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THE EVOLUTION OF HARM – EFFECT OF SEXUAL CONFLICTS AND POPULATION SIZE

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ABSTRACT

Conflicts of interest between mates can lead to the evolution of male traits that reduce female fitness and that drive coevolution between the sexes. The rate of adaptation depends on the intensity of selection and its efficiency, which depends on drift and genetic variability. This leads to the largely untested prediction that coevolutionary adaptations such as those driven by sexual conflict should evolve faster in large populations. We tested this prediction using the bruchid beetle *Callosobruchus maculatus*, a species where harm inflicted by males is well documented. Whilst most experimental evolution studies remove sexual conflict, we reintroduced it in populations where it had been experimentally removed. Both population size and standing genetic variability were manipulated in a factorial experimental design. After 90 generations of relaxed conflict (monogamy), the reintroduction of sexual conflicts for 30 generations

favoured males that harmed females and females more resistant to the genital damage inflicted by males. Males evolved to become more harmful when population size was large rather than when initial genetic variation was enriched. Our study shows that sexual selection can create conditions where males can benefit from harming females and that selection may tend to be more intense and effective in larger populations.

KEYWORDS

Experimental evolution, sexual selection, *Callosobruchus maculatus*, genital damage, population size

Sexual conflict occurs when the evolutionary interests of males and females differ (Parker 1979), and can result in the evolution of traits beneficial to individuals but harmful to their mates (Arnqvist and Rowe 2005). Extreme examples of this phenomenon occur when male reproductive behaviour harms females via traits such as toxic substances transferred in the ejaculate (Chapman et al. 1995; Eady et al. 2007; Rice 1996) or damaging intromittent organs (Blanckenhorn et al. 2002; Crudgington and Siva-Jothy 2000; Stutt and Siva-Jothy 2001).

Two hypotheses have been proposed to explain the evolution of harm. First, the collateral harm hypothesis (Hosken et al. 2003; Morrow et al. 2003) suggests that harm is a side effect of adaptations beneficial in male-male competition (Lessells 2006; Parker 1979). For example, in *Drosophila melanogaster* genotypes that have superior sperm defence capabilities reduce female longevity (Civetta and Clark 2000). Alternatively, the adaptive harm hypothesis posits that harm benefits males more directly because of the reduction of female survival. For example, injuries could deter females from subsequently re-mating and/or alter female perceptions of their health status resulting in increased resource reallocation to reproduction. Theoretical treatments support this "terminal investment" hypothesis (Johnstone and Keller 2000; Lessells 2005), even when damage decreases the re-mating interval (Lessells 2005). However, empirical support for these models is lacking (Hosken et al. 2003; Morrow et al. 2003).

The bruchid beetle (*Callosobruchus maculatus*) is a species where harm inflicted by males is well documented. Male bruchid beetles have a complex aedeagus, the internal

sac of which is covered with spines that puncture the female genital tract during copulation (Crudgington and Siva-Jothy 2000). Despite comparative evidence supporting the notion that the spines are involved in male-female antagonistic coevolution at the interspecific level (Rönn et al. 2007), evidence for an association between sexual selection and genital damage is scarce at the intraspecific level. Hotzy and Arnqvist (2009) demonstrated a correlation between spine length and male success in sperm competition across populations, but no such relationship was found in two other studies investigating why male bruchid beetles harm their mates (Edvardsson and Tregenza 2005; Morrow et al. 2003). Here we use an experimental evolution approach to further assess the potential link between harm and sexual selection.

Experimental evolution is a powerful tool that can be used to assess the evolution of harm and female resistance to it. This approach has been used to eliminate sexual conflict (and drastically reduce sexual selection) by enforcing monogamy. Males evolving under monogamy should evolve to become more benign to their partners since male and female fitness are simultaneously maximized, while monogamous females should become more susceptible to harm because selection on counteradaptations to reduce harm is relaxed (assuming that female resistance is costly). These predictions have been supported in experimental populations of *Drosophila melanogaster* (Holland and Rice 1999; Pitnick et al. 2001a; Pitnick et al. 2001b). Similarly, enforced monogamy in the fly *Sepsis cynipsea* enhanced female survival (Martin and Hosken 2003a) and monogamous populations of *Scathophaga stercoraria* had higher fitness than polyandrous lines (Martin et al. 2004). In an experiment where natural selection and sexual selection were manipulated

simultaneously, Fricke and Arnqvist (2007) showed that, when reared on standard diets, monogamous selection lines of *Callosobruchus maculatus* produced more offspring. Recent studies have employed sex ratio biasing, to manipulate sexual conflict and sexual selection. In *D. pseudoobscura*, male biased populations (with more scope for sexual selection) did not differ greatly from monogamous lines (Crudgington et al. 2005), and Wigby and Chapman (2004) found no difference in the male harming ability of *D. melanogaster* lines with different sex ratios.

Following the publication of the first experimental evolution studies aimed at understanding the role of sexual selection by manipulating the mating regime, Snook (2001) and then Wigby and Chapman (2004) argued that altering the sex ratio or population density can result in differences in effective population size, so that different treatments experience different levels of drift and inbreeding. Additionally, because monogamous lines often have a smaller population size, differences in population sizes can be confounded with treatment. However, while these criticisms are in principle sound, they were refuted for the specific studies initially criticized (Rice et al. 2005; and see Reuter et al. 2008). More recently, Snook et al. (2009) raised additional concerns about inbreeding and genetic variation when population size is manipulated. The authors stress that a lack of genetic drift and higher genetic variability could result in more efficient selection in large populations. Beyond the effect of drift and genetic variability, theoretical models also suggest that sexually antagonistic coevolution is more likely in large populations (Gavrilets 2000). Higher densities might favour more intense sexual conflicts, due for example to interference from other males, through physical harm to

females, seminal fluid toxicity or polyspermy (Arnqvist 1997; Arnqvist and Nilsson 2000; Gavrilets et al. 2001). Population size could therefore affect evolution via sexual conflict in two ways: either because sexually antagonistic coevolution is more likely in large populations, or because selection is more efficient in large populations (Robertson 1970). The later could result from the fact that large populations harbour greater levels of standing genetic variation and experience more mutations and little drift (Schultz and Lynch 1997; Willi et al. 2006). While there is evidence consistent with population size effects on sexually antagonistic evolution (Gay et al. 2009; Hosken et al. 2009; Martin and Hosken 2003b), there have been few attempts to document the relative effects of the potential causal factors involved (but see Ödeen and Florin (2000) regarding selection efficiency). Here we use a fully factorial experimental design where both population size and standing genetic variability are manipulated to disentangle the effect of intensified sexual conflicts from the effect of increased genetic diversity, in a context of reintroduced conflicts.

Starting with populations in which monogamy has been enforced for 90 generations, we reintroduced sexual conflict and sexual selection by allowing free mate choice and multiple mating. We established replicate populations differing in size and standing genetic variability. After 30 generations of reintroduced sexual conflict and sexual selection, we preliminarily tested for effects of inbreeding in small and low variability populations. Then we examined whether genital damage evolved in response to the reintroduction of sexual conflict (1), by comparing the extent of genital damage in females mated to males from polygamous (conflict) lines compared to the monogamous

(relaxed conflict) lines from which they had been established 30 generations previously. Then we examined whether sexual conflict resulted in more rapid evolution in larger populations or those with greater initial genetic variation (2), by comparing the evolution of adaptations to polygamy across our lines. Additionally, we assessed the costs of damage (3) by evaluating associations between level of damage and female longevity and lifetime reproductive success. Finally, we tested the two hypotheses about why males harm females (4): Are damaging males better at accelerating female oviposition or deterring females to re-mate (adaptive harm hypothesis) or are they better at sperm competition (collateral harm hypothesis)? We simultaneously tested for an effect of population size and genetic variability on male manipulative ability (5).

Material and methods

STUDY SPECIES AND EXPERIMENTAL DESIGN

Two replicate monogamous lines were established from an ancestral *C. maculatus* population (Niamey, Niger) cultured on black eyed-beans (*Vigna unguiculata*) at 27°C, 32 % RH and 16L:8D photoperiod. Each generation we isolated beans carrying eggs in 48-well cell culture plates in order to collect virgin beetles immediately post-emergence. Virgins (< 24h post eclosion) were subsequently paired and each pair was placed in a 40mm Petri dish and observed until copulation had ceased. From these monogamous pairs, 60 singly mated females were transferred together to approximately 400 beans for oviposition.

After 90 generations of enforced monogamy, polygamy was re-established in new populations established from the two lines by placing 60 newly emerged adults of each

sex from each line on 400 beans. A third polygamous line was created by combining 30 males and 30 females from each of the monogamous lines. In this crossed population, genetic variability should be greater, because 90 generations of isolation and drift is likely to have promoted genetic differentiation and some loss of diversity from the two monogamous lines. These three polygamous lines were allowed to expand exponentially for two generations, before we established 16 experimental populations. The crossed population (with enriched genetic diversity) seeded eight lines at two different densities (four small populations size = 50 individuals, four large populations size = 5000 individuals). Each of the two other polygamous lines was used separately to start another four polygamous lines with basal genetic variability, two small (50 individuals) and two large (5000) (Fig. 1). This generated four treatments (small population size and basal genetic variability; small population size and enriched genetic variability; large population size and basal genetic variability; large population size and enriched genetic variability) each with 4 replicates. Males and females were housed together for their entire lifespan in all 16 lines. We continued to maintain the monogamous populations, as above.

To retain a constant population size and ratio of resources to beetles, we sieved and weighed the newly emerging adults each generation and placed another 50 (for the small populations), or 5000 (for the large ones) individuals on new black-eyed beans. Small populations were provided with 40g of beans in a cylindrical container 10cm wide and 4cm deep, large populations were provided with 4kg of beans in a rectangular container 30cm x 20cm x 13cm deep. Half of the populations for our genetic variability treatment are derived from each monogamous line. Comparison between the basal genetic

variability populations created from monogamous line 1 and monogamous line 2 revealed male-induced damage, LRS, female re-mating rate, oviposition speed and P2 to be equivalent, although the populations derived from monogamous line 1 lived significantly longer than those derived from monogamous line 2 (12 days versus 11). We accounted for this difference in the analysis of longevity (see below).

To reduce possible maternal and phenotypic effects, we standardized selection one generation prior to the assay (generation 30) for all populations by housing beetles individually under standardised conditions - single mating and one egg per bean (this is in excess of what a single larva can consume (Cope and Fox 2003)) - for one generation. Prior to beetle emergence, we isolated these beans in 'virgin chambers' (48-Well cell culture plates, VWR International Ltd, Lutterworth, UK). Beans were checked every 24h for emerging virgin adults (generation 31).

TEST FOR INBREEDING DEPRESSION

In our experiment, the small populations are potentially susceptible to inbreeding during experimental evolution. Inbreeding can lead to inbreeding depression affecting life history traits (e.g. fecundity and longevity) (Charlesworth and Charlesworth 1987; DeRose and Roff 1999) and competitive male mating ability (Sharp 1984). These effects could potentially confound our predictions (see below). We looked for evidence of inbreeding depression in fecundity, lifetime reproductive success and longevity by crossing males and females between replicate populations and comparing their performance to matings between males and females from within replicate populations (the potentially inbred populations). We assessed those treatments most likely to suffer

inbreeding depression, namely the populations of small census size and basal initial standing genetic variation. We also assessed the large populations with basal initial standing genetic variation as this allowed us to determine the potential impact of population size and initial genetic variance on inbreeding depression. We analysed these data using a general linear model including population size, crossing status (within or between replicate crosses) and their interaction. Elytra length (a measure of body size) was included as a covariate in the analysis of fecundity and lifetime reproductive success, whilst fecundity was included as a covariate in the analysis of longevity.

MALE OFFENCE AND FEMALE RESISTANCE: DAMAGE, LONGEVITY AND LIFETIME REPRODUCTIVE SUCCESS

Both males and females are likely to influence the amount of damage suffered by females during copulation. To isolate the damaging effect of males from the susceptibility of females, we used the two monogamous lines as testers. Four types of crosses were performed: (1) between males from the polygamous populations and tester females (male offence assay - $♀_{M}♂_{P}$); (2) between males and females from the same polygamous population (female resistance assay - $♀_{P}♂_{P}$); (3) between females from the polygamous populations and tester males ($♀_{P}♂_{M}$); (4) a control cross between tester males and females ($♀_{M}♂_{M}$). For each assay, 20 crosses were performed for each replicate (x4) of each treatment (x4) (= 1280 crosses).

