



The evolution of harm - Effect of sexual conflicts and population size

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Complete List of Authors:	Gay, Laurène; UMR DIA-PC 1097, Structure and Evolution of Genetic Diversity Hosken, David; University of Exeter Cornwall campus, Center for Ecology and Conservation Eady, Paul; University of Lincoln, Biological Sciences Vasudev, Ram; University of Lincoln, Biological Sciences Tregenza, Tom; University of Exeter Cornwall campus, Center for Ecology and Conservation
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4 1 **THE EVOLUTION OF HARM – EFFECT OF SEXUAL CONFLICTS**
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6 2 **AND POPULATION SIZE**
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11 4 **Laurène Gay,^{1,2} David J. Hosken,¹ Paul Eady,³ Ram Vasudev,³ Tom Tregenza^{1,4}**

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13 5 *¹Centre for Ecology and Conservation, University of Exeter, Tremough Campus, Penryn,*
14
15 6 *UK*

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17 7 *²Structure and Evolution of Genetic Diversity UMR DIA-PC 1097, Domaine de*
18
19 8 *Melgueil, 34130 Mauguio, France*

20
21 9 *³Department of Biological Sciences, University of Lincoln, UK*

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23 10 *⁴T.Tregenza@exeter.ac.uk*
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33 12 **ABSTRACT**

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35 13 Conflicts of interest between mates can lead to the evolution of male traits that reduce
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37 14 female fitness and that drive coevolution between the sexes. The rate of adaptation
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39 15 depends on the intensity of selection and its efficiency, which depends on drift and
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41 16 genetic variability. This leads to the largely untested prediction that coevolutionary
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43 17 adaptations such as those driven by sexual conflict should evolve faster in large
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45 18 populations. We tested this prediction using the bruchid beetle *Callosobruchus*
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47 19 *maculatus*, a species where harm inflicted by males is well documented. Whilst most
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49 20 experimental evolution studies remove sexual conflict, we reintroduced it in populations
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51 21 where it had been experimentally removed. Both population size and standing genetic
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53 22 variability were manipulated in a factorial experimental design. After 90 generations of
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55 23 relaxed conflict (monogamy), the reintroduction of sexual conflicts for 30 generations
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3 1 favoured males that harmed females and females more resistant to the genital damage
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5 2 inflicted by males. Males evolved to become more harmful when population size was
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8 3 large rather than when initial genetic variation was enriched. Our study shows that sexual
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10 4 selection can create conditions where males can benefit from harming females and that
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12 5 selection may tend to be more intense and effective in larger populations.
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20 8 **KEYWORDS**
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22 9 Experimental evolution, sexual selection, *Callosobruchus maculatus*, genital damage,
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1 Sexual conflict occurs when the evolutionary interests of males and females differ
2 (Parker 1979), and can result in the evolution of traits beneficial to individuals but
3 harmful to their mates (Arnqvist and Rowe 2005). Extreme examples of this phenomenon
4 occur when male reproductive behaviour harms females via traits such as toxic
5 substances transferred in the ejaculate (Chapman et al. 1995; Eady et al. 2007; Rice 1996)
6 or damaging intromittent organs (Blanckenhorn et al. 2002; Crudgington and Siva-Jothy
7 2000; Stutt and Siva-Jothy 2001).

8
9 Two hypotheses have been proposed to explain the evolution of harm. First, the
10 collateral harm hypothesis (Hosken et al. 2003; Morrow et al. 2003) suggests that harm is
11 a side effect of adaptations beneficial in male-male competition (Lessells 2006; Parker
12 1979). For example, in *Drosophila melanogaster* genotypes that have superior sperm
13 defence capabilities reduce female longevity (Civetta and Clark 2000). Alternatively, the
14 adaptive harm hypothesis posits that harm benefits males more directly because of the
15 reduction of female survival. For example, injuries could deter females from
16 subsequently re-mating and/or alter female perceptions of their health status resulting in
17 increased resource reallocation to reproduction. Theoretical treatments support this
18 “terminal investment” hypothesis (Johnstone and Keller 2000; Lessells 2005), even when
19 damage decreases the re-mating interval (Lessells 2005). However, empirical support for
20 these models is lacking (Hosken et al. 2003; Morrow et al. 2003).

