched these predictions. However, they also showed that the quantitative changes in mating system parameters across sex ratios are not parallel. Thus, the rates of female single and multiple mating differed little between an even and intermediate female-biased population sex ratio (4:1), but dropped significantly if sex ratio bias is increased further (10:1). Male mating rate showed a different pattern, and increased already with intermediate female bias. Male sperm depletion, finally, showed a pattern similar to that of female mating rate, with substantial sperm limitation occurring only at extreme female bias.

The differential changes in mating system parameters in response to the sex ratio allows us to disentangle the effects of sperm competition and of male mating rate on the evolution of male reproductive morphology (in particular, testis size). If males evolved predominantly in response to the intensity of sperm competition, we would expect to observe larger testes in the 1:1 and 4:1 treatments compared to the 10:1 treatment. If, in contrast, the evolution of male reproductive morphology is mainly driven by male mating rate and fertilization capacity, then the largest testes should be observed in males from populations with the most extreme female bias. It is this latter pattern we observed in our selection lines. After 28 generations of evolution, male flies from lines evolving under a 10:1 sex ratio showed evidence of significantly increased testis size, relative to flies evolving under the 1:1 or 4:1 female bias. The restriction of an evolutionary response to the most extreme sex ratio regime suggests that the response is driven by selection on males to increase their fertilization capacity and to avoid sperm depletion, because sperm limitation is only prominent in the 10:1 treatment (cf. Table 1). This interpretation is supported by the fact that the observed response in testis size was associated with a difference in the rate with which males fertilized females; the proportion of females fertilized within four days was higher in lines evolved under 10:1 high-N_e regime than those of the 1:1 regime, when both were subjected to a female-biased population sex ratio in our essay of fertilization capacity. It is important to note in this context that we infer male sperm depletion indirectly from the occurrence of low female fertility. Our measure therefore potentially confounds actual male sperm depletion with effects of male ejaculate tailoring. Indeed, males in female-biased populations might respond to the ready availability of virgin females by transferring ejaculates of reduced size to spread their sperm resources more widely, hence resulting in lower female fertility. The existence of such male strategies would imply that the extent of male sperm limitation required to favor increased investment in testis size is in fact small.

Earlier experimental studies of testis size evolution in insects were based on imposing either monogamy or polyandry on laboratory populations, while aiming at keeping male mating rate low enough to be ignored (Holland and Rice 1999; Hosken and Ward 2001). These experiments consistently found that testis size was larger in polyandrous lines, in which sperm competition was present (Hosken et al. 2001; Hosken and Ward 2001; Pitnick et al. 2001). Our data do not contradict these findings. However, they show that when both key variables, sperm competition and male mating rate, are varied simultaneously in different directions, it is the latter variable that dominates the evolutionary response. Importantly, our experiment manipulated sperm competition intensity and mating rate via an alteration of the operational sex ratio from 1:1 to 10:1. This means that the conclusions of male mating rate being the dominant force (although necessarily limited to the range of conditions examined) are valid for those combinations of the two variables that occur "naturally" due to changes in the mating system. Conditions under which sperm competition is the more important variable could undoubtedly be created artificially by precise and independent experimental manipulation of sperm competition and mating rate.

Our results contrast with the finding that male mating rate per se does not affect testis size in primates (Harcourt et al. 1981) and birds (Birkhead and Møller 1992; but see Pitcher et al. 2005). This discrepancy suggests that the importance of male mating rate varies between taxonomic groups. Such variation could arise because groups of species differ in the degree to which the amount of sperm received limits fecundity. Thus, male mating rate is expected to be of minor importance in species in which females produce relatively few offspring per reproductive season. In this case, a tiny number of sperm is sufficient to successfully fertilize the eggs from which offspring develop. In contrast, male mating rate may affect the evolution of testis size more in species in which large ejaculates are required to successfully fertilize large clutches of eggs. Hence in general, one might expect that male mating rate has a stronger impact on testis size evolution in insects (which produce many offspring per clutch) than vertebrates (which usually produce few). Given the effect of such differences in life history between species and taxonomic groups, caution should be used when assessing the effect of sperm competition on the evolution of testis size in correlative studies.

