

Within-group male relatedness reduces harm to females in

Drosophila

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17 **Resolving the mechanisms that switch competition to cooperation is key to**
18 **understand biological organization¹. This is particularly relevant for intrasexual**
19 **competition, which often leads to males harming females². Recent theory**
20 **proposes that kin selection may modulate female harm by relaxing competition**
21 **among relatives³⁻⁵. We experimentally manipulated the relatedness of groups of**
22 **male *Drosophila melanogaster* competing over females to demonstrate that, as**
23 **expected, within group relatedness inhibits male competition and female harm.**
24 **Females exposed to three brothers unrelated to the female had higher lifetime**
25 **reproductive success and slower reproductive ageing compared to females**
26 **exposed to triplets of males unrelated to each other. Triplets of brothers also**
27 **fought less with each other, courted females less intensively and lived longer than**
28 **triplets of unrelated males. However, associations among brothers may be**
29 **vulnerable to invasion by minorities of unrelated males: when two brothers were**
30 **matched with an unrelated male, the latter sired on average twice as many**
31 **offspring as either brother. These results demonstrate that relatedness can**
32 **profoundly affect fitness through its modulation of intrasexual competition, as**
33 **flies plastically adjust sexual behaviour in a way consistent with kin selection**
34 **theory.**

35
36 We first tested the effect of relatedness of males within a group on female fitness, by
37 quantifying different aspects of fitness and life-history (experiment 1) in females
38 exposed to male triplets. Males were unrelated to the female and either full-sib
39 brothers of each other (AAA) or unrelated to each other (ABC), and were replaced
40 weekly until female death. Consistent with expectations³⁻⁵, we found that females
41 exposed to AAA-males had significantly higher lifetime reproductive success than
42 females exposed to ABC-males (Fig. 1a). This was due to the fact that while total

43 female lifespan did not differ on average between treatments ($F_{1, 119} = 1.66$, $P = 0.2$),
 44 females exposed to AAA-males had significantly longer reproductive lifespan (from
 45 eclosion to last egg-laying day⁶, Fig. 1b), and female reproductive lifespan was
 46 positively correlated with female lifetime reproductive success ($F_{1, 117} = 484.59$, $P <$
 47 0.001). Two non-mutually exclusive mechanisms might cause this. First, high-
 48 fecundity females might die faster when exposed to ABC-males, leading to an
 49 average higher productivity of AAA-replicates ('selective death'). Second, individual
 50 females might suffer a steeper rate of age-dependent decline in reproductive output
 51 when exposed to ABC- rather than AAA-males ('reproductive ageing'). We found no
 52 evidence of 'selective death': across both treatments (AAA and ABC) females
 53 characterized by a relatively low (rather than high) initial oviposition rate died
 54 significantly faster than high-fecundity females ($F_{1, 117} = 11.038$, $P = 0.0012$;
 55 treatment*oviposition rate interaction, $F_{1, 117} = 0.224$, $P = 0.64$), which does not
 56 support the prediction that high-fecundity females die faster in ABC- compared to
 57 AAA-trials. In contrast, we found robust support for 'reproductive ageing': the rate of
 58 offspring production declined with age significantly faster for females exposed to
 59 ABC-males than for females exposed to AAA-males (Fig. 1c). This was partly due to
 60 the fact that offspring egg-to-adult viability declined significantly faster as females
 61 aged in the ABC- than the AAA-treatment (Fig. 1d). We explored the generality of
 62 these results by estimating rate-sensitive female fitness costs under different intrinsic
 63 rates of population growth⁶, and confirmed that exposure to ABC-males resulted in
 64 relative fitness costs, both for individual females and entire female cohorts, that were
 65 particularly pronounced in contracting or stable populations (Fig. S1). Experiment 1
 66 therefore indicates that relatedness within male groups promotes female lifetime
 67 reproductive success largely by delaying reproductive ageing.
 68