Virgin females and males (all <24h post eclosion) were paired and each pair (10 pairs x 4 treatments x 4 replicates x 4 crossings) was placed in a 40mm Petri dish and observed

until copulation had ceased. Mated females were then placed on 10 beans for 24 hours and then moved to another 60 beans for the remainder of their lives. We measured fecundity in the first 24 hours of oviposition by directly counting eggs laid. Longevity was estimated by recording female mortality every 24 hours. After their natural death, females were dissected and the number of damage points (scars) in their genital tracts determined. For 25 females we also measured the area covered by scars and found that it was highly correlated with the number of scars (log-linear regression, $R^2 = 0.68$). Female elytra length was measured as a proxy for body size.

MANIPULATION OF RE-MATING AND OVIPOSITION

We measured the ability of males to deter females from subsequently re-mating (male defence) by mating monogamous tester females with males from the polygamous populations and then exposing them to monogamous tester males ($♀_M$-$♂_P$-$♂_M$). We also measured male offence - the ability of males to induce previously mated females to re-mate - by mating monogamous tester females with monogamous tester males and then exposing them to males from the polygamous populations ($♀_M$-$♂_M$-$♂_P$). For each assay, 10 females were paired and subsequently offered a chance to re-mate, following 24h of oviposition. Earlier studies revealed that over 80% of females will re-mate 24 h after their initial copulation (Eady et al. 2004; Edvardsson and Tregenza 2005) but in a pilot experiment we found lower re-mating rates in our lines that were maintained monogamous for 90 generations. We thus estimated that 24 h is a time point at which one might be able to distinguish differences in female re-mating propensity between

populations. Females were transferred to a 40mm Petri dish with a new virgin male (from the appropriate line) and were observed for 30 minutes to see if they copulated.

We measured the ability of males from the polygamous populations to stimulate female fecundity by counting eggs laid during the female refractory period using males from the 16 polygamous lines mated to 10 monogamous tester females. Again, mated females were placed on 10 beans for 24 hours and then moved to another 60 beans for the remainder of their lifespan. We subsequently counted the number of offspring produced during the first 24h after mating and over their entire lifespan, and then used the proportion of offspring produced in 24h relative to the lifetime reproductive success as a measure of male manipulation. Because both female re-mating rate and last male sperm precedence are high in this species (Eady et al. 2004; Edvardsson and Tregenza 2005), the benefits to any additional stimulation of oviposition beyond the first 24 hours will probably be enjoyed by rival males and as such we did not assess them here.

SPERM COMPETITION

We used a standard sperm competition experiment - where females are mated with two males - to test the hypothesis that harmful males are more successful at sperm competition. Males from the polygamous populations were competed against black tester males from a separate polygamous line with both mating to a black tester female. The black phenotype is a naturally occurring polymorphism and this co-dominant marker was used to score offspring. Offspring sired by brown males (with black females) are

phenotypically intermediate (dark brown body colour and brown legs and antennae) and readily discernable from offspring from a black x black pair (Eady 1991).

Virgin black females and black males were paired in individual 40mm Petri dishes and observed until copulation began. After copulation ceased, males were removed and females were allowed to oviposit for 20 hours on 5 beans. Females were then transferred back to individual 40mm Petri dishes with a virgin brown male from one of the polygamous populations. We repeated this for at least 20 females per replicate (4) per treatment (4). For each pair, we recorded whether copulation occurred successfully within 30 minutes. After copulation with the focal (brown) male ceased, each black female was transferred to a 90mm Petri dish containing 80 beans and allowed to oviposit until death. Eggs laid prior to the second mating were counted (first 20 h), as were the total number of offspring after two successive matings, and offspring phenotype (hybrid or black) was recorded. P2 - the proportion of offspring sired by the second (focal = brown) male was calculated as the proportion of intermediate offspring. The experiment was repeated at generation 32 to increase the sample size. We accounted for this by including a generation factor in the analytical models. Additionally, to ascertain confidence in our co-dominant phenotypic marker, we estimated the repeatability of our paternity estimates by re-measuring P2 blind to the first measurement for 20 randomly chosen females. P2 repeatability was calculated following Lessells & Boag (1987), and was high (r = 0.996).

STATISTICAL ANALYSES

Analyses were performed in R. To avoid pseudoreplication, we performed all analyses on population means. We also used mixed effect models adding replicate as a random effect and obtained similar results, but only the results using the population means are presented here. All traits (damage, longevity, fecundity, lifetime reproductive success and elytra length) were normally distributed (Kolmogorov-Smirnov test, all $P > 0.05$). Additionally, residuals did not deviate significantly from normality (Kolmogorov-Smirnov test, all $P > 0.05$), and were not autocorrelated (Durbin-Watson test, all $P > 0.05$), and errors were homoscedastic (Breusch-Pagan test, all $P > 0.05$).

Cost of damage

We used a general linear model to test the effect of population size, genetic variability and their interaction on genital damage inflicted by polygamous line males. Female type (monogamous or polygamous) was used as a third factor. We examined whether genital damage evolved with the reintroduction of sexual conflict and sexual selection by testing for an effect of male and female type (from a polygamous or monogamous line) on the amount of damage sustained by a female, using data from four assays ($♀_M♂_P$, $♀_P♂_P$, $♀_P♂_M$ and $♀_M♂_M$). We also examined the cost of damage by testing for a negative relationship between damage and longevity or damage and LRS using linear models. We included population size, genetic variability and female type in the model, as well as elytra length as a covariate for LRS and 24h fecundity as a covariate for longevity to account for life history trade offs. To account for the difference in longevity between the populations of the low variability treatment derived from the two monogamous lines, we

added a third level to the factor "genetic variability" (i.e. we replaced basal/enriched variability with basal from M1/basal from M2/enriched).

Effect of damage on re-mating, oviposition and sperm competition

Harm could be beneficial for males if it deters females from re-mating, if it accelerates the oviposition rate or if it provides an advantage in sperm competition. We tested these hypotheses using generalized linear models with the number of damage points (scars) in females' genital tracts as an explanatory variable. For re-mating and sperm competition (P2), a binomial error distribution was used. We corrected for overdispersion using a quasi-binomial model when the ratio of residual deviance by residual degrees of freedom was larger than one. The number of eggs laid by the female in the first 24h (between both mating occasions) was used as a covariate for re-mating and P2, elytra length was used as a covariate for all three variables.

Effect of population size and genetic variability on male manipulative ability

To ascertain how population size and genetic variability influence the evolution of males' ability to affect female reproduction, we compared re-mating rates, oviposition rate and P2 between our experimental populations that differ in the level of damage inflicted by males. For re-mating, we estimated an index of male manipulation by combining the assays of male defence ($\female_M$-$\male_P$-$\male_M$) and male offence ($\female_M$-$\male_M$-$\male_P$): male manipulation was estimated as the difference between the proportion of females re-mating in the offence experiment minus the proportion that re-mated in the defence experiment. We tested the effect of population size, genetic variability and their interaction on this re-

mating manipulation-index and on oviposition speed using a linear model with female tester line as a covariate. For sperm competition, we used a generalized linear mixed model with a quasi-binomial error distribution to test for the effect of population size, genetic variability and their interaction on P2, the number of offspring sired by the second of two males to mate with a female (see Sperm competition above). The number of eggs laid in the first 20 h and a generation factor were included as covariates.

Results

TEST FOR INBREEDING DEPRESSION

There was no evidence for inbreeding depression in small and low variability populations. We found no significant effect of the interaction between population size and crossing status (within or between replicate crosses) (Fecundity: $F_{7,1}$=0.5 p=0.480; longevity: $F_{7,1}$=0.04 p=0.837; LRS: $F_{7,1}$=0.5 p=0.517). Fecundity and longevity were not significantly different in crosses within or between replicate populations (Fig. 2a: $F_{9,1}$=0.9 p=0.360 and Fig. 2b: $F_{8,1}$=0.01 p=0.909). Population size also had no effect on these fitness measures, suggesting that inbreeding depression was either absent or was similar across experimental populations (Fig. 2a: $F_{10,1}$ = 2.8 p = 0.125 and Fig. 2b: $F_{9,1}$ = 0.4 p = 0.533). Lifetime reproductive success (LRS) was also equivalent in the within or between replicate crosses (Fig. 2c; $F_{9,1}$ = 1.6 p = 0.234), but population size had an effect with small populations having lower LRS than large populations (Fig. 2c; $F_{10,1}$ = 8.6 p = 0.015). When the analysis was restricted to small populations only, fecundity, longevity

and LRS within and between replicate crosses remained equivalent. These results suggest that population size influenced LRS, but this was not the result of inbreeding depression.

GENITAL DAMAGE EVOLVES IN RESPONSE TO THE REINTRODUCTION OF SEXUAL CONFLICT

Females mated to males from the monogamous populations sustained less damage than those mated to males from the polygamous populations (monogamous males: 29 points of damage ± 2; polygamous males: 39 ± 2; $F_{48,1}$=12 p = 0.0009; Fig. 3). However, the susceptibility of females did not seem to have evolved in the 30 generations after the reintroduction of sexual conflict (monogamous females mated to polygamous males: 38 points of damage ± 2; polygamous females mated to polygamous males: 33 ± 2; $F_{47,1}$=0.2 p = 0.675; Fig. 3). There was no significant interaction between male and female type ($F_{46,1}$=0.02 p = 0.872).

DAMAGE EVOLVES FASTER IN LARGER RATHER THAN MORE DIVERSE POPULATIONS

As there was no difference between monogamous or polygamous females in susceptibility to damage, we analysed the effect of population size and genetic variability on damage using all the crosses involving males from polygamous populations (♀$_M$♂$_P$ and ♀$_P$♂$_P$). Males from large populations inflicted more damage to females (large population: 44 points of damage ± 2; small population: 33 ± 2; $F_{30,1}$ = 15.5 p = 0.0005; Fig. 4). There was no significant effect of population genetic variability ($F_{29,1}$ = 1.8 p =

0.189) or of female type (monogamous: 39 ± 2; polygamous: 38 ± 2; $F_{28,1} = 0.3$ p = 0.597).

GENITAL DAMAGE IS COSTLY

The number of damage points in a female's reproductive tract was negatively associated with female longevity (Fig. 5, slope = -0.04 days/damage point; $F_{30,1} = 5.5$ p = 0.027, Table 1). Furthermore, females from the polygamous populations tended to outlive females from monogamous populations (M: 10.9 days ± 0.2; P: 11.7 ± 0.3; $F_{30,1} = 4.6$ p = 0.040, Table 1, Fig. 5). This was also reflected in the LRS results, where females from polygamous populations had greater LRS (M: 69 offspring ± 2; P: 78 ± 2; $F_{26,1} = 8.7$ p = 0.006, Table 2). LRS was also influenced by an interaction between the number of scars in the female tract and polygamous line population size ($F_{26,1} = 7.0$ p = 0.014, Table 2). More scaring in females from larger populations resulted in lower LRS, but for females from smaller populations the association between genital damage and LRS was flat or even positive (Fig. 6). Note that when we removed one outlier from the analysis (the one small population with very low LRS and damage), the interaction between the number of scars and population size remained significant (p = 0.028): in large populations, the relationship between damage and LRS remained negative but was flat in small populations.

EFFECT OF DAMAGE ON RE-MATING, OVIPOSITION AND SPERM COMPETITION

We tested three hypotheses relating to the function of male-induced genital damage (delayed female re-mating, elevation of female oviposition rate and increased success in sperm competition) using generalized linear models with damage as an explanatory variable, elytra length and the number of eggs laid in the first 24h as covariates (for re-mating and P2 only). We found no significant effect of damage on female re-mating (χ^2_{13} = 0.80 p = 0.37) or oviposition rate (proportion of offspring produced within the first 24 hours following mating, $F_{1,15}$ = 1.6 p = 0.224) and males from more damaging populations were not more successful at sperm competition (χ^2_{13} = 0.32 p = 0.571).

