Transgenerational effects of sexual interactions and sexual conflict: non-sires boost the fecundity of females in the following generation

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Full methods

(a) Study populations and lines

Our focal flies were sourced from an outbred lab-reared population of *D. melanogaster* (provided by Stephen Robinson and Jason Kennington, from the University of Western Australia, who in turn obtained it from William Rice, from the University of California, Santa Barbara) fixed for a recessive autosomal mutation encoding brown eyes (LH_M-bw). This mutation had been backcrossed into a full replicate of the outbred wild type population over numerous generations [1]. We also sourced flies from a wild type outbred population (CH), provided by Carla Sgro, from Monash University, Melbourne), originally derived from 60 inseminated females collected at Coffs Harbour, New South Wales Australia, and cultured in our laboratory for one year prior to the experiments [2, 3]. This CH population provided the 'tester' males, described in the experiment below.

We also generated lines of flies of standardized genotype, by crossing two near-isogenic lines (each produced by 16 generations of full-sibling pair mating), non-reciprocally, and using the F1 offspring. These F1 flies were used as 'tester flies' in subsequent assays of reproductive success of both daughters and sons, described below. F1 offspring of these crosses exhibit high levels of genome-wide heterozygosity, but with no genetic variance across flies from within a given sex and cross. These crosses similarly sourced tester flies for the experiment. One of these *S*tandardized *H*eterozygote *L*ines (SHL-wt) was generated by crossing two near-isogenic lines created using wild-type flies from an outbred population collected in the Swan Valley, Western Australia. A second standardized heterozygote line (SHL-bw) was generated by crossing near-isogenic lines originally derived from the LH_M-bw population. Crosses between these lines were performed using 4-5 days old virgin individuals.

Throughout the study flies were reared in 10-dram vials on a potato-dextrose-agar media, with live yeast added to the media surface. At least once every two days, adult flies were transferred to new vials containing fresh media. Experimental males and females from all populations and lines used in the study were separated within seven hours of adults emerging from pupal cases, to ensure their virginity prior to exposure to experimental treatments. Flies were anaesthetised under light CO_2 during virgin collection. All outbred and isogenic lines were kept at a constant environment (25˚C, 12: 12 light: dark cycle), and ~ 150 eggs per vial.

(b) Treatments applied to focal mothers

Focal LH_M-bw mothers were collected as virgins and exposed to the mating treatments when they were four days old. They were transferred into vials (hence Replicate) in groups of eight and were given *ad libitum* access to yeast. Twelve four day old LH_M-bw males were then added to each vial and males and females were allowed to mate for two hours. *D. melanogaster* females, kept on early-life discrete generation culturing regimes, virtually always mate once and only once when housed with a 50% excess of males during two hours [2, 4]. At the end of this period males were removed from the mating vials and each respective group of mothers (Replicate) were subjected to a treatment comprising five levels of sexual interaction and lasting for 10 days.