69 We then investigated the signature of within-group relatedness on male competition.
 70 Relatedness can influence the way males compete over access to mating opportunities
 71 (pre-copulatory competition) and/or the way their ejaculates compete over
 72 fertilization (post-copulatory competition)⁴. For example, when females mate in a
 73 patch, then disperse to a new patch where they mate again, pre-copulatory
 74 competition occurs locally and post-copulatory competition, globally. We tested the
 75 effect of male relatedness within a group on male pre-copulatory competition
 76 (experiment 2), by measuring how males respond to changes in within-group male
 77 relatedness. We assembled male triplets that comprised three full-sib brothers (AAA
 78 treatment), two full-sib brothers and an unrelated male (AAB), or three males
 79 unrelated to each other (ABC), exposed each triplet to a single female unrelated to the
 80 males, and maintained vials without replacement. We detected no difference in
 81 mating rates across treatments ($\chi^2_2 = 0.071$, $P = 0.965$; mating rate [number of
 82 matings per 100 scans] estimate \pm se: AAA = 0.70 ± 0.158 , AAB = 0.76 ± 0.214 , ABC
 83 = 0.83 ± 0.260). However, consistent with expectations, fighting was more common
 84 in triplets of unrelated males (ABC) than in AAA- and AAB-triplets (Fig 2a). ABC-
 85 males also courted the female more intensely than AAA-triplets (Fig. 2b). We further
 86 confirmed the effect of within-group male relatedness on male behavior using the first
 87 axis of a Principal Component Analysis, summarizing different aspects of male
 88 fighting and courting (see Full Methods). Within-group relatedness was also
 89 associated with variation in male longevity. First, AAA-males lived on average longer
 90 than ABC-males (Fig. 2c). Second, survival analysis by means of a Cox proportional
 91 hazards model detected significant overall treatment effects in male mortality risk
 92 across treatments (Fig. 2d). While this experiment was not designed to test treatment
 93 effects on female fitness because males were allowed to co-age with females, and we

found no significant differences in female lifespan or reproductive success between females exposed to AAA- and ABC-males, the trends for females exposed to ABC-males to suffer shorter reproductive lifespan and lower lifetime reproductive success were in line with the findings of experiment 1 (Table S1). We next tested whether within-group relatedness also influences the intensity of male post-copulatory competition. For example, competing with relatives may inhibit male ejaculate investment in accessory gland products (Acps) such as the *Drosophila* sex peptide, which boosts female egg-laying rates and inhibits female re-mating, hence delaying sperm competition^{7, 8}, but can also induce contribute to female harm and reproductive ageing under certain conditions^{9, 10}. We tested this idea (experiment 3) by monitoring mating duration with the first male, latency to re-mate with a new male, and egg-laying rates in females, which were first mated to a male from the AAA-treatment, a male from the ABC-treatment or a control male kept in isolation. We found no difference in the mating duration, re-mating latency or egg-laying rate of the females first mated to AAA- vs. ABC-males (Table S2). These results suggest that within-group relatedness is associated with longer male lifespan and relaxes the key aspects of pre- (rather than post-) copulatory competition in this species: courtship and fighting.

To study how groups of relatives interact with unrelated competitors, we assembled (experiment 4) triplets comprising two brothers and one male unrelated to them (i.e. AAB), replicated across three different genetic stocks (wild-type *-wt-*, and two homozygous recessive mutants *-sepia (sep)*¹¹ and *sparkling (spa)*¹²- each backcrossed into the wild-type Dahomey population^{9, 13, 14}) and exposed to a single female double homozygous recessive for both *sep* and *spa*. This design enabled us to test whether males behaved differentially towards related (A) or unrelated (B) competitors, and to

assign offspring paternity to A- or B-males in each trial. We found no evidence of differential behavioural interactions (Table S3). An A-male was just as likely to fight with his brother than with the unrelated B-male (mean \pm se proportion of all fights that were direct to the B-male = 0.51 \pm 0.07; effect of relatedness: $z = 0.20$, $P = 0.84$). Similarly, the unrelated of the three males (B) did not court (0.34 \pm 0.03, difference from expected 0.33: $z = 0.20$, $P = 0.84$) or mate with the female more frequently than each of the two brothers (0.38 \pm 0.07, difference from expected 0.33: $z = 0.63$, $P = 0.53$). However, the unrelated B-male sired on average twice as many offspring as either A-male (Fig. 3, Table S3, S4), suggesting that a minority of unrelated competitors may gain a disproportionate share of reproductive success.

Sexual selection favours males that outcompete each other over access to females or their ova to a point that often harms female fitness², with pronounced repercussions for the population as a whole, reducing productivity and even leading to local extinctions^{15, 16}, a process akin to the tragedy of the commons¹⁷. In structured populations however, where local rivals can be genetically related to each other, harming females impacts the inclusive fitness of a male by reducing the reproductive success of his male relatives, and kin selection should discourage female harm by relaxing competition among related males³⁻⁵. Our study provides experimental support for these expectations in *D. melanogaster*. A proximate explanation is that elevated rates of harassment and male-male fighting, induced by low within-group male relatedness, impose cumulative costs on females and accelerate their reproductive ageing¹³. By mating with genetically different (i.e. unrelated) males, females could also incur higher immunological costs¹⁸. We found little evidence that differential female harm is mediated by post-copulatory traits, suggesting that post-copulatory male competition may occur on a more global scale than pre-copulatory competition⁴.