EFFECT OF POPULATION SIZE AND GENETIC VARIABILITY ON MALE MANIPULATIVE ABILITY (RE-MATING, OVIPOSITION RATE AND SPERM COMPETITION)

We compared oviposition in the 24 hours after mating across the treatments and found no effect of population size (Table 3, Fig. 7a), but an effect of genetic variability: males from lines with basal genetic variability seem to accelerate female oviposition (35% of offspring are produced during the first 24 hours ± 2%) compared to males from the enriched genetic variability lines (30 ± 1%; $F_{30,1}$ = 6.1 p = 0.020, Table 3). In this analysis, there was also a difference between the two monogamous lines used as testers, with one having significantly elevated oviposition in the 20 hours after mating (Table 3).

There was no effect of population size or standing genetic variability on the index of male manipulation of female re-mating, which implies that all males were equally good at

inducing previously mated females to re-mate and at deterring females from subsequently re-mating (Table 4, Fig. 7b).

Both population size and initial genetic variability influenced male success in sperm competition. Males from small populations with basal initial genetic variability were the best competitors (Fig. 7c, large population: P2 = 0.73 ± 0.03; small pop. P2 = 0.82 ± 0.02, $F_{29,1}$ = 9.9 p = 0.004; enriched variability population: P2 = 0.75 ± 0.03; basal variability: P2 = 0.81 ± 0.02, $F_{29,1}$ = 4.8 p = 0.037; Table 5).

Discussion

While most other experimental evolution studies have investigated the consequences of removing sexual conflict, this is the first that has reintroduced conflict into experimental populations and assessed the microevolutionary consequences. After 90 generations of monogamy, the reintroduction of sexual selection and sexual conflict for 30 generations resulted in the evolution of more damaging males. However, there was no evidence that female susceptibility to this damage (frequency of scaring) evolved during this time. In spite of this, the response of females to damage did evolve, with females evolving under polygamy typically having greater LRS and longevity at any given level of damage. Furthermore, large population size rather than high initial genetic variation allowed males to evolve faster and become more harmful. In addition, we provide evidence that genital damage is costly for females. It unequivocally reduced female longevity and tended to reduce lifetime reproductive success, although this latter effect was complicated by an

interaction with population size (see discussion below). Overall, these results suggest that sexual conflicts favours males that inflict costly genital damage to females and that the evolution of harm was more pronounced in large populations, either because selection was more efficient or because large population size intensified sexual conflicts and favoured sexually antagonistic coevolution. This implies that sexual selection creates conditions where males benefit from harming females in *C. maculatus*.

Mean damage levels were not associated with female oviposition rate or propensity to re-mate. Our results thus provide no support for the adaptive harm hypothesis. This is in agreement with previous work: Edvardsson and Tregenza (2005) manipulated copulation duration to elevate female damage (Crudgington 2001) and also found no benefits to harming males via delayed re-mating or increased rate of offspring production. Consequently, and despite theoretical support, there is still no empirical evidence for the adaptive harm hypothesis, whether the mechanism involved is terminal investment or delayed re-mating (Edvardsson and Tregenza 2005; Hosken et al. 2003; Morrow et al. 2003), and our results serve to reinforce this. Males from populations with basal genetic variability were better at stimulating female oviposition in the first 24 hours. This could be because favourable gene combinations were broken up by mixing of the two monogamous lines to create the populations with enriched genetic variability, although more work is needed to determine whether epistatic interactions can explain this finding.

If harm does not benefit males directly, it could be a side-effect of some other male adaptation to male-male competition (the collateral harm hypothesis), with the obvious

candidate being sperm competitive ability. However, we found no evidence supporting the idea that males from more damaging populations are more successful in sperm competition. P2 is a composite trait that is likely to be influenced by an unknown number of male derived chemicals and behaviours, so that the prediction of the effect of population size might be less straightforward than for simpler traits such as genital damage. Nevertheless, in the dung fly *S. cynipsea* more damaging males were not more competitive (Teuschl et al. 2007) and our findings are in agreement with results from Edvardsson and Tregenza (2005) who failed to find an effect of damage on P2. In contrast, Hotzy and Arnqvist (2009) found that across 13 geographically distinct populations of *C. maculatus*, male genital armature and the harm males inflict upon females were positively correlated with male success in sperm competition. This discrepancy between *C. maculatus* studies could result from the fact that the balance between the advantage in sperm competition and the cost of harming females is "contingent upon mating system, female life histories and sperm competition regime" (Hotzy and Arnqvist 2009), which may differ when looking within rather than across populations, and certainly could differ across studies. Our results, in conjunction with Edvardsson's (2005), suggest that the damage inflicted by the spines is not associated with male success in sperm competition, but the damage they inflict did evolve after only 30 generations of restored polygamy. Perhaps a direct measure of spininess would be more revealing (e.g. Hotzy & Arnqvist, 2009), but perhaps the spines serve other purposes too, such as anchoring males firmly during copulation (Edvardsson and Tregenza 2005). Using spines as an anchor could be beneficial for males if female

kicking behaviour was a way to exert mate choice or to avoid being dislodged by competing males before ejaculate transfer (Simmons 2001).

Like the damage inflicted by males which evolved after 30 generations in our polygamous lines, females have also evolved resistance to harm. It is interesting that the number of scars inflicted by males did not differ in females evolving under polygamy or monogamy, but the effects did. Damage inflicted by males could increase female investment in immunocapacity, as has been suggested in other insects (Reinhardt and Siva-Jothy 2007). As a result, the LRS and longevity of females evolving under polygamy were on average higher. Our longevity results are straightforward: increased damage leads to reduced longevity and females from polygamous populations always live longer than monogamous females at any given level of damage. Similarly, LRS of females from monogamous populations always tended to be lower across damage levels. Nevertheless, LRS results are somewhat more complicated in that the damage effect only shows up in an interaction with the population size of the male. When males are from larger populations, more damage equates to lower LRS, but when males are from smaller populations more damage does not reduce LRS. This could reflect a lower cost per scar of male damage in small populations, coupled with lower numbers of scars. Only males from large populations seem to have evolved beyond a threshold where damage becomes costly (in terms of LRS). It is unlikely that the lack of cost in small populations is due to higher female resistance because neither monogamous nor polygamous females suffered reduced LRS when mated to males from small populations. Greater sensitivity to damage in large populations (as suggested by this interaction effect of damage and population size

on LRS) is consistent with more intense sexual conflicts and sexually antagonistic coevolution in large populations: as females evolve resistance to male damage, antagonistic coevolution will favour males that inflict more harm. If coevolution is more likely to happen in large populations, we expect more harmful males (as observed: large males inflict more scars), but also more resistant females (higher LRS in large populations), which in return escalates towards more costly damage. These findings are generally consistent with a previous comparative analysis within the seed beetles (Coleoptera: Bruchidae) which also provided evidence for male-female coevolution. In species where males had evolved more harmful genitalia, females had evolved a more robust copulatory tract (Rönn et al. 2007). This observation is congruent with sexually antagonistic coevolution, which we also found within our group of experimental populations, and experimental evolution of similar durations has documented evolution in female resistance/susceptibility in other taxa (Martin and Hosken 2003a).

Despite manipulating population size for 30 generations, we found no evidence for inbreeding depression in smaller populations. This could result from purging of deleterious mutations over the 90 generations of monogamy when population size was relatively small (between 100 and 150 individuals for each of the two monogamous lines), assuming that inbreeding depression is primarily due to the expression of deleterious recessives and not to loss of heterozygosity in *C. maculatus*. Alternatively, population sizes of this order may escape serious inbreeding over this time frame. Recent results suggest that the spectrum of deleterious mutations contains a high proportion of very small effect mutation (<<1%) (Estes et al. 2004) such that even large finite

populations will gradually accumulate deleterious recessive alleles, but such small effects may not be detectable over the 30 generations of our study. Since it appears that the lower LRS of our small populations was not due to inbreeding depression, it must have arisen from another property of small population sizes. The potential alternatives are the independent fixation of mutations that are not associated with inbreeding depression, such as dominant mutations. These may accumulate due to stronger drift, a lower number of new mutations resulting in lower genetic variability to fuel evolutionary change, or less intense conflicts between males and females reducing the strength of sexual selection. The effects of genetic drift are taken into account by using replicates for each treatment: a major role of drift seems unlikely given that the responses in all replicate populations were in the same direction. Alternatively, the evolution of small populations could have been constrained by the lack of genetic variability. We designed our experimental to disentangle the effect of population size from that of genetic variability: if the higher genetic variability in large populations was crucial for the observed microevolution, we would expect to see a significant effect of initial genetic variability as well as an effect of population size, which we did not. This argues against the hypothesis that the large populations evolved faster because of their higher standing genetic variability. It is worth noting that our design relies on the assumption that genetic variability is indeed higher in the crossed populations (with enriched genetic variability) than in the two monogamous lines. However, it does seem likely that genetic variation will be structured predominantly between, rather than within lines after 90 generations of isolation at a relatively small population size. The lack of inbreeding effects observed could slightly weaken this assumption, unless it results from an efficient purge of

deleterious mutations, as suggested above. Three broad explanations therefore remain for the patterns we detect: (1) larger populations experience a larger number of new mutations; (2) selection is more efficient in large populations; (3) sexual selection (including that driven by sexual conflict) is more intense in larger populations and sexually antagonistic coevolution is favoured, as discussed in the Introduction. Although our population sizes are sufficiently large for us to expect new mutations, some of which may affect conflict adaptations, 30 generations is a short time for such new mutations to become fixed. Hence the most likely explanation for the patterns we observe seems to be the potential for larger populations to evolve faster through an increased intensity of sexual conflicts combined with more efficient selection with larger effective size (Robertson 1970). This is in accordance with theoretical models predicting that sexually antagonistic coevolution is more likely in large populations (Gavrilets 2000; Gavrilets et al. 2001).

Our experimental design manipulated population size and standing genetic variability simultaneously and independently. It thus contributes empirical data relevant to debates on the effect of population size and inbreeding in experimental evolution, in particular experimental sexual selection. Effective population size is a key parameter in these experimental evolution studies, firstly because the experimental manipulation of mating systems or sex ratio can lead to different effective population sizes between treatments and confound effects (Snook et al. 2009). Secondly, small populations may lack the influx of new beneficial mutations, but slightly deleterious mutations are more likely to get fixed. Finally, small populations suffer less intense conflicts. Consequently, effective

population size can have a major influence on the outcome of experimental evolution (Martin and Hosken 2003a). For example, our experiment suggests that some evolutionary trajectories might only occur if effective population size is sufficiently large. Similarly, Reuter *et al*. (2008) showed that predicted patterns of sexual selection can be constrained by low effective population size. Ödeen and Florin (2000) further suggested that low effective population size could constrain the evolution of assortative mating and thereby limit the power of experimental tests of sympatric or parapatric speciation. Moreover, sexual selection itself changes effective population size and as the intensity of selection increases and male mating success becomes more skewed, populations experiencing sexual selection will have smaller effective population sizes. Classically, effective population size is estimated as $(4n_m n_f)/(n_m + n_f)$, where n_m is male number and n_f is female number (Hartl 2000). If the number of males contributing genes to offspring is low, then the effective population size is also reduced (assuming that n_f is constant). As a result, we suggest that attempting to manipulate population size in order to remove this feature of sexual selection (Snook et al. 2009) is only justified where there is an explicit aim to focus on other effects of selection. Where this is not the case we suggest that maintaining large census sizes when possible is the best approach, if only because selection is always more efficient in large populations (Willi et al. 2006). In particular, it can be misleading to focus on maintaining equal effective population sizes if the increased work load and/or limited space constrain replicates to small census size.

In conclusion, this study is the first attempt at reversing experimental evolution under sexual conflicts. Reintroducing sexual selection and sexual conflict for 30 generations

into previously monogamous populations resulted in the evolution of more harmful males, and female resistance to harm also evolved. Damage was costly for females, in terms of longevity and lifetime reproductive success, but the benefits to males are unclear. It seems unlikely that the aedeagal spines which damage females evolved solely to harm, and further research is needed to assess whether damage is associated with benefits during non-sperm competition forms of male-male competition in these populations. Finally, population size affected the evolutionary responses we detected, but not via an inbreeding effect, suggesting sexual selection was more effective in our larger populations.

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Figure 1. Diagram of the experimental design. 90 generations of relaxed sexual selection and sexual conflicts (monogamy, in grey) was followed by 30 generations of restored polygamy (in black). In parallel, the two monogamous lines were maintained to be used as testers. At generation 90, the two monogamous lines were crossed. Generations 91 and 92 were population expansion. At generation 92, the four treatments were set up by manipulating population size (large or small) and using the enhanced genetic variability of the crossed line to form four treatments: large population size enriched genetic variability, large population size basal genetic variability, small population size enriched genetic variability and small population size basal genetic variability, with four replicates for each treatment (16 lines in total). All lines were standardized for mating rate and larval density at generation 122 and 123.