In treatment level 1, focal mothers received no further exposure to males. This level is referred to as *Baseline*, since all females in the experiment first experienced this 2 h exposure to males (one mating) prior to their subsequent assignment to the other levels. In levels 2 and 3, focal mothers were exposed to a new group of 12 LH_M-bw virgin males. However, males used in level 2 had had their genitals cauterised using a fine tungsten wire probe connected to a 6V, 1A power source, leaving them able to harass the females but unable to mate with them. This procedure precludes copulation by males without impairing their pre-copulatory sexual behaviour [2]. We refer to this as the *Pre-cop only* level (Table 1). In level 3, prospective mothers were exposed to a group of 12 LH_M-bw virgin males whose genitals were fully intact. Mothers assigned to this level experienced therefore effects of ongoing male pre-copulatory activity, several copulations, and post-copulatory effects associated with the receipt of multiple ejaculates from males from the LH_M-bw genetic background. We refer to this level as the *Pre+Post-cop* level. In treatment level 4 each replicate of prospective mothers was exposed to a group of males consisting of 6 LH_M-bw virgin males and 6 CH cauterized virgin males. Thus, in level 4 females were subjected to pre-copulatory effects by males from the two genetic backgrounds but post-copulatory effects (receipt of ejaculates) from just the LH_M-bw genetic background. Level 4 is referred to as *Pre-cop interacting phenotypes* level. Finally, in level 5, each group of eight prospective mothers was exposed to 6 LH_M-bw virgin males and 6 CH virgin males. Level 5 was used to test for cross-generational effects arising from exposure (at the pre- and post-copulatory stages) to a higher diversity of male genotypes (sourced from two distinct global populations), compared to level 3. Level 5 and the use of the brown-eye morphological marker allowed us to inspect transgenerational effects that are in essence indirect genetic effects on offspring fitness traits, because despite mothers in treatment level 5 producing a mixture of brown (sired by LH_M-bw males) and red eyed flies (sired by CH males), the traits were measured only in brown-eyed offspring (i.e., those produced by LH_M-bw females and sired by LH_M-bw, not by CH males). Thus, any effects seen in the offspring generated in level 5, compared to 3, would arise from mothers interacting sexually (at both pre- and post-copulatory phases of reproduction) with males from different populations and not by Mendelian genetic effects tied to the sire. We refer to level 5 as *Pre+Post-cop interacting phenotypes* level (see Table 1). In total, 70 maternal vials (= Replicates), each containing a group of 8 mothers were allocated among the treatment levels in two independent sampling blocks. Sample sizes are denoted in Table 1. During their experimental exposure, focal mothers were transferred every second day to fresh vials with live yeast added to the media surface *ad libitum*, and males that died or escaped during the experiment were replaced with fresh males.

(c) Collection of offspring

On the fifth and tenth days of the treatment, we collected the eggs laid by each group of mothers so that effects of maternal age could be examined. Flies were transferred to new vials and the eggs laid over a period of 32 hours by each group of eight focal mothers were collected and transferred to vials at a maximum density of 25 eggs per vial. Within each maternal age category (5 or 10 days of sexual treatment), these eggs were all of the same age (within 32 h) regardless of the maternal sexual treatment level from which they were sourced. This therefore precludes variance in egg-age

effects from confounding our interpretations. These vials, denoted 'juvenile vials', were the vials in which the focal offspring were reared, prior to their eclosion as adults and subsequent redistribution into vials of adults for the assays described below. The number of pupal cases that had formed in each of these vials was recorded eight days after the laying period and used to estimate juvenile viability for each treatment level replicate x maternal age vial. Given the cap on larval density in the juvenile vials at very low levels, there was little scope for density-dependent effects to influence our interpretations. In fact, maternal clutch viabilities were unanimously high (mean ± SE = 0.92 ± 0.006, n=134 measures of juvenile viability distributed among treatment levels and maternal ages). Nonetheless, we also included juvenile viability as a covariate in our subsequent statistical analyses to account for any potential confounds arising from density-dependent effects at the larval stage. Focal sons and daughters were collected as virgins and stored by sex in groups of 10 until they entered the offspring fitness assays when they were four days old.

The focal offspring assayed for fitness were distributed in groups that shared the same set of eight mothers (Replicate) that had been exposed to the maternal sexual treatment, a fact that it is properly accounted for in the analyses. Also, as noted before, females in treatment level 5 produced brown-eyed offspring (from matings with LH_M-bw males) and red-eyed offspring (from matings with CH males), but only brown-eyed offspring were collected from level 5. Thus, any additional effects seen in the offspring of level 5 as compared to level 3 offspring can be traced to additional effects of mothers mating with CH males (e.g., indirect genetic effects, and in particular interacting phenotypes on offspring fitness arising from the receipt of ejaculates from CH males in addition to ejaculates from LH_M-bw males).