In contrast to testes, accessory gland size did not diverge significantly between the treatments of our experiment, despite the presence of heritable genetic variance for the trait in the

Dahomey base stock (Linklater and Chapman, unpubl. data). The lack of a selective response could imply that selection on accessory gland size is negligible across our experimental treatments. Alternatively, the lack of selective response could be attributed to the simultaneous action of two opposing evolutionary forces. On the one hand, reduced sperm competition could select for a reduction in accessory gland size or accessory gland protein transfer. This is supported by a recent study by Linklater et al. (2007) comparing the ejaculate delivery patterns between the male- and female-biased fruitfly lines established by Wigby and Chapman (2004). The results of this study suggest that low levels of sperm competition in female-biased populations could select for a decreased expenditure of accessory gland fluid and smaller gland size (Linklater et al. 2007). However, this could be counterbalanced by selection for larger glands imposed by increased mating rate observed in *Drosophila* (Bangham et al. 2002) and stalk-eyed flies (Rogers et al. 2005). Thus, in our experiments the conditions of low sperm competition and high mating rate may have led to opposing selective forces canceling each other out.

The observed response in male reproductive morphology to the sex ratio treatments of our experiment was nonlinear. Phenotypic changes were only observed with the most extreme female bias (10:1), whereas the treatment with intermediate bias remained similar to the 1:1 control. This pattern is in agreement with results obtained by Wigby and Chapman (2004) and Linklater et al. (unpubl. ms.) who did not observe any change in testis or accessory gland size in populations of *D. melanogaster* after at least 86 generations of evolution under a 3:1 female-biased sex ratio. The absence of a response to selection at intermediate levels of female bias begs the question of what constrains the evolution of testis size, or, in other words, what character(s) trades off with testis size? The absence of an evolutionary response in accessory gland size in our lines implies that testes and accessory glands are not traded off against each other, a suggestion supported by comparative data across *Drosophila* species (Kraaijeveld and Chapman, unpubl. ms.). Instead, testis size resources could be traded off against resources spent in the acquisition of mates, in the form of energetically costly courtship or mate searching. Accordingly, testis size would not be expected to evolve in the intermediate sex ratios where few females are virgin (see Table 1) and courtship is an important determinant of male fitness. In the 10:1 treatments, in contrast, unmated females are abundant and provide males with less competitive access to mating opportunities.

GENETIC DRIFT

Our experiment has also demonstrated that random genetic drift can prevent, or significantly slow the rate of, adaptive evolution. Although male reproductive traits of the 10:1 treatment with a high effective population size (275 females : 28 males) diverged from those in the 1:1 and 4:1 treatments, a similar change was not observed in the treatment with a 10:1 sex ratio but a low effective size (91 females : 9 males). This result is not surprising because stochastic effects in small populations have long been known to affect the efficacy of selection (Wright 1931). However, the result is interesting nevertheless, because it contributes empirical data relevant to a recent debate on the effect of inbreeding in experiments using the monogamy/polyandry approach. In these experiments, polyandry lines usually comprise of a larger total number of individuals than monogamy lines, because of the presence of extra males. In Holland and Rice's (1999) study, for example, polyandry lines were based on 300 males and 100 females per generation, compared to 100 males and 100 females in monogamy lines. Wigby and Chapman (2004) suggested that in studies using the monogamy/polyandry approach, divergence between treatments could be caused by differences in the rate of inbreeding. This proposition was countered by Rice and Holland (2005), who argued that the expected rate of accumulation of deleterious alleles was too low to lead to a significant decrease in fitness over the course of a selection experiment. Our empirical data on body size support the suggestion of Rice and Holland (2005). We observed no significant change in size between treatments, in particular not between the 10:1 high- and low-N_e treatments. Because body size is a trait affected by inbreeding (Radwan and Drewniak 2001), this result suggests that deleterious alleles had not accumulated in our lines, including the 10:1 low-N_e treatment whose effective population size is considerably smaller than that of Holland and Rice's (1999) monogamy lines. The fact that we observed a response to selection in the 10:1 high-N_e treatment (with an N_e of 100 following Crow and Kimura 1970, p. 350) also demonstrates that adaptive evolution readily occurs in populations with an effective size smaller than that of Holland and Rice's monogamy lines (in which $N_e = 200$).