Figure 2. Test of the effect of inbreeding in the experimental lines with low genetic variability, small or large population size. Inbreeding depression was assessed in terms of (a) fecundity (number of eggs laid in the first 24 hours), (b) longevity (days) or (c) lifetime reproductive success (total number of offspring that emerged). Bars and error bars stand for means and standard errors respectively.

Figure 3. Genital damage (measured as the mean number of scars in the female genital tract) suffered by females from monogamous or polygamous lines mated to males from monogamous or polygamous lines. White bars indicate polygamous line males and standard errors are shown.

Figure 4. Effect of male population size (large or small) and initial genetic variability (basal or enriched) on genital damage (mean number of scars) inflicted by polygamous males to females (monogamous tester $♂_P♀_M$ or line females $♂_P♀_P$) with standard errors.

Figure 5. Effect of genital damage (measured as the number of scars in the female genital tract) on female longevity (in days). Damage is inflicted by polygamous males on females from monogamous (crosses and dotted line) or polygamous lines (circles and solid line) ($♂_P$ x $♀_M$ or $♀_P$).

Figure 6. Effect of genital damage (number of scars) on female lifetime reproductive success (total number of offspring that emerged) in lines of small (triangles and solid line) or large (crosses and dotted line) population size, when males from polygamous lines are mated to females from either monogamous (tester) or polygamous lines ($♂_P$ x $♀_M$ or $♀_P$).

Figure 7. Effect of male population size (large or small) and initial genetic variability (basal or enriched) on (a) oviposition speed measured as the mean percentage of offspring produced by a female that hatched from eggs laid in the first 24 hours following mating, (b) the mean index of male manipulation of female re-mating (see text) and (c) the success of a male in sperm competition P2, measured as the mean proportion of offspring sired by that male when he was the 2nd male to mate. Error bars stand for standard errors.

Table 1. Effect of genital damage on female longevity when males from polygamous lines are mated to females from either monogamous (tester) or polygamous lines (♂$_P$ x ♀$_M$ or ♀$_P$). To account for the difference in longevity between populations of the low variability treatment derived from the two monogamous line, we added a third level to the factor "genetic variability" (i.e. we replaced basal/enriched variability with basal from M1/basal from M2/enriched). Significant results are shown in bold.

Longevity	deviance	df	F	p
Pop size* variability (basalM1/basalM2/enriched)	0.10	2	0.06	0.945
Damage * variability (basalM1/basalM2/enriched)	0.41	2	0.26	0.776
Damage * pop size	0.20	1	0.27	0.606
Damage * female type (M/P)	0.26	1	0.36	0.556
Elytra length (body size)	0.09	1	0.12	0.728
Pop size	1.25	1	1.85	0.186
Fecundity	1.70	1	2.43	0.131
Variability (basalM1/basalM2/enriched)	3.71	2	2.52	0.100
Female type (M/P)	**3.77**	**1**	**4.63**	**0.040**
Damage	**4.44**	**1**	**5.45**	**0.027**
Error	15.90	18		

Table 2. Effect of genital damage on female lifetime reproductive success when males from polygamous lines are mated to females from either monogamous (tester) or polygamous lines (♂P x ♀M or ♀P). Significant results are shown in bold.

LRS	MS	df	F	p
Damage * female type (M/P)	17.36	1	0.3	0.582
Damage* pop size	**397.8**	**1**	**7.0**	**0.014**
Damage * variability	46.1	1	0.9	0.361
Pop size * variability	39.0	1	0.7	0.404
Pop size	**491.7**	**1**	**8.6**	**0.007**
Variability	41.6	1	0.8	0.384
Elytra length (body size)	173.6	1	3.3	0.081
Female type (M/P)	**497.9**	**1**	**8.7**	**0.006**
Damage	11.0	1	0.2	0.651
Error	1166.8	21		

Table 3. Effect of population size, genetic variability and their interaction on female oviposition speed when males from polygamous lines are mated to monogamous tester females. The line of the tester female (monogamous) was included as a covariate. Significant results are shown in bold.

Oviposition speed	MS	df	F	p
Pop size * variability	27.8	1	0.7	0.401
Elytra length (body size)	0.03	1	0.0008	0.978
Pop size	0.8	1	0.02	0.880
Variability	**212.9**	**1**	**6.1**	**0.020**
Tester female	**399.8**	**1**	**11.4**	**0.002**
Error	992.2	26		

Table 4. Effect of population size, genetic variability and their interaction on male manipulation of female re-mating, estimated as the difference between a male's ability to induce previously mated females to re-mate and to deter females from subsequently re-mating. The line of the tester female (monogamous) was included as a covariate.

Index of male manipulation of female re-mating	MS	df	F	p
Pop size * variability	0.27	1	1.9	0.179
Elytra length (body size)	0.01	1	0.1	0.842
Pop size	0.04	1	0.3	0.581
Variability	0.08	1	0.6	0.455
Tester female	0.04	1	0.3	0.586
Error	3.65	26		

Table 5. Effect of population size and initial genetic variability on P2, the success of a male in sperm competition. Significant results are shown in bold.

P2	Deviance	df	F	p
Pop size * variability	4.4	1	1.2	0.288
Fecundity 24h	1.9	1	0.5	0.478
Pop size	**36.0**	**1**	**9.9**	**0.004**
Variability	**17.3**	**1**	**4.8**	**0.037**
Generation	**117.3**	**1**	**32.4**	**<0.001**
error	99.4	26		

Figure 1.

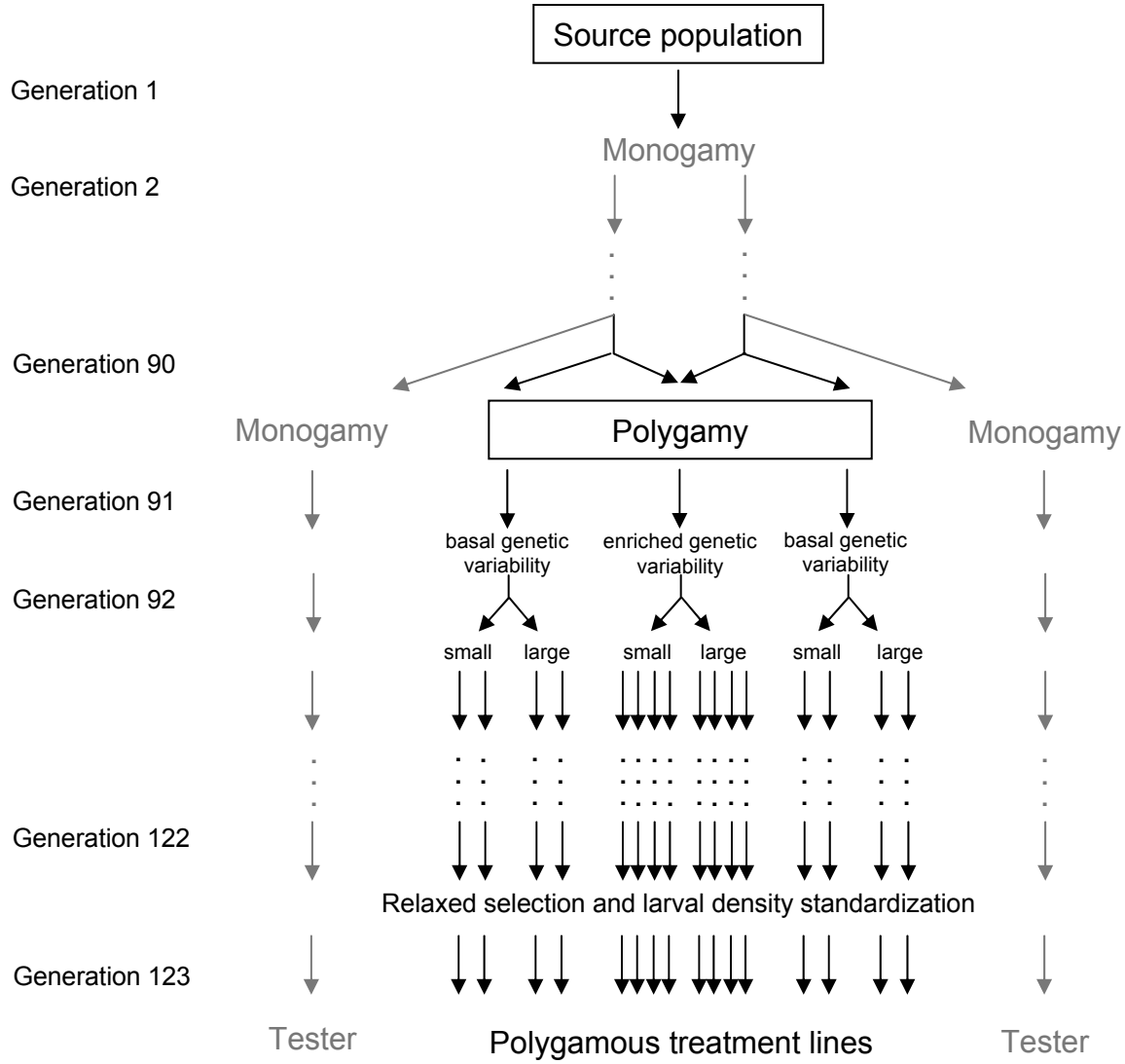

Source population
Generation 1
Monogamy
Generation 2
Generation 90
Monogamy
Polygamy
Monogamy
Generation 91
basal genetic variability
enriched genetic variability
basal genetic variability
Generation 92
small
large
small
large
small
large
Generation 122
Relaxed selection and larval density standardization
Generation 123
Tester
Polygamous treatment lines
Tester

Figure 2.

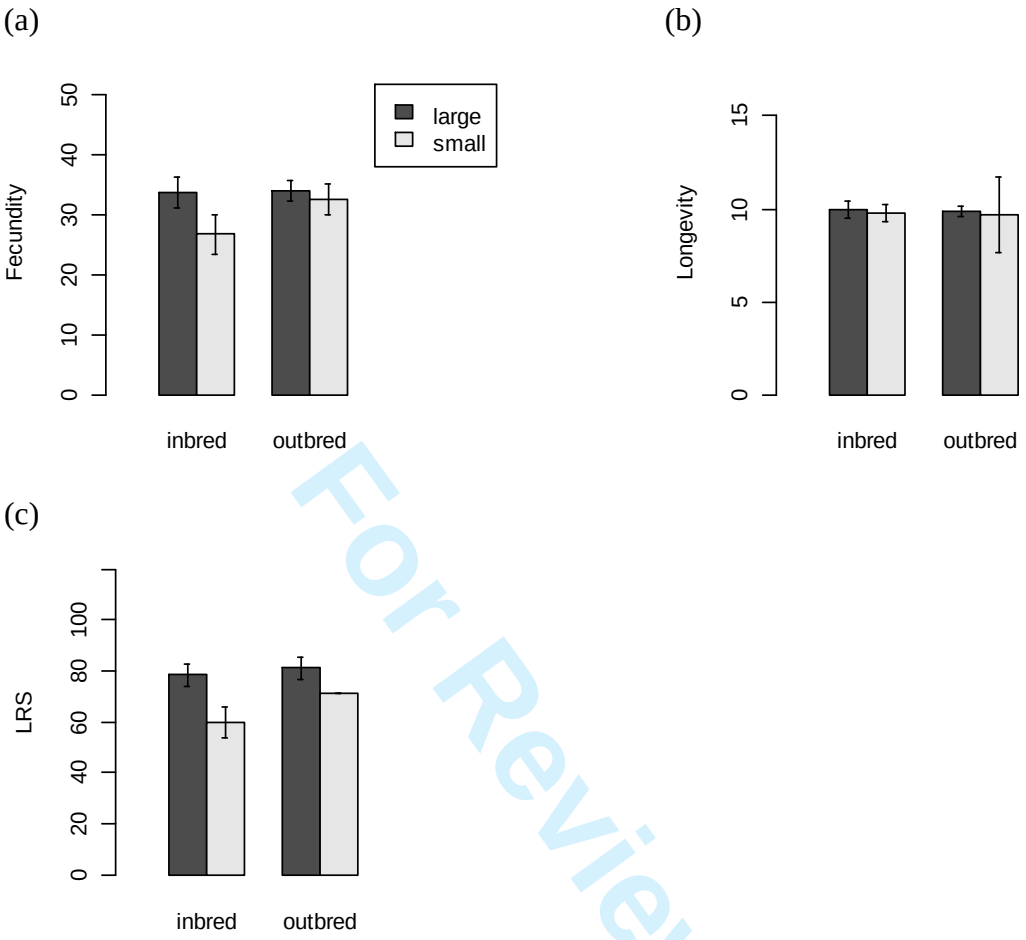


Figure 3.