It would therefore appear that in the evolutionary past, the structure of natural *D. melanogaster* populations generated sufficient opportunity for the evolution of kin-selected sexual behaviours. Natural fly populations display limited dispersal and a tendency for local aggregations^{19, 20}, and although the extent to which different lab-adapted populations have retained kin-biased sexual behaviour is unclear, evidence of differential sexual responses based on kinship have been shown in some fly lab populations including our own study population¹⁴.

Although insects have inspired a large body of literature documenting how relatedness among group members structures social interactions, this work has largely focused on the particular case of eusociality^{1, 21, 22}. However, the influence of relatedness transcends eusociality and can modulate fundamental aspects of social behaviour more broadly. Sexual cooperation among related males has been observed in different animal societies²³⁻²⁷, however the fitness consequences for females have previously received little attention. While the possibility that sexual selection results in males harming females is well established², we currently lack a framework to understand the high variability in female harm observed across and within taxa⁵. Our study indicates that variation in relatedness and conditional behavioural responses to kin are potentially key factors underpinning such diversity. Whilst the genetic make-up of social groups was proposed as a modulator of female harm^{28, 29}, it was only recently that kin selection was explicitly applied to sexually-selected female harm³⁻⁵. This process is reminiscent of the way kin selection modulates virulence in pathogens³⁰. In both female harm and virulence, selfishness leads to a tragedy of the commons, which is inhibited by the relatedness of local competitors^{5, 30}. As in other cooperative systems¹, we found that minorities of selfish unrelated rivals may be able to invade and persist in groups of male relatives. This may be due to a number of

mechanisms, including an imperfect kin recognition system¹, e.g. males might respond to the average relatedness of the group because they are unable to recognize their relatedness to individual group members. Although it is difficult to extrapolate these experimental findings to the complexities of natural populations (e.g. variable patterns of relatedness among the offspring of polyandrous females), these results indicate that the benefits of relaxed competition among relatives may be dynamic, diminishing rapidly as populations become less viscous, a result consistent with our finding that the benefits of within-group male relatedness are higher in contracting populations. In conclusion, we present an experimental demonstration that genetic relatedness of social groups modulates the switch between sexual competition and cooperation. Future work should investigate the generality of these results and further resolve underpinning proximate mechanisms and evolutionary dynamics.

METHODS SUMMARY

Across experiments, male triplets were set-up by collecting recently eclosed (virgin) adult males from controlled 24h pairings of one-week-old (virgin) pairs of flies. Families were brought up in the same vials. Triplets consisted of three full-sib males (AAA), two full-sib males and one unrelated male (AAB), or three unrelated males (ABC). Male triplets were set up between 48-72h before the beginning of a trial, which begun by introducing a 48/72h-old virgin female (unrelated to any of the males in the triplet) into a vial with a male triplet.

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Author information. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to T.P. (tommaso.pizzari@zoo.ox.ac.uk).

Figure Legends

Figure 1| The effect of male-male relatedness on female fitness. (a) Female lifetime reproductive success was higher in the high-male relatedness (AAA) than in the low-male relatedness treatment (ABC, $F_{1,119} = 4.11$, $P = 0.045$). This difference was highly significant when we included female reproductive lifespan and its interaction with treatment as factors in the analysis ($F_{1,117} = 20.83$, $P < 0.001$). (b) Female reproductive lifespan was longer (ABC, $F_{1,119} = 6.55$, $P = 0.012$) and the probability to cease reproducing at any given time was lower ($\chi^2_2 = 3.95$, $P = 0.047$; $n_{AAA} = 63$, $n_{ABC} = 62$) in the high-male relatedness (AAA) than in the low-male relatedness treatment (ABC). (c) Female reproductive rates declined more sharply in individual females exposed to ABC- rather than to AAA-male (average number of offspring produced by AAA- and ABC-females over successive days of their life: treatment, $\chi^2_1 = 4.11$, $P = 0.043$; day, $\chi^2_1 = 1570.8$, $P < 0.001$; treatment*day interaction, $\chi^2_1 = 7.55$, $P = 0.006$). (d) Offspring viability (egg to adult survival) declined more sharply over time in females exposed to ABC- rather than AAA-males (treatment*week interaction: $\chi^2_1 = 9.23$, $P = 0.002$, estimated difference in viability drop AAA-ABC, mean \pm se: estimate = -0.231 ± 0.075). Vertical bars = se, horizontal bar with asterisk = significant comparisons; $n_{AAA} = 61$, $n_{ABC} = 60$, unless stated otherwise.