The comparison between the two 10:1 treatments is of further interest because it allows us to get a rough estimate of the strength of selection acting on loci determining male reproductive morphology. The force of selection is predicted to override random genetic drift whenever the selection coefficient s exceeds the reciprocal of effective population size, i.e. $|s| > 1/N_e$ (Li 1978). For the male-limited characters we are considering this becomes $|s/2| > 1/N_e$, because the genes encoding them are exposed to drift in every generation (being transmitted by both males and females) but are under selection only half of the time (with half of the genes in the offspring generation being of paternal origin). Knowing that selection was efficient in the 10:1 high-N_e treatment ($|s/2| > 1/N_{e,h}$), but inefficient in the 10:1 low-N_e treatment ($|s/2| < 1/N_{e,l}$), we obtain $0.02 < |s| < 0.06$ (Crow and Kimura 1970, p. 350). Although these figures are only rough estimates (the condition $|s| > 1/N_e$ assumes a large population and weak selection), they do suggest that sex ratio bias can exert significant selection pressures on reproductive traits.

Indeed, the bounds above are likely to be underestimates, because in our experimental evolution setup flies did not only adapt to the mating system but also to the general rearing conditions. Thus, Hill–Robertson interference (Hill and Robertson 1966) between the loci coding for reproductive morphology and those implicated in general life-history characters is bound to increase genetic drift in loci coding for a particular set of traits, leading to a lower realized N_e than that used in our calculations.

EFFECTS OF NATURAL SEX RATIO DISTORTION

Our experiment also sheds light on the immediate and long-term effects of naturally occurring sex ratio distortion. Numerous wild populations are subject to sex ratio distortion toward females, caused either by the presence of selfish nuclear genetic elements, such as sex-chromosome drivers (Jaenike 2001), or cytoplasmic factors (e.g., *Wolbachia* in arthropods (Werren 1997) or cytoplasmic male sterility in plants (Budar and Pelletier 2001)). Due to the potentially extreme and persistent sex ratio bias (Dyson and Hurst 2004), affected populations can suffer a severe reduction in overall productivity and hence an increased risk of extinction (Charlat et al. 2003).

The present study provides several insights into the effects of natural sex ratio distortion. One important finding is that selection acting on individuals within such populations lead to increased male fertilization capacity. Thus, adaptation to sex ratio distortion tends to stabilize the affected populations by increasing their overall productivity. This corroborates the findings of a correlative analysis on several woodlice species infected with *Wolbachia*. Moreau and Rigaud (2003) found that mating capacity was higher in five species in which *Wolbachia* induced a female-biased sex ratio by feminizing males than in two species in which *Wolbachia* causes cytoplasmic incompatibility, which does not affect the sex ratio. Our results support the interpretation that this difference in male mating capacity represents an adaptation to the population sex ratio.

We also identified factors that might prevent or slow down evolutionary change in response to sex ratio distortion. First, the assessment of treatment effects (Table 1) showed that female multiple mating can occur despite moderate female bias. The resulting sperm competition is expected to favor the transfer of large ejaculates (Parker 1990) and thereby prevent evolution toward an even partitioning of sperm between many subsequent matings. In this way, selection arising from elevated levels of sperm competition contributes to sperm limitation in females and a resulting decrease in population productivity. There is evidence for such a scenario in populations of the butterfly *Hypolimnas bolina*. Charlat et al. (2007) studied male and female mating behavior in southeast Asian island populations, in which varying levels of infection with a male-killing *Wolbachia* cause differences in the population sex ratio. They showed that moderate female bias leads to increased rates of female mating in response to male sperm depletion. Charlat et al. (2007) proposed that the resulting sperm competition prevents the evolution of sperm partitioning and thereby maintains sperm limitation and, in turn, female remating and sperm competition. *Hypolimnas bolina* differs from *D. melanogaster* in that female multiple mating is normally rare and only occurs as the result of a facultative female response to male sperm depletion in female-biased populations. However, our data on the effect of female bias on the mating system show that in "naturally" multiply mating species, facultative female responses are not required to prevent the evolution of sperm partitioning, because high rates of female multiple mating can be maintained even in the face of moderate sex ratio distortion.