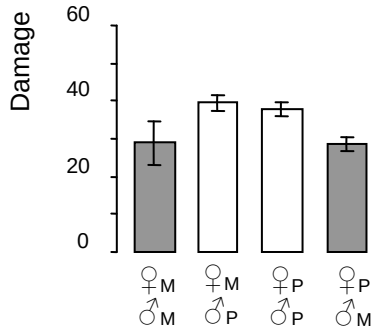


Figure 4.

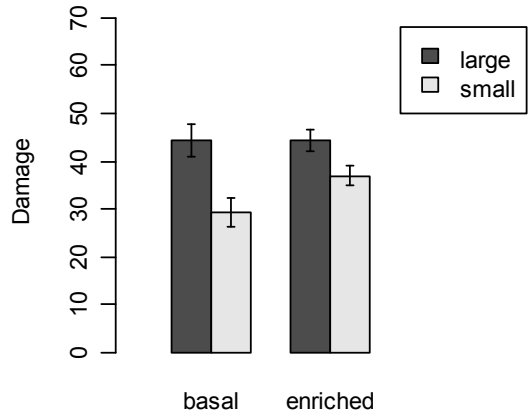


Figure 5.

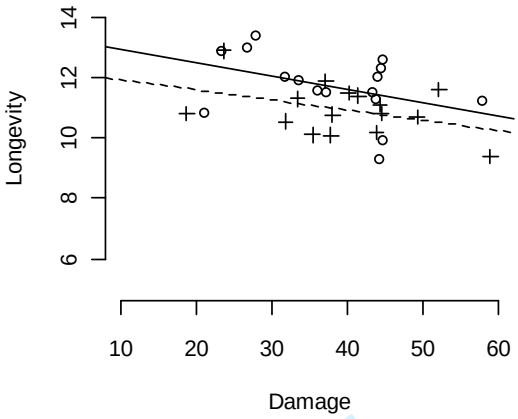


Figure 6.

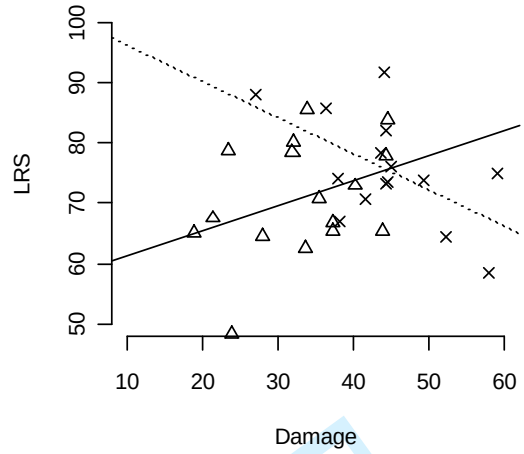
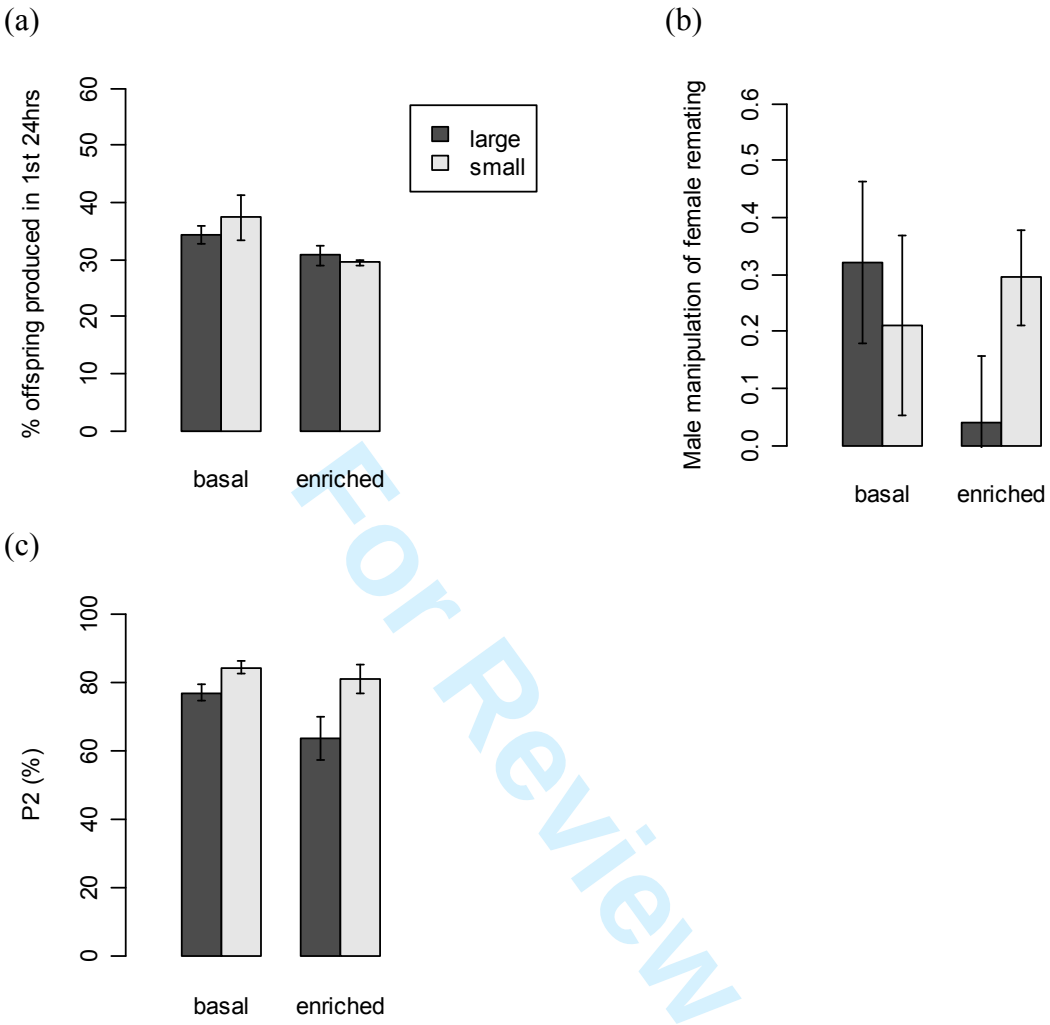


Figure 7.



THE EVOLUTION OF HARM – EFFECT OF SEXUAL CONFLICTS AND POPULATION SIZE

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ABSTRACT

Conflicts of interest between mates can lead to the evolution of male traits that reduce female fitness and that drive coevolution between the sexes. The rate of adaptation depends on the intensity of selection and its efficiency, which depends on drift and genetic variability. This leads to the largely untested prediction that coevolutionary adaptations such as those driven by sexual conflict should evolve faster in large populations. We tested this prediction using the bruchid beetle *Callosobruchus maculatus*, a species where harm inflicted by males is well documented. Whilst most experimental evolution studies remove sexual conflict, we reintroduced it in populations where it had been experimentally removed. Both population size and standing genetic variability were manipulated in a factorial experimental design. After 90 generations of relaxed conflict (monogamy), the reintroduction of sexual conflicts for 30 generations

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favoured males that harmed females and females more resistant to the genital damage inflicted by males. Males evolved to become more harmful when population size was large rather than when initial genetic variation was enriched. Our study shows that sexual selection can create conditions where males can benefit from harming females and that selection may tend to be more intense and effective in larger populations.

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KEYWORDS

Experimental evolution, sexual selection, *Callosobruchus maculatus*, genital damage, population size

Sexual conflict occurs when the evolutionary interests of males and females differ (Parker 1979), and can result in the evolution of traits beneficial to individuals but harmful to their mates (Arnqvist and Rowe 2005). Extreme examples of this phenomenon occur when male reproductive behaviour harms females via traits such as toxic substances transferred in the ejaculate (Chapman et al. 1995; Eady et al. 2007; Rice 1996) or damaging intromittent organs (Blanckenhorn et al. 2002; Crudgington and Siva-Jothy 2000; Stutt and Siva-Jothy 2001).

Two hypotheses have been proposed to explain the evolution of harm. First, the collateral harm hypothesis (Hosken et al. 2003; Morrow et al. 2003) suggests that harm is a side effect of adaptations beneficial in male-male competition (Lessells 2006; Parker 1979). For example, in *Drosophila melanogaster* genotypes that have superior sperm defence capabilities reduce female longevity (Civetta and Clark 2000). Alternatively, the adaptive harm hypothesis posits that harm benefits males more directly because of the reduction of female survival. For example, injuries could deter females from subsequently re-mating and/or alter female perceptions of their health status resulting in increased resource reallocation to reproduction. Theoretical treatments support this "terminal investment" hypothesis (Johnstone and Keller 2000; Lessells 2005), even when damage decreases the re-mating interval (Lessells 2005). However, empirical support for these models is lacking (Hosken et al. 2003; Morrow et al. 2003).

The bruchid beetle (*Callosobruchus maculatus*) is a species where harm inflicted by males is well documented. Male bruchid beetles have a complex aedeagus, the internal

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sac of which is covered with spines that puncture the female genital tract during copulation (Crudgington and Siva-Jothy 2000). Despite comparative evidence supporting the notion that the spines are involved in male-female antagonistic coevolution at the interspecific level (Rönn et al. 2007), evidence for an association between sexual selection and genital damage is scarce at the intraspecific level. Hotzy and Arnqvist (2009) demonstrated a correlation between spine length and male success in sperm competition across populations, but no such relationship was found in two other studies investigating why male bruchid beetles harm their mates (Edvardsson and Tregenza 2005; Morrow et al. 2003). Here we use an experimental evolution approach to further assess the potential link between harm and sexual selection.

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Experimental evolution is a powerful tool that can be used to assess the evolution of harm and female resistance to it. This approach has been used to eliminate sexual conflict (and drastically reduce sexual selection) by enforcing monogamy. Males evolving under monogamy should evolve to become more benign to their partners since male and female fitness are simultaneously maximized, while monogamous females should become more susceptible to harm because selection on counteradaptations to reduce harm is relaxed (assuming that female resistance is costly). These predictions have been supported in experimental populations of *Drosophila melanogaster* (Holland and Rice 1999; Pitnick et al. 2001a; Pitnick et al. 2001b). Similarly, enforced monogamy in the fly *Sepsis cynipsea* enhanced female survival (Martin and Hosken 2003a) and monogamous populations of *Scathophaga stercoraria* had higher fitness than polyandrous lines (Martin et al. 2004). In an experiment where natural selection and sexual selection were manipulated

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simultaneously, Fricke and Arnqvist (2007) showed that, when reared on standard diets, monogamous selection lines of *Callosobruchus maculatus* produced more offspring. Recent studies have employed sex ratio biasing, to manipulate sexual conflict and sexual selection. In *D. pseudoobscura*, male biased populations (with more scope for sexual selection) did not differ greatly from monogamous lines (Crudgington et al. 2005), and Wigby and Chapman (2004) found no difference in the male harming ability of *D. melanogaster* lines with different sex ratios.