Figure 2| The effect of male-male relatedness on males. (a) Triplets of unrelated males (ABC) had a significantly higher frequency of male-male fighting than triplets of brothers (i.e. AAA, proportion of focal scans in which male-male fighting was observed, $\chi^2_2 = 14.46$, $P < 0.001$; Tukey, ABC-AAA, $z = 3.73$, $P < 0.001$, ABC-AAB, $z = 2.92$, $P = 0.01$, $n_{AAA}=47$, $n_{AAB}=47$, $n_{ABC}=45$). (b) Compared to triplets of brothers (AAA), triplets of unrelated males (ABC) were characterised by higher courting intensity (i.e. number of courting males when courting was observed, $\chi^2_2 = 5.01$, $P = 0.081$; Tukey ABC-AAA: $z = 2.38$, $p = 0.045$; $n_{AAA}=47$, $n_{AAB}=47$, $n_{ABC}=45$). (c) Male longevity was significantly lower in unrelated triplets (ABC) than among full-sib brothers (AAA, $F_{2,128} = 3.77$, $P = 0.026$; Estimated differential lifespan for ABC, mean \pm se: -5.62 ± 2.63 , $t = -2.139$, $P = 0.034$; $n_{AAA}=43$, $n_{AAB}=44$, $n_{ABC}=45$). (d) We found significant differences in male mortality risk across treatments ($\chi^2_2 = 10.47$, $P = 0.005$), and post-hoc direct comparisons between the treatments indicated that this effect was due to males in unrelated triplets (ABC) being more likely to die than in AAA- ($\chi^2_2 = 9.55$, $P = 0.002$) and AAB-triplets ($\chi^2_2 = 6.66$, $P = 0.010$; $n_{AAA} = n_{AAB} = n_{ABC} = 47$). Vertical bars = se, horizontal bar with asterisk = significant post-hoc comparisons.

Figure 3| Unrelated males outcompete brothers. Proportion of offspring sired by the unrelated male (B) in male triplets in which two brothers were matched with an unrelated male (AAB, $n = 54$). The B-male sired on average half of the offspring produced by the female, leaving the two brothers to share the other half. This distribution of paternity deviated significantly from an equalitarian distribution of paternity across the three males (i.e. 0.33, dashed line, $z = 3.99$, $P < 0.001$), and was independent of male stock (i.e. *sep*, *spa*). Vertical bars = se.

FULL METHODS

Experiments 1-3 used a lab-adapted, wild-type, Dahomey stock of *D. melanogaster*, maintained outbred since 1970³⁰. Experiment 4 used males from three different stocks: wild-type, and two homozygous recessive mutants, sepia (*sep*) and sparkling (*spa*), each backcrossed into the wild-type Dahomey population for at least five generations. Females for experiment 4 were from the same stocks and were double homozygous recessive for *sep* and *spa*. Flies were maintained at 25°C with overlapping generations to minimise selection on replication rate and life span. Across experiments, families were set up from eggs raised at a standard density (~100 flies per bottle)³⁰. Virgins were aged for one week before pairing for 24h to produce experimental flies, which were all 48-72 hours-post-eclosion at the beginning of trials. Families developed in the same vials. Triplets consisted of three full-sib males (AAA), two full-sib males and one unrelated male (AAB), or three unrelated males (ABC). Male triplets were set up between 48-72h before the beginning of a trial, which begun by introducing a 48/72h-old virgin female (unrelated to any of the males in the triplet) into a vial with a male triplet. Sample sizes were estimated from prior experiments, flies were haphazardly allocated to experimental groups in all experiments, behavioural observations were conducted by an observer blind to vial treatments, and animals were only excluded from analyses due to missing data. We checked that data met all necessary assumptions before running tests, including evidence for over- or under-dispersion. The potential influence of extreme outliers ($\alpha=0.01-0.05$) was explored by substituting extreme outliers for the next non-outlier value³¹, however this did not affect the qualitative outcome (direction and significance) of statistical tests. All reported *P*-values are two-sided