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

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3 1 sac of which is covered with spines that puncture the female genital tract during
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5 2 copulation (Crudginton and Siva-Jothy 2000). Despite comparative evidence supporting
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7 3 the notion that the spines are involved in male-female antagonistic coevolution at the
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9 4 interspecific level (Rönn et al. 2007), evidence for an association between sexual
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11 5 selection and genital damage is scarce at the intraspecific level. Hotzy and Arnqvist
12
13 6 (2009) demonstrated a correlation between spine length and male success in sperm
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15 7 competition across populations, but no such relationship was found in two other studies
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17 8 investigating why male bruchid beetles harm their mates (Edvardsson and Tregenza
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19 9 2005; Morrow et al. 2003). Here we use an experimental evolution approach to further
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21 10 assess the potential link between harm and sexual selection.
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29 12 Experimental evolution is a powerful tool that can be used to assess the evolution of
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31 13 harm and female resistance to it. This approach has been used to eliminate sexual conflict
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33 14 (and drastically reduce sexual selection) by enforcing monogamy. Males evolving under
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35 15 monogamy should evolve to become more benign to their partners since male and female
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37 16 fitness are simultaneously maximized, while monogamous females should become more
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39 17 susceptible to harm because selection on counteradaptations to reduce harm is relaxed
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41 18 (assuming that female resistance is costly). These predictions have been supported in
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43 19 experimental populations of *Drosophila melanogaster* (Holland and Rice 1999; Pitnick et
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45 20 al. 2001a; Pitnick et al. 2001b). Similarly, enforced monogamy in the fly *Sepsis cynipsea*
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47 21 enhanced female survival (Martin and Hosken 2003a) and monogamous populations of
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49 22 *Scathophaga stercoraria* had higher fitness than polyandrous lines (Martin et al. 2004).
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51 23 In an experiment where natural selection and sexual selection were manipulated
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1 simultaneously, Fricke and Arnqvist (2007) showed that, when reared on standard diets,
2 monogamous selection lines of *Callosobruchus maculatus* produced more offspring.
3 Recent studies have employed sex ratio biasing, to manipulate sexual conflict and sexual
4 selection. In *D. pseudoobscura*, male biased populations (with more scope for sexual
5 selection) did not differ greatly from monogamous lines (Crudginton et al. 2005), and
6 Wigby and Chapman (2004) found no difference in the male harming ability of *D.*
7 *melanogaster* lines with different sex ratios.

8
9 Following the publication of the first experimental evolution studies aimed at
10 understanding the role of sexual selection by manipulating the mating regime, Snook
11 (2001) and then Wigby and Chapman (2004) argued that altering the sex ratio or
12 population density can result in differences in effective population size, so that different
13 treatments experience different levels of drift and inbreeding. Additionally, because
14 monogamous lines often have a smaller population size, differences in population sizes
15 can be confounded with treatment. However, while these criticisms are in principle
16 sound, they were refuted for the specific studies initially criticized (Rice et al. 2005; and
17 see Reuter et al. 2008). More recently, Snook et al. (2009) raised additional concerns
18 about inbreeding and genetic variation when population size is manipulated. The authors
19 stress that a lack of genetic drift and higher genetic variability could result in more
20 efficient selection in large populations. Beyond the effect of drift and genetic variability,
21 theoretical models also suggest that sexually antagonistic coevolution is more likely in
22 large populations (Gavrilets 2000). Higher densities might favour more intense sexual
23 conflicts, due for example to interference from other males, through physical harm to

1 females, seminal fluid toxicity or polyspermy (Arnqvist 1997; Arnqvist and Nilsson
2 2000; Gavrilets et al. 2001). Population size could therefore affect evolution via sexual
3 conflict in two ways: either because sexually antagonistic coevolution is more likely in
4 large populations, or because selection is more efficient in large populations (Robertson
5 1970). The later could result from the fact that large populations harbour greater levels of
6 standing genetic variation and experience more mutations and little drift (Schultz and
7 Lynch 1997; Willi et al. 2006). While there is evidence consistent with population size
8 effects on sexually antagonistic evolution (Gay et al. 2009; Hosken et al. 2009; Martin
9 and Hosken 2003b), there have been few attempts to document the relative effects of the
10 potential causal factors involved (but see Ödeen and Florin (2000) regarding selection
11 efficiency). Here we use a fully factorial experimental design where both population size
12 and standing genetic variability are manipulated to disentangle the effect of intensified
13 sexual conflicts from the effect of increased genetic diversity, in a context of reintroduced
14 conflicts.