Following the publication of the first experimental evolution studies aimed at understanding the role of sexual selection by manipulating the mating regime, Snook (2001) and then Wigby and Chapman (2004) argued that altering the sex ratio or population density can result in differences in effective population size, so that different treatments experience different levels of drift and inbreeding. Additionally, because monogamous lines often have a smaller population size, differences in population sizes can be confounded with treatment. However, while these criticisms are in principle sound, they were refuted for the specific studies initially criticized (Rice et al. 2005; and see Reuter et al. 2008). More recently, Snook et al. (2009) raised additional concerns about inbreeding and genetic variation when population size is manipulated. The authors stress that a lack of genetic drift and higher genetic variability could result in more efficient selection in large populations. Beyond the effect of drift and genetic variability, theoretical models also suggest that sexually antagonistic coevolution is more likely in large populations (Gavrilets 2000). Higher densities might favour more intense sexual conflicts, due for example to interference from other males, through physical harm to

females, seminal fluid toxicity or polyspermy (Arnqvist 1997; Arnqvist and Nilsson 2000; Gavrilets et al. 2001). Population size could therefore affect evolution via sexual conflict in two ways: either because sexually antagonistic coevolution is more likely in large populations, or because selection is more efficient in large populations (Robertson 1970). The later could result from the fact that large populations harbour greater levels of standing genetic variation and experience more mutations and little drift (Schultz and Lynch 1997; Willi et al. 2006). While there is evidence consistent with population size effects on sexually antagonistic evolution (Gay et al. 2009; Hosken et al. 2009; Martin and Hosken 2003b), there have been few attempts to document the relative effects of the potential causal factors involved (but see Ödeen and Florin (2000) regarding selection efficiency). Here we use a fully factorial experimental design where both population size and standing genetic variability are manipulated to disentangle the effect of intensified sexual conflicts from the effect of increased genetic diversity, in a context of reintroduced conflicts.

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Starting with populations in which monogamy has been enforced for 90 generations, we reintroduced sexual conflict and sexual selection by allowing free mate choice and multiple mating. We established replicate populations differing in size and standing genetic variability. After 30 generations of reintroduced sexual conflict and sexual selection, we preliminarily tested for effects of inbreeding in small and low variability populations. Then we examined whether genital damage evolved in response to the reintroduction of sexual conflict (1), by comparing the extent of genital damage in females mated to males from polygamous (conflict) lines compared to the monogamous

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(relaxed conflict) lines from which they had been established 30 generations previously. Then we examined whether sexual conflict resulted in more rapid evolution in larger populations or those with greater initial genetic variation (2), by comparing the evolution of adaptations to polygamy across our lines. Additionally, we assessed the costs of damage (3) by evaluating associations between level of damage and female longevity and lifetime reproductive success. Finally, we tested the two hypotheses about why males harm females (4): Are damaging males better at accelerating female oviposition or deterring females to re-mate (adaptive harm hypothesis) or are they better at sperm competition (collateral harm hypothesis)? We simultaneously tested for an effect of population size and genetic variability on male manipulative ability (5).

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Material and methods

STUDY SPECIES AND EXPERIMENTAL DESIGN

Two replicate monogamous lines were established from an ancestral *C. maculatus* population (Niamey, Niger) cultured on black eyed-beans (*Vigna unguiculata*) at 27°C, 32 % RH and 16L:8D photoperiod. Each generation we isolated beans carrying eggs in 48-well cell culture plates in order to collect virgin beetles immediately post-emergence. Virgins (< 24h post eclosion) were subsequently paired and each pair was placed in a 40mm Petri dish and observed until copulation had ceased. From these monogamous pairs, 60 singly mated females were transferred together to approximately 400 beans for oviposition.

After 90 generations of enforced monogamy, polygamy was re-established in new populations established from the two lines by placing 60 newly emerged adults of each

sex from each line on 400 beans. A third polygamous line was created by combining 30 males and 30 females from each of the monogamous lines. In this crossed population, genetic variability should be greater, because 90 generations of isolation and drift is likely to have promoted genetic differentiation and some loss of diversity from the two monogamous lines. These three polygamous lines were allowed to expand exponentially for two generations, before we established 16 experimental populations. The crossed population (with enriched genetic diversity) seeded eight lines at two different densities (four small populations size = 50 individuals, four large populations size = 5000 individuals). Each of the two other polygamous lines was used separately to start another four polygamous lines with basal genetic variability, two small (50 individuals) and two large (5000) (Fig. 1). This generated four treatments (small population size and basal genetic variability; small population size and enriched genetic variability; large population size and basal genetic variability; large population size and enriched genetic variability) each with 4 replicates. Males and females were housed together for their entire lifespan in all 16 lines. We continued to maintain the monogamous populations, as above.

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To retain a constant population size and ratio of resources to beetles, we sieved and weighed the newly emerging adults each generation and placed another 50 (for the small populations), or 5000 (for the large ones) individuals on new black-eyed beans. Small populations were provided with 40g of beans in a cylindrical container 10cm wide and 4cm deep, large populations were provided with 4kg of beans in a rectangular container 30cm x 20cm x 13cm deep. Half of the populations for our genetic variability treatment are derived from each monogamous line. Comparison between the basal genetic

variability populations created from monogamous line 1 and monogamous line 2 revealed male-induced damage, LRS, female re-mating rate, oviposition speed and P2 to be equivalent, although the populations derived from monogamous line 1 lived significantly longer than those derived from monogamous line 2 (12 days versus 11). We accounted for this difference in the analysis of longevity (see below).

To reduce possible maternal and phenotypic effects, we standardized selection one generation prior to the assay (generation 30) for all populations by housing beetles individually under standardised conditions - single mating and one egg per bean (this is in excess of what a single larva can consume (Cope and Fox 2003)) - for one generation. Prior to beetle emergence, we isolated these beans in 'virgin chambers' (48-Well cell culture plates, VWR International Ltd, Lutterworth, UK). Beans were checked every 24h for emerging virgin adults (generation 31).

TEST FOR INBREEDING DEPRESSION

In our experiment, the small populations are potentially susceptible to inbreeding during experimental evolution. Inbreeding can lead to inbreeding depression affecting life history traits (e.g. fecundity and longevity) (Charlesworth and Charlesworth 1987; DeRose and Roff 1999) and competitive male mating ability (Sharp 1984). These effects could potentially confound our predictions (see below). We looked for evidence of inbreeding depression in fecundity, lifetime reproductive success and longevity by crossing males and females between replicate populations and comparing their performance to matings between males and females from within replicate populations (the potentially inbred populations). We assessed those treatments most likely to suffer

inbreeding depression, namely the populations of small census size and basal initial standing genetic variation. We also assessed the large populations with basal initial standing genetic variation as this allowed us to determine the potential impact of population size and initial genetic variance on inbreeding depression. We analysed these data using a general linear model including population size, crossing status (within or between replicate crosses) and their interaction. Elytra length (a measure of body size) was included as a covariate in the analysis of fecundity and lifetime reproductive success, whilst fecundity was included as a covariate in the analysis of longevity.

MALE OFFENCE AND FEMALE RESISTANCE: DAMAGE, LONGEVITY AND LIFETIME REPRODUCTIVE SUCCESS

Both males and females are likely to influence the amount of damage suffered by females during copulation. To isolate the damaging effect of males from the susceptibility of females, we used the two monogamous lines as testers. Four types of crosses were performed: (1) between males from the polygamous populations and tester females (male offence assay - $♀_M♂_P$); (2) between males and females from the same polygamous population (female resistance assay - $♀_P♂_P$); (3) between females from the polygamous populations and tester males ($♀_P♂_M$); (4) a control cross between tester males and females ($♀_M♂_M$). For each assay, 20 crosses were performed for each replicate (x4) of each treatment (x4) (= 1280 crosses).

Virgin females and males (all <24h post eclosion) were paired and each pair (10 pairs x 4 treatments x 4 replicates x 4 crossings) was placed in a 40mm Petri dish and observed

until copulation had ceased. Mated females were then placed on 10 beans for 24 hours and then moved to another 60 beans for the remainder of their lives. We measured fecundity in the first 24 hours of oviposition by directly counting eggs laid. Longevity was estimated by recording female mortality every 24 hours. After their natural death, females were dissected and the number of damage points (scars) in their genital tracts determined. For 25 females we also measured the area covered by scars and found that it was highly correlated with the number of scars (log-linear regression, $R^2 = 0.68$). Female elytra length was measured as a proxy for body size.

MANIPULATION OF RE-MATING AND OVIPOSITION

We measured the ability of males to deter females from subsequently re-mating (male defence) by mating monogamous tester females with males from the polygamous populations and then exposing them to monogamous tester males ($♀_M$-$♂_P$-$♂_M$). We also measured male offence - the ability of males to induce previously mated females to re-mate - by mating monogamous tester females with monogamous tester males and then exposing them to males from the polygamous populations ($♀_M$-$♂_M$-$♂_P$). For each assay, 10 females were paired and subsequently offered a chance to re-mate, following 24h of oviposition. Earlier studies revealed that over 80% of females will re-mate 24 h after their initial copulation (Eady et al. 2004; Edvardsson and Tregenza 2005) but in a pilot experiment we found lower re-mating rates in our lines that were maintained monogamous for 90 generations. We thus estimated that 24 h is a time point at which one might be able to distinguish differences in female re-mating propensity between

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populations. Females were transferred to a 40mm Petri dish with a new virgin male (from the appropriate line) and were observed for 30 minutes to see if they copulated.

We measured the ability of males from the polygamous populations to stimulate female fecundity by counting eggs laid during the female refractory period using males from the 16 polygamous lines mated to 10 monogamous tester females. Again, mated females were placed on 10 beans for 24 hours and then moved to another 60 beans for the remainder of their lifespan. We subsequently counted the number of offspring produced during the first 24h after mating and over their entire lifespan, and then used the proportion of offspring produced in 24h relative to the lifetime reproductive success as a measure of male manipulation. Because both female re-mating rate and last male sperm precedence are high in this species (Eady et al. 2004; Edvardsson and Tregenza 2005), the benefits to any additional stimulation of oviposition beyond the first 24 hours will probably be enjoyed by rival males and as such we did not assess them here.

SPERM COMPETITION

We used a standard sperm competition experiment - where females are mated with two males - to test the hypothesis that harmful males are more successful at sperm competition. Males from the polygamous populations were competed against black tester males from a separate polygamous line with both mating to a black tester female. The black phenotype is a naturally occurring polymorphism and this co-dominant marker was used to score offspring. Offspring sired by brown males (with black females) are

phenotypically intermediate (dark brown body colour and brown legs and antennae) and readily discernable from offspring from a black x black pair (Eady 1991).

Virgin black females and black males were paired in individual 40mm Petri dishes and observed until copulation began. After copulation ceased, males were removed and females were allowed to oviposit for 20 hours on 5 beans. Females were then transferred back to individual 40mm Petri dishes with a virgin brown male from one of the polygamous populations. We repeated this for at least 20 females per replicate (4) per treatment (4). For each pair, we recorded whether copulation occurred successfully within 30 minutes. After copulation with the focal (brown) male ceased, each black female was transferred to a 90mm Petri dish containing 80 beans and allowed to oviposit until death. Eggs laid prior to the second mating were counted (first 20 h), as were the total number of offspring after two successive matings, and offspring phenotype (hybrid or black) was recorded. P2 - the proportion of offspring sired by the second (focal = brown) male was calculated as the proportion of intermediate offspring. The experiment was repeated at generation 32 to increase the sample size. We accounted for this by including a generation factor in the analytical models. Additionally, to ascertain confidence in our co-dominant phenotypic marker, we estimated the repeatability of our paternity estimates by re-measuring P2 blind to the first measurement for 20 randomly chosen females. P2 repeatability was calculated following Lessells & Boag (1987), and was high (r = 0.996).

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STATISTICAL ANALYSES

Analyses were performed in R. To avoid pseudoreplication, we performed all analyses on population means. We also used mixed effect models adding replicate as a random effect and obtained similar results, but only the results using the population means are presented here. All traits (damage, longevity, fecundity, lifetime reproductive success and elytra length) were normally distributed (Kolmogorov-Smirnov test, all $P > 0.05$). Additionally, residuals did not deviate significantly from normality (Kolmogorov-Smirnov test, all $P > 0.05$), and were not autocorrelated (Durbin-Watson test, all $P > 0.05$), and errors were homoscedastic (Breusch-Pagan test, all $P > 0.05$).

Cost of damage

We used a general linear model to test the effect of population size, genetic variability and their interaction on genital damage inflicted by polygamous line males. Female type (monogamous or polygamous) was used as a third factor. We examined whether genital damage evolved with the reintroduction of sexual conflict and sexual selection by testing for an effect of male and female type (from a polygamous or monogamous line) on the amount of damage sustained by a female, using data from four assays ($♀_M♂_P$, $♀_P♂_P$, $♀_P♂_M$ and $♀_M♂_M$). We also examined the cost of damage by testing for a negative relationship between damage and longevity or damage and LRS using linear models. We included population size, genetic variability and female type in the model, as well as elytra length as a covariate for LRS and 24h fecundity as a covariate for longevity to account for life history trade offs. To account for the difference in longevity between the populations of the low variability treatment derived from the two monogamous lines, we

added a third level to the factor "genetic variability" (i.e. we replaced basal/enriched variability with basal from M1/basal from M2/enriched).