Experiment 1

Experiment 1 was designed to quantify the impact of within-group male relatedness on female fitness. We placed a single virgin female with three virgin males under two different social treatments ($n_{AAA}=63$, $n_{ABC}=62$; 1 ABC vial was excluded because one male in the triplet died before introducing the experimental female): (a) all three males were full-siblings (AAA), or (b) all three males were from different families (ABC). To avoid male co-ageing, we replaced male triplets by fresh young triplets (48-72h old) every 7 days. For each female, all new triplets were always constructed from the same families used to construct previous triplets. In order to achieve this, parental pairs were crossed 16d before introducing each batch of triplets; to minimize ageing, parental flies were isolated in vials containing standard sugar-yeast medium (but no live yeast) and maintained in a chamber at 20°C. Each parental family contributed males to only one male triplet (i.e. 3 males to a AAA triplet or one male to an ABC triplet; 252 parental families were used overall). To avoid sampling biases, we only used males from families that produced at least 3 males following each cross. Experimental foursomes (i.e. male triplet + experimental female) were changed to a fresh vial with live yeast 24h after triplets were introduced, which allowed us to estimate fecundity and egg-to-adult viability during the first 24h after having exposed experimental females to a set of novel triplet of males. Apart from that, foursomes were changed to a new fresh vial with live yeast every three days, and collected eggs were incubated at standard conditions for 12-15d after oviposition, at which time we counted emerging offspring. Offspring were hence collected in three batches per week in which the first batch consisted of offspring from day 1, the second of offspring from days 2-4, and the third of offspring from days 5-7. Vials were checked daily for female mortality until female death, at which time males were discarded. Vials in which the date of death of one of the individuals is unknown due to unexpected

contingencies (e.g. they escape during a change of vial) were eliminated from linear lifespan models but were included in the demographic survival analysis as ‘right-censored individuals’ up until the date the individual disappeared³². We quantified female lifespan (to the nearest day), the number of offspring each female produced per batch, egg-to-adult viability (only for offspring collected on day one each week; i.e. 24h after the introduction of each new male triplet), and lifetime reproductive success (total number of offspring). We also calculated w_{pop} and w_i as rate-sensitive fitness measures (see below). To generate daily offspring counts, offspring emerging from days 2-4 and 5-7 each week were assumed to follow a linear pattern of increase or decrease in number from the known count in day 1 of that week to the known count of day 1 of the next week⁶. We used linear models to test for differences in female lifespan, reproductive lifespan and lifetime reproductive fitness, for which analyses we excluded 2 AAA and 2 ABC females (right-censored, see above; final sample size: $n_{AAA}=61$, $n_{ABC}=60$). We also ran a Cox proportional hazards survival model (which included right-censored females) to look at differences in mortality risk and in the risk of ceasing to reproduce. To test for ‘selective death’, we examined whether early fecundity (i.e. fecundity during the first 24h), treatment, and the interaction between the two explained standardised female lifespan or standardised female reproductive lifespan. To examine ‘reproductive ageing’, we tested for an interaction effect between treatment and time (day) on variation in reproductive rate (i.e. offspring produced per day) with a Generalized Linear Mixed Model (GLMM) in which we included female reproductive lifespan, treatment, day and treatment*day interaction as fixed factors, and female identity as a random factor. We also tested for a treatment*week interaction in our egg to adult viability estimates of week 1 and week 2 (most flies had died by week three so we only included these two time points in the analysis). Values of w_{pop} and w_i were calculated from the fitness index developed by Charlesworth³³. Values of r were taken in the range of -0.4 to 0.4 as suggested for laboratory populations of *D. melanogaster*⁶. Values of w_{pop} were used to determine the relative costs (Cr) of decreasing within-group male relatedness for different values of r defined as: $Cr = w_{pop\ ABC} / w_{pop\ AAA}$ (³⁴). To facilitate comparisons with other studies, offspring counts were halved to take into account each female’s genetic contribution³⁵.

Experiment 2

In experiment 2 we followed the same focal male triplet along with its associated experimental female until the first male in the vial died (see below). For this experiment, we added a third treatment with two full-siblings and one unrelated male (AAB). The underlying rationale was to include a treatment with both related and unrelated males as behavioural responses might vary in this treatment (e.g. related males may cooperate or be more aggressive against the unrelated male). In this experiment, the design was paired: all the A-males belonged to the same family and therefore each family of males were represented thrice (AAA, AAB and ABC; one set). We set up 47 sets of male AAA/AAB/ABC trios by balancing the order in which trios reflecting different within-group male relatedness treatments were set-up. Systematic behavioural observations begun 24h after the beginning of the experiment, and were conducted every day for the first 5 days and then every second day for the next 5 days (i.e. days 2-6, 8 and 10). Observations started after lights on and lasted for a total of three hours, during which vials were scanned ca. every 10 minutes by a single observer that was blind to the treatment of each vial. We quantified matings, courtship events directed at the female³⁶, and the frequency of male-male aggressive