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

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

12 *Material and methods*

13 **STUDY SPECIES AND EXPERIMENTAL DESIGN**

14 Two replicate monogamous lines were established from an ancestral *C. maculatus*
15 population (Niamey, Niger) cultured on black eyed-beans (*Vigna unguiculata*) at 27°C, 32
16 % RH and 16L:8D photoperiod. Each generation we isolated beans carrying eggs in 48-
17 well cell culture plates in order to collect virgin beetles immediately post-emergence.
18 Virgins (< 24h post eclosion) were subsequently paired and each pair was placed in a
19 40mm Petri dish and observed until copulation had ceased. From these monogamous
20 pairs, 60 singly mated females were transferred together to approximately 400 beans for
21 oviposition.

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

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

17 To retain a constant population size and ratio of resources to beetles, we sieved and
18 weighed the newly emerging adults each generation and placed another 50 (for the small
19 populations), or 5000 (for the large ones) individuals on new black-eyed beans. Small
20 populations were provided with 40g of beans in a cylindrical container 10cm wide and
21 4cm deep, large populations were provided with 4kg of beans in a rectangular container
22 30cm x 20cm x 13cm deep. Half of the populations for our genetic variability treatment
23 are derived from each monogamous line. Comparison between the basal genetic

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3 1 variability populations created from monogamous line 1 and monogamous line 2 revealed
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5 2 male-induced damage, LRS, female re-mating rate, oviposition speed and P2 to be
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8 3 equivalent, although the populations derived from monogamous line 1 lived significantly
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10 4 longer than those derived from monogamous line 2 (12 days versus 11). We accounted
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12 5 for this difference in the analysis of longevity (see below).
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15 6 To reduce possible maternal and phenotypic effects, we standardized selection one
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17 7 generation prior to the assay (generation 30) for all populations by housing beetles
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19 8 individually under standardised conditions - single mating and one egg per bean (this is in
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21 9 excess of what a single larva can consume (Cope and Fox 2003)) - for one generation.
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23 10 Prior to beetle emergence, we isolated these beans in 'virgin chambers' (48-Well cell
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25 11 culture plates, VWR International Ltd, Lutterworth, UK). Beans were checked every 24h
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27 12 for emerging virgin adults (generation 31).
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34 **TEST FOR INBREEDING DEPRESSION**

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36 15 In our experiment, the small populations are potentially susceptible to inbreeding during
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38 16 experimental evolution. Inbreeding can lead to inbreeding depression affecting life
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40 17 history traits (e.g. fecundity and longevity) (Charlesworth and Charlesworth 1987;
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42 18 DeRose and Roff 1999) and competitive male mating ability (Sharp 1984). These effects
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44 19 could potentially confound our predictions (see below). We looked for evidence of
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46 20 inbreeding depression in fecundity, lifetime reproductive success and longevity by
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48 21 crossing males and females between replicate populations and comparing their
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50 22 performance to matings between males and females from within replicate populations
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52 23 (the potentially inbred populations). We assessed those treatments most likely to suffer
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3 1 inbreeding depression, namely the populations of small census size and basal initial
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5 2 standing genetic variation. We also assessed the large populations with basal initial
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7 3 standing genetic variation as this allowed us to determine the potential impact of
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9 4 population size and initial genetic variance on inbreeding depression. We analysed these
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11 5 data using a general linear model including population size, crossing status (within or
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13 6 between replicate crosses) and their interaction. Elytra length (a measure of body size)
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15 7 was included as a covariate in the analysis of fecundity and lifetime reproductive success,
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17 8 whilst fecundity was included as a covariate in the analysis of longevity.
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10 **MALE OFFENCE AND FEMALE RESISTANCE: DAMAGE, LONGEVITY AND** 11 **LIFETIME REPRODUCTIVE SUCCESS**

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

21
22 Virgin females and males (all <24h post eclosion) were paired and each pair (10 pairs
23 x 4 treatments x 4 replicates x 4 crossings) was placed in a 40mm Petri dish and observed

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

9

10 **MANIPULATION OF RE-MATING AND OVIPOSITION**

11 We measured the ability of males to deter females from subsequently re-mating (male
12 defence) by mating monogamous tester females with males from the polygamous
13 populations and then exposing them to monogamous tester males ($\text{♀}_M\text{-}\text{♂}_P\text{-}\text{♂}_M$). We also
14 measured male offence - the ability of males to induce previously mated females to re-
15 mate - by mating monogamous tester females with monogamous tester males and then
16 exposing them to males from the polygamous populations ($\text{♀}_M\text{-}\text{♂}_M\text{-}\text{♂}_P$). For each assay,
17 10 females were paired and subsequently offered a chance to re-mate, following 24h of
18 oviposition. Earlier studies revealed that over 80% of females will re-mate 24 h after their
19 initial copulation (Eady et al. 2004; Edvardsson and Tregenza 2005) but in a pilot
20 experiment we found lower re-mating rates in our lines that were maintained
21 monogamous for 90 generations. We thus estimated that 24 h is a time point at which one
22 might be able to distinguish differences in female re-mating propensity between