Effect of damage on re-mating, oviposition and sperm competition

Harm could be beneficial for males if it deters females from re-mating, if it accelerates the oviposition rate or if it provides an advantage in sperm competition. We tested these hypotheses using generalized linear models with the number of damage points (scars) in females' genital tracts as an explanatory variable. For re-mating and sperm competition (P2), a binomial error distribution was used. We corrected for overdispersion using a quasi-binomial model when the ratio of residual deviance by residual degrees of freedom was larger than one. The number of eggs laid by the female in the first 24h (between both mating occasions) was used as a covariate for re-mating and P2, elytra length was used as a covariate for all three variables.

Effect of population size and genetic variability on male manipulative ability

To ascertain how population size and genetic variability influence the evolution of males' ability to affect female reproduction, we compared re-mating rates, oviposition rate and P2 between our experimental populations that differ in the level of damage inflicted by males. For re-mating, we estimated an index of male manipulation by combining the assays of male defence (♀_{M}-♂_{P}-♂_{M}) and male offence (♀_{M}-♂_{M}-♂_{P}); male manipulation was estimated as the difference between the proportion of females re-mating in the offence experiment minus the proportion that re-mated in the defence experiment. We tested the effect of population size, genetic variability and their interaction on this re-

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mating manipulation-index and on oviposition speed using a linear model with female tester line as a covariate. For sperm competition, we used a generalized linear mixed model with a quasi-binomial error distribution to test for the effect of population size, genetic variability and their interaction on P2, the number of offspring sired by the second of two males to mate with a female (see Sperm competition above). The number of eggs laid in the first 20 h and a generation factor were included as covariates.

Results

TEST FOR INBREEDING DEPRESSION

There was no evidence for inbreeding depression in small and low variability populations. We found no significant effect of the interaction between population size and crossing status (within or between replicate crosses) (Fecundity: $F_{7,1}$=0.5 p=0.480; longevity: $F_{7,1}$=0.04 p=0.837; LRS: $F_{7,1}$=0.5 p=0.517). Fecundity and longevity were not significantly different in crosses within or between replicate populations (Fig. 2a: $F_{9,1}$=0.9 p=0.360 and Fig. 2b: $F_{8,1}$=0.01 p=0.909). Population size also had no effect on these fitness measures, suggesting that inbreeding depression was either absent or was similar across experimental populations (Fig. 2a: $F_{10,1}$ = 2.8 p = 0.125 and Fig. 2b: $F_{9,1}$ = 0.4 p = 0.533). Lifetime reproductive success (LRS) was also equivalent in the within or between replicate crosses (Fig. 2c; $F_{9,1}$ = 1.6 p = 0.234), but population size had an effect with small populations having lower LRS than large populations (Fig. 2c; $F_{10,1}$ = 8.6 p = 0.015). When the analysis was restricted to small populations only, fecundity, longevity

and LRS within and between replicate crosses remained equivalent. These results suggest that population size influenced LRS, but this was not the result of inbreeding depression.

GENITAL DAMAGE EVOLVES IN RESPONSE TO THE REINTRODUCTION OF SEXUAL CONFLICT

Females mated to males from the monogamous populations sustained less damage than those mated to males from the polygamous populations (monogamous males: 29 points of damage ± 2; polygamous males: 39 ± 2; $F_{48,1}$=12 p = 0.0009; Fig. 3). However, the susceptibility of females did not seem to have evolved in the 30 generations after the reintroduction of sexual conflict (monogamous females mated to polygamous males: 38 points of damage ± 2; polygamous females mated to polygamous males: 33 ± 2; $F_{47,1}$=0.2 p = 0.675; Fig. 3). There was no significant interaction between male and female type ($F_{46,1}$=0.02 p = 0.872).

DAMAGE EVOLVES FASTER IN LARGER RATHER THAN MORE DIVERSE POPULATIONS

As there was no difference between monogamous or polygamous females in susceptibility to damage, we analysed the effect of population size and genetic variability on damage using all the crosses involving males from polygamous populations ($♀_M♂_P$ and $♀_P♂_P$). Males from large populations inflicted more damage to females (large population: 44 points of damage ± 2; small population: 33 ± 2; $F_{30,1}$ = 15.5 p = 0.0005; Fig. 4). There was no significant effect of population genetic variability ($F_{29,1}$ = 1.8 p =

0.189) or of female type (monogamous: 39 ± 2; polygamous: 38 ± 2; $F_{28,1}$ = 0.3 p = 0.597).

GENITAL DAMAGE IS COSTLY

The number of damage points in a female's reproductive tract was negatively associated with female longevity (Fig. 5, slope = -0.04 days/damage point; $F_{30,1}$ = 5.5 p = 0.027, Table 1). Furthermore, females from the polygamous populations tended to outlive females from monogamous populations (M: 10.9 days ± 0.2; P: 11.7 ± 0.3; $F_{30,1}$ = 4.6 p = 0.040, Table 1, Fig. 5). This was also reflected in the LRS results, where females from polygamous populations had greater LRS (M: 69 offspring ± 2; P: 78 ± 2; $F_{26,1}$ = 8.7 p = 0.006, Table 2). LRS was also influenced by an interaction between the number of scars in the female tract and polygamous line population size ($F_{26,1}$ = 7.0 p = 0.014, Table 2). More scaring in females from larger populations resulted in lower LRS, but for females from smaller populations the association between genital damage and LRS was flat or even positive (Fig. 6). Note that when we removed one outlier from the analysis (the one small population with very low LRS and damage), the interaction between the number of scars and population size remained significant (p = 0.028): in large populations, the relationship between damage and LRS remained negative but was flat in small populations.

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EFFECT OF DAMAGE ON RE-MATING, OVIPOSITION AND SPERM COMPETITION

We tested three hypotheses relating to the function of male-induced genital damage (delayed female re-mating, elevation of female oviposition rate and increased success in sperm competition) using generalized linear models with damage as an explanatory variable, elytra length and the number of eggs laid in the first 24h as covariates (for re-mating and P2 only). We found no significant effect of damage on female re-mating (χ^2_{13} = 0.80 p = 0.37) or oviposition rate (proportion of offspring produced within the first 24 hours following mating, $F_{1,15}$ = 1.6 p = 0.224) and males from more damaging populations were not more successful at sperm competition (χ^2_{13} = 0.32 p = 0.571).

EFFECT OF POPULATION SIZE AND GENETIC VARIABILITY ON MALE MANIPULATIVE ABILITY (RE-MATING, OVIPOSITION RATE AND SPERM COMPETITION)

We compared oviposition in the 24 hours after mating across the treatments and found no effect of population size (Table 3, Fig. 7a), but an effect of genetic variability: males from lines with basal genetic variability seem to accelerate female oviposition (35% of offspring are produced during the first 24 hours ± 2%) compared to males from the enriched genetic variability lines (30 ± 1%; $F_{30,1}$ = 6.1 p = 0.020, Table 3). In this analysis, there was also a difference between the two monogamous lines used as testers, with one having significantly elevated oviposition in the 20 hours after mating (Table 3).

There was no effect of population size or standing genetic variability on the index of male manipulation of female re-mating, which implies that all males were equally good at

inducing previously mated females to re-mate and at deterring females from subsequently re-mating (Table 4, Fig. 7b).

Both population size and initial genetic variability influenced male success in sperm competition. Males from small populations with basal initial genetic variability were the best competitors (Fig. 7c, large population: P2 = 0.73 ± 0.03; small pop. P2 = 0.82 ± 0.02, $F_{29,1}$ = 9.9 p = 0.004; enriched variability population: P2 = 0.75 ± 0.03; basal variability: P2 = 0.81 ± 0.02, $F_{29,1}$ = 4.8 p = 0.037; Table 5).

Discussion

While most other experimental evolution studies have investigated the consequences of removing sexual conflict, this is the first that has reintroduced conflict into experimental populations and assessed the microevolutionary consequences. After 90 generations of monogamy, the reintroduction of sexual selection and sexual conflict for 30 generations resulted in the evolution of more damaging males. However, there was no evidence that female susceptibility to this damage (frequency of scaring) evolved during this time. In spite of this, the response of females to damage did evolve, with females evolving under polygamy typically having greater LRS and longevity at any given level of damage. Furthermore, large population size rather than high initial genetic variation allowed males to evolve faster and become more harmful. In addition, we provide evidence that genital damage is costly for females. It unequivocally reduced female longevity and tended to reduce lifetime reproductive success, although this latter effect was complicated by an

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interaction with population size (see discussion below). Overall, these results suggest that sexual conflicts favours males that inflict costly genital damage to females and that the evolution of harm was more pronounced in large populations, either because selection was more efficient or because large population size intensified sexual conflicts and favoured sexually antagonistic coevolution. This implies that sexual selection creates conditions where males benefit from harming females in *C. maculatus*.

Mean damage levels were not associated with female oviposition rate or propensity to re-mate. Our results thus provide no support for the adaptive harm hypothesis. This is in agreement with previous work: Edvardsson and Tregenza (2005) manipulated copulation duration to elevate female damage (Crudgington 2001) and also found no benefits to harming males via delayed re-mating or increased rate of offspring production. Consequently, and despite theoretical support, there is still no empirical evidence for the adaptive harm hypothesis, whether the mechanism involved is terminal investment or delayed re-mating (Edvardsson and Tregenza 2005; Hosken et al. 2003; Morrow et al. 2003), and our results serve to reinforce this. Males from populations with basal genetic variability were better at stimulating female oviposition in the first 24 hours. This could be because favourable gene combinations were broken up by mixing of the two monogamous lines to create the populations with enriched genetic variability, although more work is needed to determine whether epistatic interactions can explain this finding.

If harm does not benefit males directly, it could be a side-effect of some other male adaptation to male-male competition (the collateral harm hypothesis), with the obvious

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candidate being sperm competitive ability. However, we found no evidence supporting the idea that males from more damaging populations are more successful in sperm competition. P2 is a composite trait that is likely to be influenced by an unknown number of male derived chemicals and behaviours, so that the prediction of the effect of population size might be less straightforward than for simpler traits such as genital damage. Nevertheless, in the dung fly *S. cynipsea* more damaging males were not more competitive (Teuschl et al. 2007) and our findings are in agreement with results from Edvardsson and Tregenza (2005) who failed to find an effect of damage on P2. In contrast, Hotzy and Arnqvist (2009) found that across 13 geographically distinct populations of *C. maculatus*, male genital armature and the harm males inflict upon females were positively correlated with male success in sperm competition. This discrepancy between *C. maculatus* studies could result from the fact that the balance between the advantage in sperm competition and the cost of harming females is "contingent upon mating system, female life histories and sperm competition regime" (Hotzy and Arnqvist 2009), which may differ when looking within rather than across populations, and certainly could differ across studies. Our results, in conjunction with Edvardsson's (2005), suggest that the damage inflicted by the spines is not associated with male success in sperm competition, but the damage they inflict did evolve after only 30 generations of restored polygamy. Perhaps a direct measure of spininess would be more revealing (e.g. Hotzy & Arnqvist, 2009), but perhaps the spines serve other purposes too, such as anchoring males firmly during copulation (Edvardsson and Tregenza 2005). Using spines as an anchor could be beneficial for males if female

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kicking behaviour was a way to exert mate choice or to avoid being dislodged by competing males before ejaculate transfer (Simmons 2001).