events³⁷, which were operationally defined as either a charging or boxing event as previously described^{38, 39}. We used these behavioural data to estimate: (a) mating rate (proportion of scans where mating was observed), (b) mated (whether a female mated or not during the 3h observation period), (c) courtship rate, (proportion of scans where courtship was observed), (d) courtship intensity (number of courting males when courting was observed), and (e) aggression rate (proportion of scans where male aggression was observed). We excluded two ABC triplets from this analysis because in one triplet one male died before the end of the first observation period, and the other triplet was lost during manipulation. In contrast to experiment 1, experimental vials were not supplemented with live yeast to maximize female survival during the first 10d of behavioural observations. Flies were transferred to a new fresh vial after the end of behavioural observations every day for the first two weeks of the experiment, and every second day thereafter. Vials were kept and checked daily for mortality until the first male in the vial died. In most vials, females died before the first male, in which case we discarded the female and retained the males until the first one of them died. We tested for treatment differences in male lifespan (i.e. first male to die in each vial) by fitting a linear model with treatment and the days males outlived the female as fixed factors. The latter variable was included to control for the fact that males that coexist with females that die soon may experience a more benign environment. We excluded 4 AAA, 3 AAB and 2 ABC males from this analysis because they were lost during manipulations (e.g. while changing them to fresh vials). We also fitted a Cox proportional hazards survival model (with ‘days outlived’ as covariate) to test for differences in mortality risk across treatments, including the males lost during manipulations as ‘right-censored’ individuals (i.e. individuals that are taken into account for demographic analysis until the day they disappear³²). Differences in reproductive behaviors across treatments were analyzed using a time-explicit analysis by fitting five separate GLMMs with treatment, day and its interaction as fixed factors and female identity as a random factor; we used Gaussian error distributions for all the variables except for ‘mated’, which was modeled with a binomial error distribution. Given there were no treatment differences in the variation of behavioural rates with time, we complemented this analysis pooling behavioural data across days and testing for treatment effects on the averaged values of courtship rate, courtship intensity and fighting rate, and on the total number of matings. We fitted Generalised Linear Models (GLMs) with Gaussian error distributions for courtship rate and fighting rate, with Poisson error distribution for total number of matings (which allowed us to test for over-/under-dispersion of data), and with Gamma error distribution for courtship intensity (data positively skewed due to positive extreme outliers). These analyses confirmed results from the time-explicit analysis (total number of matings: $F_{2, 136} = 0.026$, $P=0.974$, residual deviance/residual degrees of freedom = 1.21; courtship rate: $F_{2, 136} = 1.136$, $P=0.324$; courtship intensity: $F_{2, 136} = 5.583$, $P=0.005$, ABC estimate \pm se=0.04 \pm 0.02, $t=2.05$, $P=0.042$; fighting rate: $F_{2, 136} = 6.872$, $P=0.00$, ABC estimate \pm se=0.02 \pm 0.006, $t=3.50$, $P<0.001$). For courtship intensity, substituting extreme outliers for the next non-outlier value³¹ ($\alpha=0.1$) was effective in transforming positively skewed courtship intensity data to a normal distribution, and a GLM with Gaussian error distribution on this data also showed a significant treatment effect (courtship intensity: $F_{2, 136} = 3.056$, $P = 0.05$, ABC estimate \pm se=0.06 \pm 0.02, $t=2.45$, $P=0.015$). Finally, because male fighting rate and courtship intensity were positively correlated across triplets ($F_{1,133}=25.250$, $P<0.001$), and because the strength of such correlation was greater in ABC-triplets (treatment*courtship intensity interaction term, $F_{2,133}=4.071$, $P=0.019$;

ABC*courtship intensity, estimate \pm se=0.083 \pm 0.038, $t=2.22$, $P=0.028$; fighting rate ~ courtship intensity simple effects for: AAA, $F_{1,45}=4.206$, $P=0.046$, $F_{adj}=0.065$; AAB, $F_{1,45}=0.463$, $P=0.45$, $F_{adj}=-0.012$; ABC, $F_{1,43}=15.54$, $P<0.001$, $F_{adj}=0.248$) we performed a Principal Component Analysis (PCA) on averaged data of male fighting and both measures of male courting (i.e. courtship rate and courtship intensity). Given there were no treatment differences in the variation of behavioural rates with time, we subsequently used data averaged across days to look at correlations between behavioural measures, and to run the PCA. The first axis (PC1) explained over 62% of the variance and captured a concordant proportion of variation in courting rate, courting intensity, and fighting intensity (Loadings=0.582, 0.598 and 0.550, respectively), so we retained this variable as a combined measure of male-male competition. We confirmed that PC1 significantly varied with within-group male relatedness ($\chi^2_2=6.675$, $P=0.036$), which was driven by higher values of PC1 in ABC- than in AAA-triplets (Tukey, $z=2.539$, $P=0.033$).