The comparison between the 10:1 high- and low-N_e treatments in our experiment also suggests that adaptive evolution can be limited by increased levels of genetic drift in populations affected by sex ratio distortion. Sex ratio bias increases genetic drift (reduces N_e) because the members of the rare sex contribute large parts of the next generation's gene pool (Crow and Kimura 1970, p. 350). In populations in which sex ratio is distorted by cytoplasmic elements, a further reduction of N_e occurs due to limitations on genetic exchange between the infected and the uninfected parts of the population (Engelstädter and Hurst 2007). Male-killing parasites, for example, turn infected females into genetic sinks by preventing them from producing sons who could mate with females from other matrilines. As a consequence, adaptation in infected populations is hampered by the fact that advantageous mutations arising within the infected part of the population cannot readily spread to fixation (Engelstädter and Hurst 2007). Due to the joint action of these two effects, the effective size of populations infected with sex ratio distorters can be very small. In the butterfly *H. bolina*, for example, the effective size of populations with a high male-killer prevalence may be as low as 50 (based on Engelstädter and Hurst 2007 and assuming a numerical population size of 5000), a figure that is close to the N_e of our 10:1 low-N_e treatment. Accordingly, high levels of genetic drift are a genuine problem in the wild, even in numerically large populations.

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APPENDIX

In this appendix, we outline the Bayesian approach used to estimate female mating rates. The experiment used to measure mating rates in the experimental treatments (see main text for details) consisted of pairing females with two types of males, one homozygous for a recessive eye-color mutant scarlet, the other homozygous for the corresponding wild-type allele. Because females were also homozygous *scarlet*, we could directly measure the number of females that mated with both a wild-type and a *scarlet* male (and produced two types of offspring). However, it was not possible to determine which proportion of the females producing offspring with only one eyecolor had mated multiply with males of the same type, rather than with a single male. For this reason, a statistical estimation procedure is needed.

For the purpose of our estimation, we assume that females either do not mate, mate once, or mate twice. This assumption seems reasonable, given the mating biology of *D. melanogaster* and the period of time available for mating (four days). We further assume that successive matings of a same female are independent, as are matings of different females. Thus, a female will mate with a wild-type male with a constant probability p_w. We estimate the probability m_1 that a female mates at least once (as opposed to not at all) and the probability m_2 that a female that mates actually mates twice, as well as the probability p_w that a mating is achieved by a wild-type male. This latter parameter is of little interest for the purpose of this study, but its inclusion allows us to render our estimations independent of possible fitness differences between the two male genotypes.

In Bayesian inference a plausibility (or probability) is assigned to each value that an unknown parameter θ may take and that is consistent with the observed data. The probability distribution of a continuous parameter θ given the dataset D is obtained by applying Bayes' rule

$$\Pr(\theta|D) = \frac{\Pr(\theta)\Pr(D|\theta)}{\int_\theta \Pr(\theta)\Pr(D|\theta)d\theta}, \tag{A1}$$

where $\Pr(\theta)$ is the prior probability of the parameter before observing the dataset D and $\Pr(D|\theta)$ is the so-called likelihood function, namely the probability of observing D given the parameter θ (Jaynes 2003). In our case, the data for one replicate of the experiment consist of a series of values $D = \{N_0, N_w, N_s, N_{ws}\}$, where N_0 is the number of females producing no offspring, N_w the number of females producing only wild-type offspring, N_s the number of females producing only *scarlet* offspring and N_{ws} the number of females producing both types of offspring. The likelihood function is therefore an equation describing the probability of observing a dataset D given the probabilities m_1, m_2, and p_w. In the following, we will construct the likelihood function based on the biology underlying the experiment.

We start by deriving a likelihood function for the number of females that produce offspring as a function of the probability of mating at least once, m_1. From our data, we know the number of females N_r that have produced offspring ($N_r = N_w + N_s + N_{ws}$) among the total number N_t of females that were included in the experimental replicate ($N_t = N_0 + N_w + N_s + N_{ws}$). Based on the assumption of independent matings, we can use the binomial distribution with probability m_1 to obtain the likelihood function for the number of mated females in the form

$$\Pr(N_r = n|m_1) = \frac{N_t!}{n!(Nt-n)!} m_1^n (1-m_1)^{N_{t-n}}. \tag{A2}$$

To obtain a likelihood function for the different classes of mated females, we first use the probabilities m_2 and p_w to express the probability of observing N_w and N_{ws} females with, respectively, wild-type and mixed progeny. This probability can be written as

$$\begin{aligned}&\Pr(N_w = i, N_{ws} = j|m_2, p_w)\\ &= \sum_{h=0}^{N} \Pr(N_w = i, N_{ws} = j|N_2 = h, m_2, p_w)\\ &\times \Pr(N_2 = h|m_2, p_w).\end{aligned} \tag{A3}$$