(d) Daughter's reproductive success and sons' sperm competitiveness

When daughters were 4 days old, each was provided an individual vial. One 3 to 4 day old (mean=3.6, SE=0.02, n=589) male from a brown-eyed standardized heterozygote tester line (SHL-bw) was added to each vial for two hours to ensure single mating (see above), and discarded after this period. Daughters were then allowed to oviposit for 40 hours, across two vials (20 h per vial), and the number of eggs laid across the 40 h period counted. The number of adult offspring that had eclosed from each of these vials was recorded 14 days after the collection period began (denoted daughter productivity). The number of eggs produced in the 40 hours following mating was used as an estimate of daughter's fecundity, while the proportion of adults eclosing from these eggs provided a measure of egg-to-adult viability (daughter fertility).

Four day old virgin focal sons (all LH_M-bw) were assayed in sperm competition trials as the second mates of once-mated females. First, 3-4 days old (mean=3.81, SE=0.0166, n=551) standardized heterozygote brown-eyed tester females (the F1 from SHL-bw) were mated (2 hours; groups of 4 females to 6 males) with 3-4 days old (mean=3.49, SE=0.0213, n=551) standardized heterozygous red-eyed tester competitor males (F1 from SHL-wt). Standardization of tester males in the sperm competition trials was conducted to minimize sampling variance affecting the estimation of competitive reproductive success for focal males (see [5]). The males were then discarded and the females transferred individually to new vials. Then one focal son was added to each of these vials and allowed to mate with the tester female for 30 hours, after which the focal son was discarded. Each female was then provided with a new vial with live yeast added and allowed 20 hours to lay eggs. Wild type (red-eyed) competitor males were used to ensure that offspring fathered by the competitor male would have red eyes and offspring fathered by the focal son would have brown eyes. The number of offspring sired by each male was recorded 14 days later. When paternity attributable to the red eye tester male was zero, we then checked for the presence of red eye offspring in the 'remating vial' in which the tester female had been placed with the focal males for 30 hours. When red eye paternity in this vial was also zero, then we assumed that the red eye male had not mated with the female at all, and the data point was excluded from analysis.

(e) Statistical analyses

We used R 3.0.3 [6] for all statistical analyses. Multilevel linear and generalized linear models were fitted with the *lmer* and *glmer* functions, respectively, from the *lme4* package [7]. Explanatory variables in all analyses were (a) Treatment level (fixed factor with 5 levels), (b) Maternal age at oviposition (fixed factor with two levels), and (c) larval viability of the juvenile vial, a covariate (centred after log transformation) consisting of the egg-to-adult viability score (proportion) calculated for each treatment level replicate x maternal age (n juvenile vials = 134). In addition, all models included (d) Block (2 levels), entered as a random factor to account for the multilevel structure of the data [8], and (e) Replicate, a random factor which consisted of flies sharing individual vials within each Treatment level and Block (total number of replicates across treatment levels = 70).

We ran a different analysis for each of the dependent variables:
(i) Daughter's productivity (number of adults produced by daughters, n = 589 daughters) was analysed with a Poisson model. To account for overdispersion an observation-level random effect was also included in the model [9].
(ii) Daughter's fecundity was inspected in a two-step analysis. First, we analysed the probability of daughters' laying at least one egg (yes/no response; n = 589 daughters) using a model with binomial error structure and logit-link function. Second, for those females laying at least one egg (n = 465), we ran a linear mixed model (this measure of fecundity followed a Gaussian distribution).
(iii) Daughter's fertility (n = 465) was investigated with a model with binomial distribution and using the command cbind to compose the response variable as a binomial vector comprising the number of eggs successfully reaching and unsuccessfully reaching the adult stage. An observation-level random effect was also included in the model to account for overdispersion.
(iv) Son's fertilization success in competitive contexts (sperm competition phenotype as second mates) was analysed with a model with binomial error structure and logit-link function. The response variable was a binomial vector (number of offspring sired by the son, number of offspring sired by rival male), and overdispersion was accounted for in the model with an observation-level random effect. Sample size for this analysis was 527 sons after excluding 24 cases where P2 = 0 (in *D. melanogaster* zero fertilization success by the last male to mate with a female likely suggests a failed insemination, given strong second male sperm precedence in this species (P2 ~ 80%). We also ran this analysis without excluding these cases and results are qualitatively similar (not shown).