Like the damage inflicted by males which evolved after 30 generations in our polygamous lines, females have also evolved resistance to harm. It is interesting that the number of scars inflicted by males did not differ in females evolving under polygamy or monogamy, but the effects did. Damage inflicted by males could increase female investment in immunocapacity, as has been suggested in other insects (Reinhardt and Siva-Jothy 2007). As a result, the LRS and longevity of females evolving under polygamy were on average higher. Our longevity results are straightforward: increased damage leads to reduced longevity and females from polygamous populations always live longer than monogamous females at any given level of damage. Similarly, LRS of females from monogamous populations always tended to be lower across damage levels. Nevertheless, LRS results are somewhat more complicated in that the damage effect only shows up in an interaction with the population size of the male. When males are from larger populations, more damage equates to lower LRS, but when males are from smaller populations more damage does not reduce LRS. This could reflect a lower cost per scar of male damage in small populations, coupled with lower numbers of scars. Only males from large populations seem to have evolved beyond a threshold where damage becomes costly (in terms of LRS). It is unlikely that the lack of cost in small populations is due to higher female resistance because neither monogamous nor polygamous females suffered reduced LRS when mated to males from small populations. Greater sensitivity to damage in large populations (as suggested by this interaction effect of damage and population size

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on LRS) is consistent with more intense sexual conflicts and sexually antagonistic coevolution in large populations: as females evolve resistance to male damage, antagonistic coevolution will favour males that inflict more harm. If coevolution is more likely to happen in large populations, we expect more harmful males (as observed: large males inflict more scars), but also more resistant females (higher LRS in large populations), which in return escalates towards more costly damage. These findings are generally consistent with a previous comparative analysis within the seed beetles (Coleoptera: Bruchidae) which also provided evidence for male-female coevolution. In species where males had evolved more harmful genitalia, females had evolved a more robust copulatory tract (Rönn et al. 2007). This observation is congruent with sexually antagonistic coevolution, which we also found within our group of experimental populations, and experimental evolution of similar durations has documented evolution in female resistance/susceptibility in other taxa (Martin and Hosken 2003a).

Despite manipulating population size for 30 generations, we found no evidence for inbreeding depression in smaller populations. This could result from purging of deleterious mutations over the 90 generations of monogamy when population size was relatively small (between 100 and 150 individuals for each of the two monogamous lines), assuming that inbreeding depression is primarily due to the expression of deleterious recessives and not to loss of heterozygosity in *C. maculatus*. Alternatively, population sizes of this order may escape serious inbreeding over this time frame. Recent results suggest that the spectrum of deleterious mutations contains a high proportion of very small effect mutation (<<1%) (Estes et al. 2004) such that even large finite

populations will gradually accumulate deleterious recessive alleles, but such small effects may not be detectable over the 30 generations of our study. Since it appears that the lower LRS of our small populations was not due to inbreeding depression, it must have arisen from another property of small population sizes. The potential alternatives are the independent fixation of mutations that are not associated with inbreeding depression, such as dominant mutations. These may accumulate due to stronger drift, a lower number of new mutations resulting in lower genetic variability to fuel evolutionary change, or less intense conflicts between males and females reducing the strength of sexual selection. The effects of genetic drift are taken into account by using replicates for each treatment: a major role of drift seems unlikely given that the responses in all replicate populations were in the same direction. Alternatively, the evolution of small populations could have been constrained by the lack of genetic variability. We designed our experimental to disentangle the effect of population size from that of genetic variability: if the higher genetic variability in large populations was crucial for the observed microevolution, we would expect to see a significant effect of initial genetic variability as well as an effect of population size, which we did not. This argues against the hypothesis that the large populations evolved faster because of their higher standing genetic variability. It is worth noting that our design relies on the assumption that genetic variability is indeed higher in the crossed populations (with enriched genetic variability) than in the two monogamous lines. However, it does seem likely that genetic variation will be structured predominantly between, rather than within lines after 90 generations of isolation at a relatively small population size. The lack of inbreeding effects observed could slightly weaken this assumption, unless it results from an efficient purge of

deleterious mutations, as suggested above. Three broad explanations therefore remain for the patterns we detect; (1) larger populations experience a larger number of new mutations; (2) selection is more efficient in large populations; (3) sexual selection (including that driven by sexual conflict) is more intense in larger populations and sexually antagonistic coevolution is favoured, as discussed in the Introduction. Although our population sizes are sufficiently large for us to expect new mutations, some of which may affect conflict adaptations, 30 generations is a short time for such new mutations to become fixed. Hence the most likely explanation for the patterns we observe seems to be the potential for larger populations to evolve faster through an increased intensity of sexual conflicts combined with more efficient selection with larger effective size (Robertson 1970). This is in accordance with theoretical models predicting that sexually antagonistic coevolution is more likely in large populations (Gavrilets 2000; Gavrilets et al. 2001).

Our experimental design manipulated population size and standing genetic variability simultaneously and independently. It thus contributes empirical data relevant to debates on the effect of population size and inbreeding in experimental evolution, in particular experimental sexual selection. Effective population size is a key parameter in these experimental evolution studies, firstly because the experimental manipulation of mating systems or sex ratio can lead to different effective population sizes between treatments and confound effects (Snook et al. 2009). Secondly, small populations may lack the influx of new beneficial mutations, but slightly deleterious mutations are more likely to get fixed. Finally, small populations suffer less intense conflicts. Consequently, effective

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population size can have a major influence on the outcome of experimental evolution (Martin and Hosken 2003a). For example, our experiment suggests that some evolutionary trajectories might only occur if effective population size is sufficiently large. Similarly, Reuter *et al*. (2008) showed that predicted patterns of sexual selection can be constrained by low effective population size. Ödeen and Florin (2000) further suggested that low effective population size could constrain the evolution of assortative mating and thereby limit the power of experimental tests of sympatric or parapatric speciation. Moreover, sexual selection itself changes effective population size and as the intensity of selection increases and male mating success becomes more skewed, populations experiencing sexual selection will have smaller effective population sizes. Classically, effective population size is estimated as $(4n_m n_f)/(n_m + n_f)$, where n_m is male number and n_f is female number (Hartl 2000). If the number of males contributing genes to offspring is low, then the effective population size is also reduced (assuming that n_f is constant). As a result, we suggest that attempting to manipulate population size in order to remove this feature of sexual selection (Snook et al. 2009) is only justified where there is an explicit aim to focus on other effects of selection. Where this is not the case we suggest that maintaining large census sizes when possible is the best approach, if only because selection is always more efficient in large populations (Willi et al. 2006). In particular, it can be misleading to focus on maintaining equal effective population sizes if the increased work load and/or limited space constrain replicates to small census size.

In conclusion, this study is the first attempt at reversing experimental evolution under sexual conflicts. Reintroducing sexual selection and sexual conflict for 30 generations

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into previously monogamous populations resulted in the evolution of more harmful males, and female resistance to harm also evolved. Damage was costly for females, in terms of longevity and lifetime reproductive success, but the benefits to males are unclear. It seems unlikely that the aedeagal spines which damage females evolved solely to harm, and further research is needed to assess whether damage is associated with benefits during non-sperm competition forms of male-male competition in these populations. Finally, population size affected the evolutionary responses we detected, but not via an inbreeding effect, suggesting sexual selection was more effective in our larger populations.

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Figure 1. Diagram of the experimental design. 90 generations of relaxed sexual selection and sexual conflicts (monogamy, in grey) was followed by 30 generations of restored polygamy (in black). In parallel, the two monogamous lines were maintained to be used as testers. At generation 90, the two monogamous lines were crossed. Generations 91 and 92 were population expansion. At generation 92, the four treatments were set up by manipulating population size (large or small) and using the enhanced genetic variability of the crossed line to form four treatments: large population size enriched genetic variability, large population size basal genetic variability, small population size enriched genetic variability and small population size basal genetic variability, with four replicates for each treatment (16 lines in total). All lines were standardized for mating rate and larval density at generation 122 and 123.

Figure 2. Test of the effect of inbreeding in the experimental lines with low genetic variability, small or large population size. Inbreeding depression was assessed in terms of (a) fecundity (number of eggs laid in the first 24 hours), (b) longevity (days) or (c) lifetime reproductive success (total number of offspring that emerged). Bars and error bars stand for means and standard errors respectively.

Figure 3. Genital damage (measured as the mean number of scars in the female genital tract) suffered by females from monogamous or polygamous lines mated to males from monogamous or polygamous lines. White bars indicate polygamous line males and standard errors are shown.

Figure 4. Effect of male population size (large or small) and initial genetic variability (basal or enriched) on genital damage (mean number of scars) inflicted by polygamous males to females (monogamous tester ♂P♀M or line females ♂P♀P) with standard errors.

Figure 5. Effect of genital damage (measured as the number of scars in the female genital tract) on female longevity (in days). Damage is inflicted by polygamous males on females from monogamous (crosses and dotted line) or polygamous lines (circles and solid line) (♂P x ♀M or ♀P).

Figure 6. Effect of genital damage (number of scars) on female lifetime reproductive success (total number of offspring that emerged) in lines of small (triangles and solid line) or large (crosses and dotted line) population size, when males from polygamous lines are mated to females from either monogamous (tester) or polygamous lines (♂P x ♀M or ♀P).

Figure 7. Effect of male population size (large or small) and initial genetic variability (basal or enriched) on (a) oviposition speed measured as the mean percentage of offspring produced by a female that hatched from eggs laid in the first 24 hours following mating, (b) the mean index of male manipulation of female re-mating (see text) and (c) the success of a male in sperm competition P2, measured as the mean proportion of offspring sired by that male when he was the 2nd male to mate. Error bars stand for standard errors.

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Table 1. Effect of genital damage on female longevity when males from polygamous lines are mated to females from either monogamous (tester) or polygamous lines (♂$_P$ x ♀$_M$ or ♀$_P$). To account for the difference in longevity between populations of the low variability treatment derived from the two monogamous line, we added a third level to the factor "genetic variability" (i.e. we replaced basal/enriched variability with basal from M1/basal from M2/enriched). Significant results are shown in bold.

Longevity	deviance	df	F	p
Pop size* variability (basalM1/basalM2/enriched)	0.10	2	0.06	0.945
Damage * variability (basalM1/basalM2/enriched)	0.41	2	0.26	0.776
Damage * pop size	0.20	1	0.27	0.606
Damage * female type (M/P)	0.26	1	0.36	0.556
Elytra length (body size)	0.09	1	0.12	0.728
Pop size	1.25	1	1.85	0.186
Fecundity	1.70	1	2.43	0.131
Variability (basalM1/basalM2/enriched)	3.71	2	2.52	0.100
Female type (M/P)	**3.77**	**1**	**4.63**	**0.040**
Damage	**4.44**	**1**	**5.45**	**0.027**
Error	15.90	18		

Table 2. Effect of genital damage on female lifetime reproductive success when males from polygamous lines are mated to females from either monogamous (tester) or polygamous lines (♂P x ♀M or ♀P). Significant results are shown in bold.

LRS	MS	df	F	p
Damage * female type (M/P)	17.36	1	0.3	0.582
Damage* pop size	**397.8**	**1**	**7.0**	**0.014**
Damage * variability	46.1	1	0.9	0.361
Pop size * variability	39.0	1	0.7	0.404
Pop size	**491.7**	**1**	**8.6**	**0.007**
Variability	41.6	1	0.8	0.384
Elytra length (body size)	173.6	1	3.3	0.081
Female type (M/P)	**497.9**	**1**	**8.7**	**0.006**
Damage	11.0	1	0.2	0.651
Error	1166.8	21		

Table 3. Effect of population size, genetic variability and their interaction on female oviposition speed when males from polygamous lines are mated to monogamous tester females. The line of the tester female (monogamous) was included as a covariate. Significant results are shown in bold.

Oviposition speed	MS	df	F	p
Pop size * variability	27.8	1	0.7	0.401
Elytra length (body size)	0.03	1	0.0008	0.978
Pop size	0.8	1	0.02	0.880
Variability	**212.9**	**1**	**6.1**	**0.020**
Tester female	**399.8**	**1**	**11.4**	**0.002**
Error	992.2	26		

Table 4. Effect of population size, genetic variability and their interaction on male manipulation of female re-mating, estimated as the difference between a male's ability to induce previously mated females to re-mate and to deter females from subsequently re-mating. The line of the tester female (monogamous) was included as a covariate.

Index of male manipulation of female re-mating	MS	df	F	p
Pop size * variability	0.27	1	1.9	0.179
Elytra length (body size)	0.01	1	0.1	0.842
Pop size	0.04	1	0.3	0.581
Variability	0.08	1	0.6	0.455
Tester female	0.04	1	0.3	0.586
Error	3.65	26		

Table 5. Effect of population size and initial genetic variability on P2, the success of a male in sperm competition. Significant results are shown in bold.

P2	Deviance	df	F	p
Pop size * variability	4.4	1	1.2	0.288
Fecundity 24h	1.9	1	0.5	0.478
Pop size	**36.0**	**1**	**9.9**	**0.004**
Variability	**17.3**	**1**	**4.8**	**0.037**
Generation	**117.3**	**1**	**32.4**	**<0.001**
error	99.4	26		