Experiment 3

In order to test for potential differences in ejaculate allocation between AAA- and ABC-males, we conducted an experiment in which we examined how mating with males kept under different relatedness treatments influenced the key ejaculate-mediated female post-mating responses (receptivity and egg-laying rate). We set up 300 male vials ($n=100$ each) containing either: (a) three full-siblings (AAA), (b) three unrelated males (ABC), or (c) a single male (control). All males were isolated as virgins upon emergence and were kept in treatment vials for 72-96h prior to the beginning of the experiment (day 1). On day 1, after lights on, we haphazardly selected one male in each vial and aspirated it into a fresh vial containing a young (3-4d old) unrelated virgin female. Pairs were left together to mate and vials in which matings did not occur within 120 minutes were discarded ($N_{AAA}=15$, $N_{ABC}=11$, $N_{control}=26$). In vials where mating did occur, we measured mating duration. At the end of matings, we discarded the male and left the female to lay eggs until the following day. On day 2, after lights on, we aspirated females into a fresh vial with a young (6-7d old), unrelated virgin male, and monitored them for 8h or until re-mating was observed. We retained ‘old’ vials to count the eggs laid by the female and calculated egg laying rate as total eggs laid/total egg laying time (i.e. time from end of mating on day 1 until transfer into fresh vial on day 2). We discarded from the analysis 6 AAA, 8 ABC and 9 control females that did not lay eggs (final sample size: $n_{AAA}=79$, $n_{ABC}=81$, $n_{control}=65$). We used three separate GLMs to test for: (a) differences in mating duration across treatments (i.e. AAA, ABC and control), (b) the effect of within-group male relatedness on female probability to re-mate, with re-mating (i.e. re-mated or not) as a binomial response variable and mating duration, treatment and their interaction as fixed effects, and (c) to look at whether within-group male relatedness affected early egg-laying rate (i.e. during the first 24h of experiment), with egg laying rate as response and treatment, mating duration and their interaction as fixed effects.

Experiment 4

We set up AAB triplets ($n = 54$ each) using males from three different stocks: wild-type, and two homozygous recessive mutants, sepia (*sep*)¹¹ and sparkling (*spa*)¹², each backcrossed into the wild-type for five generations. Females were double homozygous recessive experimental females (*spa/sep*). Families used in one set were not used for another. Males from different families also possessed different eye colour to facilitate calculation of paternity estimates (see below). We adopted a randomised

balanced design: 54 vials of trios were set up, comprising of 18 vials where wild-type males were designated as ‘A’ males, 18 vials with *sep* males as ‘A’, and 18 vials with *spa* males as ‘A’ males. Males were marked with either red, yellow or green acrylic paint⁴⁰ in a randomised balanced design to allow identification and detailed observations of inter- and intrasexual interactions. We quantified the courtship rate, aggression rate and mating rate in two-minute spot-checks. This was done for three hours upon lights-on on the first three days of the experiment. To quantify paternity in treatment AAB, we counted the number of offspring with different eye colour. We analysed the effect of male relatedness on courtship, male-male aggression, mating and paternity share, using binomial GLMs and beta-binomial GLMs whenever we detected evidence of over- or under-dispersion⁴¹ (see Table S2). We tested the effect of male relatedness on courtship in three ways. First, we conducted a GLM with beta-binomial error distribution with the proportion of courtship attained by the B-male as the response variable and the genotypes of A- and B-males as covariates, and tested whether the parameter estimate of proportion of courtship was different from the null expectation of 0.33 with a z-test. This analysis showed that there was no effect of genotype on the proportion attained by the B-male (Table S3). Second, we then conducted another beta-binomial GLM with 3-alternative forced choices (3-AFC)⁴² to verify that the proportion of courtship attained by the B-male differed significantly from the null expectation of 0.33. Finally, we tested whether the mean of the distribution of the mean courtships for each of the six genotypic combinations differed from the null mean of 0.33 with a one-sample t-test. We tested for the effect of male relatedness on male-male aggression in a similar way: (a) one of the two A-males was randomly chosen as the focal male and the proportion of all aggression counts that he directed towards the B-male was tested against the null expectation of 0.5 with a z-test using the parameter estimate obtained from a beta-binomial GLM with the genotype of A-male and genotype of B-male as covariates, (b) a beta-binomial GLM with 2-alternative forced choices (2-AFC)⁴², and (c) with a one sample t-test comparing the mean of the distribution of mean proportion of aggressive counts across the six genotypic combinations against the null expectation of a mean of 0.5. We tested whether the proportion of mating by the B-male differed from 0.33 using a binomial GLM and z-test, and a one-sample t-test comparing the mean of the distribution of mean proportion of mating across the six genotypic combinations against the null expectation of a mean of 0.33. Finally, we tested whether the share in paternity of the B-males deviated from the null expectation of 0.33 using: (a) a z-test comparing the parameter estimate of paternity share obtained from a beta-binomial GLM with the genotype of A-male and genotype of B-male as covariates, against the null expectation of 0.33, (b) a beta-binomial GLM with 3-AFC, and (c) a one sample t-test comparing the mean of the distribution of mean paternity share across the six genotypic combinations against the null expectation of a mean of 0.33.