Here, we express $\Pr(N_w = i, N_{ws} = j \mid m_2, p_w)$ conditional on the probability that N_2 of the N_r reproducing females mated twice, which is obtained from the assumption of independent mating by the binomial distribution as

$$\Pr(N_2 = h) = \frac{N!}{h!(N-h)!} m_2^h (1-m_2)^{N-h}. \tag{A4}$$

We further refine equation (A3) by dividing the number of females producing only wild-type offspring (N_w) with those that have mated with one wild-type male ($N_{w,1}$) and those that have mated with two wild-type males ($N_{w,2}$). By conditioning on $N_{w,1}$, the number of females producing wild-type offspring and having mated only once, we have

$$\begin{aligned}&\Pr(N_w = i, N_{ws} = j|N_2 = h, m_2, pw)\\ &= \sum_{k=0}^{N-h} \Pr(N_w = i,\ N_{ws} = j|N_2 = h, N_{w,1} = k, m_2, p_w)\\ &\times \Pr(N_{w,1} = k|N_2 = h, m_2, p_w),\end{aligned} \tag{A5}$$

where

$$\begin{aligned}&\Pr(N_{w,1} = k|N_2 = h, m_2, p_w)\\ &= \frac{(N-h)!}{k!(N-h-k)!} p_w^{N-h} (1-p_w)^{N-h-k}\end{aligned} \tag{A6}$$

is again obtained from the assumption of independent mating.

Because $N_w = N_{w,1} + N_{w,2}$, we have

$$\begin{aligned}&\Pr(N_w = i, N_{ws} = j|N_2 = h, N_{w,1} = k, m_2, p_w)\\ &= \Pr(N_{w,2} = i-k, N_{ws} = j|N_2 = h, m_2, p_w),\end{aligned} \tag{A7}$$

which is the probability that j females produced mixed progeny and $i-k$ out of the i females producing only wild-type offspring do so despite having mated twice. An explicit expression for this probability can be obtained from the multinomial distribution as

$$\begin{aligned}&\Pr(N_{w,2} = i-k, N_{ws} = j|N_2 = h, m_2, p_w)\\ &= \frac{h!}{j!(i-k)!(h-i-j+k)!} p_w^{2(i-k)} (2(1-p_w)p_w)^j\\ &\times (1-p_w)^{2(h-i-j+k)}.\end{aligned} \tag{A8}$$

Combining all terms and simplifying, we finally obtain the likelihood function for the different classes of reproducing females

$$\Pr(N_w = i, N_{ws} = j | m_2, p_w) = \sum_{h=0}^{N} \sum_{k=0}^{N-h} \frac{n! x^h (1-x)^{n-h} 2^j p_w^{2i+j-k} (1-p_w)^{h-2i-j+k+n}}{j!k!(i-k)!(n-h-k)!(h-i-j+k)!}. \quad \text{(A9)}$$

Equations (A2) and (A9) provide the likelihood functions necessary to estimate the three parameters of interest, m_1, m_2, and p_w by applying Bayes' rule (eq. A1). Before doing so we have to specify a prior distribution of the parameters to be estimated. Given that we have no prior knowledge about these parameters (other than that it is physically possible to mate with either type of male), we assume all three prior distributions to be uniform (Jaynes 1968, p. 21).

The estimation for the experimental data used likelihood functions based on compound probabilities across replicates, $\prod_{rep} \Pr(D_{rep} | m_2, p_w)$. Parameters were then estimated by numerically calculating their expectations as $\hat{\theta} = \int_0^1 \theta \Pr(\theta|D) d\theta$ using the software Mathematica (Wolfram 2003). To obtain a measure of the quality of our estimation, we also calculated 95% credible intervals (the Bayesian equivalent of a confidence interval), that is, intervals (l, u) of parameter values over which the posterior probability equals 0.95 ($\Pr(l \leq \theta \leq u) = 0.95$). The boundaries u and l were chosen such that the probability of being above the credible interval is equal to the probability of being below, $\int_0^l \Pr(\theta|D) d\theta = \int_u^1 \Pr(\theta|D) d\theta = 0.025$.

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