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

3
4 We measured the ability of males from the polygamous populations to stimulate
5 female fecundity by counting eggs laid during the female refractory period using males
6 from the 16 polygamous lines mated to 10 monogamous tester females. Again, mated
7 females were placed on 10 beans for 24 hours and then moved to another 60 beans for the
8 remainder of their lifespan. We subsequently counted the number of offspring produced
9 during the first 24h after mating and over their entire lifespan, and then used the
10 proportion of offspring produced in 24h relative to the lifetime reproductive success as a
11 measure of male manipulation. Because both female re-mating rate and last male sperm
12 precedence are high in this species (Eady et al. 2004; Edvardsson and Tregenza 2005),
13 the benefits to any additional stimulation of oviposition beyond the first 24 hours will
14 probably be enjoyed by rival males and as such we did not assess them here.

15 16 **SPERM COMPETITION**

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

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

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10 Virgin black females and black males were paired in individual 40mm Petri dishes
11 and observed until copulation began. After copulation ceased, males were removed and
12 females were allowed to oviposit for 20 hours on 5 beans. Females were then transferred
13 back to individual 40mm Petri dishes with a virgin brown male from one of the
14 polygamous populations. We repeated this for at least 20 females per replicate (4) per
15 treatment (4). For each pair, we recorded whether copulation occurred successfully within
16 30 minutes. After copulation with the focal (brown) male ceased, each black female was
17 transferred to a 90mm Petri dish containing 80 beans and allowed to oviposit until death.
18 Eggs laid prior to the second mating were counted (first 20 h), as were the total number
19 of offspring after two successive matings, and offspring phenotype (hybrid or black) was
20 recorded. P2 - the proportion of offspring sired by the second (focal = brown) male was
21 calculated as the proportion of intermediate offspring. The experiment was repeated at
22 generation 32 to increase the sample size. We accounted for this by including a
23 generation factor in the analytical models. Additionally, to ascertain confidence in our co-
24 dominant phenotypic marker, we estimated the repeatability of our paternity estimates by
25 re-measuring P2 blind to the first measurement for 20 randomly chosen females. P2
26 repeatability was calculated following Lessells & Boag (1987), and was high ($r = 0.996$).

27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 **STATISTICAL ANALYSES** 54 55 56 57 58 59 60

1 Analyses were performed in R. To avoid pseudoreplication, we performed all analyses on
2 population means. We also used mixed effect models adding replicate as a random effect
3 and obtained similar results, but only the results using the population means are presented
4 here. All traits (damage, longevity, fecundity, lifetime reproductive success and elytra
5 length) were normally distributed (Kolmogorov-Smirnov test, all $P > 0.05$). Additionally,
6 residuals did not deviate significantly from normality (Kolmogorov-Smirnov test, all $P >$
7 0.05), and were not autocorrelated (Durbin-Watson test, all $P > 0.05$), and errors were
8 homoscedastic (Breusch-Pagan test, all $P > 0.05$).

9 10 *Cost of damage*

11 We used a general linear model to test the effect of population size, genetic variability
12 and their interaction on genital damage inflicted by polygamous line males. Female type
13 (monogamous or polygamous) was used as a third factor. We examined whether genital
14 damage evolved with the reintroduction of sexual conflict and sexual selection by testing
15 for an effect of male and female type (from a polygamous or monogamous line) on the
16 amount of damage sustained by a female, using data from four assays ($\text{♀}_M\text{♂}_P$, $\text{♀}_P\text{♂}_P$,
17 $\text{♀}_P\text{♂}_M$ and $\text{♀}_M\text{♂}_M$). We also examined the cost of damage by testing for a negative
18 relationship between damage and longevity or damage and LRS using linear models. We
19 included population size, genetic variability and female type in the model, as well as
20 elytra length as a covariate for LRS and 24h fecundity as a covariate for longevity to
21 account for life history trade offs. To account for the difference in longevity between the
22 populations of the low variability treatment derived from the two monogamous lines, we