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SUPPLEMENTARY RESULTS LEGENDS

Fig. S1. (a) Rate-sensitive estimates of individual female fitness over a gradient in population growth rates (r). Female fitness was estimated to be higher under high within-group male relatedness for values of r ranging from -0.1 to 0 (dark shaded area), a similar non-significant ($0.05 < P < 0.08$) pattern was extended for $r = -0.2$ and $r = 0.1$ (light shaded area). **(b)** The effect of within-group male relatedness on population fitness. The relative fitness cost of reducing within-group male relatedness at different population growth rates (r). The dashed line identifies relative fitness of 1 where reduction in within-group male relatedness has no fitness cost. Reducing within-group male relatedness is always costly over the range of population growth rates explored, but particularly so with smaller growth rates.

Table S1. Female rate-insensitive fitness measures in experiment 2. Due to the co-ageing of males in each experimental vial and to potential Coolidge effects, experiment 2 was not adequate to detect the effect of within-group male relatedness on female fitness, and we found no significant treatments affects in rate-insensitive measures of female fitness. However, fitness measures follow the same trends observed in experiment 1. Furthermore, the analysis of survival curves in experiment 2 suggests a relatively higher initial mortality in ABC- compared to AAA-vials at day 8, which is when male triplets were replaced by fresh males in experiment 1 (Survival, mean \pm se: AAA = 0.98 \pm 0.02; AAB = 0.92 \pm 0.04; ABC= 0.87 \pm 0.05).

Table S2. Female post-mating responses in experiment 3. We did not find any evidence of differences in female receptivity or egg laying rate between females mated to AAA- vs. ABC-males ($n_{AAA}=79$, $n_{ABC}=81$, $n_{control}=65$). We found a significant treatment effect on mating duration ($F_{2, 222}=17.98$, $P < 0.001$) but this was due to both AAA- and ABC-males mating for longer than control males (Tukey: Control-AAA, $t=-5.839$, $P < 0.001$; Control-ABC, $t=-3.975$, $P < 0.001$; ABC-AAA, $t=-1.023$, $P=0.251$). Similarly, we found a significant treatment effect on female re-mating propensity (Treatment effect, deviance=10.448, $P=0.005$; Interaction term, deviance=1.208, $P=0.547$), but this was again due to females mated with AAA- and ABC-males having a significantly lower probability of re-mating that control males (Tukey: AAA-Control, $t=-0.923$, $P=0.038$; ABC-Control, $t=-1.133$, $P=0.006$; ABC-AAA, $t=-0.210$, $P=0.813$). Finally, we did not find significant treatment differences in egg-laying rate (Treatment effect, deviance=5.540, $P=0.063$; Interaction term, deviance=0.476, $P=0.788$; Tukey: ABC-AAA, $z=1.532$, $P=0.275$; Control-AAA, $z=2.296$, $P=0.056$; Control-ABC, $z=0.976$, $P= 0.591$).

Table S3. Summary of statistical tests in experiment 4. Paternity share by the B-male was significantly different from 0.33. Proportion courtship and mating by B-males did not differ from 0.33 and proportion of all aggressive events performed by a focal A towards the B male did not differ from 0.5.

Table S4. Effect of genotype of A-male and genotype of B-male on the response variable. There was no effect of the genotype of either A- or B-males on any of the paternity or behavioural responses measured.