For all analysis we first fitted models with all the explanatory variables indicated above plus the interactions between Treatment level and Maternal age, Treatment level and Block, and Maternal age and Block. First, we tested random effects and interactions involving random effects with likelihood ratio tests (LRT) [10, 11] and REML. When running the LMM model (analysis of fecundity), we ran progressively simplified models by removing fixed effects one-at-a-time (starting with the highest order interactions) using the drop1 function, and testing the effect of removal of each term on the change in model deviance using likelihood ratio tests and maximum likelihood (ML). We retained only the final model of significant (alpha criterion of 0.05) fixed effects, plus the random effects. Parameter estimates (coefficients, estimates) of the fixed and random effects in the final model were then calculated using REML, while significance of the fixed effects was calculated using ML as described above [12]. The random effects (Replicate and Block) were always retained in the models to account for the hierarchichal structure of the data. None of the interactions involving random effects were significant in any analysis and they were thus not included in the final model. Differences between the levels of each significant factor were examined using Tukey's tests. We report mean ± standard error values throughout and standardized effect sizes [95% CI].

Data are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.p9h8g [13]

Supplementary results

Infertile matings

21% (124 out of 589) of daughters failed to lay eggs. This proportion fits well with the data from a review of the extent of infertile matings across 30 insect species [14], which concluded that infertile matings are more common than previously thought (range: 0%-63%, median: 22%). However, our analysis and results could be affected if the non-fecund females were not randomly distributed across the five levels of the sexual treatment. This was not the case. We ran a binomial generalized linear model on the dichotomous response variable egg-laying (yes/no response, n = 589 daughters), as detailed above in the Statistical analysis section. The proportion of daughters failing to produce eggs was 31%, 21%, 23%, 14%, and 20% for treatments 1, 2, 3, 4, and 5, respectively, but the treatment effect was not significant (LRT = 8.41, p = 0.08). Importantly, sample sizes for the number of fecund females (who laid eggs) was high across levels of the treatment: 64, 91, 88, 117, and 105 fecund daughters for treatments 1, 2, 3, 4, and 5, respectively (variation in these numbers is not only attributable to the occurrence of non-fecund daughters, but also to variation in the number of replicates set up per treatment level; see Table 1).

Maternal age effects.

Daughters' productivity was dependent on maternal age at oviposition (Likelihood ratio test, LRT = 28.265, $p < 0.001$; 44.6 ± 1.6 vs. 35.6 ± 1.6 for mothers ovipositing on the fifth and tenth days of the treatment, respectively; effect size d [95% CI] = 0.33 [0.1 , 0.49]).The probability of egg laying in daughters was also affected by the age of their mothers (which encompasses the duration they had been exposed to the treatment): 29.4% of daughters failed to lay eggs when produced by older mothers compared to 13.4% by younger mothers (LRT = 24.36, $p<0.001$, d = 0.55 [0.32, 0.78]). Maternal age effects are prevalent in nature [32-34], and given the nature of the maternal sexual treatment here, are likely to be mediated by condition-dependence. None of the other components of offspring fitness investigated (daughters' fertility, and sons' fertilization success in competitive contexts) was significantly influenced by maternal age, and there was no interaction between maternal age and treatment level for any fitness measure.

Control for density-dependent effects

In all analyses we included the egg-to-adult viability score of the vial in which the offspring produced by the treated mothers developed as larvae, to control for any potential confounds arising from density-dependent effects at the larval stage (but note that larval density was capped to 25 eggs). There was a weak positive relationship between the viability of the offspring in the juvenile vials in which the focal offspring were reared and the daughters' subsequent fecundity (LRT = 4.0233, p = 0.045, effect size computed from r, d = 0.24 [0.05, 0.42]), as well as a marginal positive relationship with the sons' fertilization success (LRT=3.66, p = 0.056, d = 0.22 [0.05, 0.39]), but viability was consistently high across treatment levels (Least squares means ± SE, 1 = 92.9 % ± 1.4, 2 = 89.1 % ± 1.30, 3 = 92.6 % ± 1.3, 4 = 92.8 % ± 1.2, 5 = 92.1 % ± 1.2).

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