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

3

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

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

14

15 *Effect of population size and genetic variability on male manipulative ability*

16 To ascertain how population size and genetic variability influence the evolution of males’
17 ability to affect female reproduction, we compared re-mating rates, oviposition rate and
18 P2 between our experimental populations that differ in the level of damage inflicted by
19 males. For re-mating, we estimated an index of male manipulation by combining the
20 assays of male defence ($\text{♀}_M\text{-}\text{♂}_P\text{-}\text{♂}_M$) and male offence ($\text{♀}_M\text{-}\text{♂}_M\text{-}\text{♂}_P$): male manipulation
21 was estimated as the difference between the proportion of females re-mating in the
22 offence experiment minus the proportion that re-mated in the defence experiment. We
23 tested the effect of population size, genetic variability and their interaction on this re-

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

7

8

9 *Results*

10 **TEST FOR INBREEDING DEPRESSION**

11 There was no evidence for inbreeding depression in small and low variability
12 populations. We found no significant effect of the interaction between population size
13 and crossing status (within or between replicate crosses) (Fecundity: $F_{7,1}=0.5$ $p=0.480$;
14 longevity: $F_{7,1}=0.04$ $p=0.837$; LRS: $F_{7,1}=0.5$ $p=0.517$). Fecundity and longevity were not
15 significantly different in crosses within or between replicate populations (Fig. 2a:
16 $F_{9,1}=0.9$ $p=0.360$ and Fig. 2b: $F_{8,1}=0.01$ $p=0.909$). Population size also had no effect on
17 these fitness measures, suggesting that inbreeding depression was either absent or was
18 similar across experimental populations (Fig. 2a: $F_{10,1} = 2.8$ $p = 0.125$ and Fig. 2b: $F_{9,1} =$
19 0.4 $p = 0.533$). Lifetime reproductive success (LRS) was also equivalent in the within or
20 between replicate crosses (Fig. 2c; $F_{9,1} = 1.6$ $p = 0.234$), but population size had an effect
21 with small populations having lower LRS than large populations (Fig. 2c; $F_{10,1} = 8.6$ $p =$
22 0.015). When the analysis was restricted to small populations only, fecundity, longevity

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

3

4 **GENITAL DAMAGE EVOLVES IN RESPONSE TO THE REINTRODUCTION** 5 **OF SEXUAL CONFLICT**

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

14

15 **DAMAGE EVOLVES FASTER IN LARGER RATHER THAN MORE DIVERSE** 16 **POPULATIONS**

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

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

4 **GENITAL DAMAGE IS COSTLY**

5 The number of damage points in a female's reproductive tract was negatively associated
6 with female longevity (Fig. 5, slope = -0.04 days/damage point; $F_{30,1} = 5.5$ $p = 0.027$,
7 Table 1). Furthermore, females from the polygamous populations tended to outlive
8 females from monogamous populations (M: 10.9 days \pm 0.2; P: 11.7 \pm 0.3; $F_{30,1} = 4.6$ $p =$
9 0.040, Table 1, Fig. 5). This was also reflected in the LRS results, where females from
10 polygamous populations had greater LRS (M: 69 offspring \pm 2; P: 78 \pm 2; $F_{26,1} = 8.7$ $p =$
11 0.006, Table 2). LRS was also influenced by an interaction between the number of scars
12 in the female tract and polygamous line population size ($F_{26,1} = 7.0$ $p = 0.014$, Table 2).
13 More scarring in females from larger populations resulted in lower LRS, but for females
14 from smaller populations the association between genital damage and LRS was flat or
15 even positive (Fig. 6). Note that when we removed one outlier from the analysis (the one
16 small population with very low LRS and damage), the interaction between the number of
17 scars and population size remained significant ($p = 0.028$): in large populations, the
18 relationship between damage and LRS remained negative but was flat in small
19 populations.

21 **EFFECT OF DAMAGE ON RE-MATING, OVIPOSITION AND SPERM**

22 **COMPETITION**

1 We tested three hypotheses relating to the function of male-induced genital damage
2 (delayed female re-mating, elevation of female oviposition rate and increased success in
3 sperm competition) using generalized linear models with damage as an explanatory
4 variable, elytra length and the number of eggs laid in the first 24h as covariates (for re-
5 mating and P2 only). We found no significant effect of damage on female re-mating (χ^2_{13}
6 = 0.80 p = 0.37) or oviposition rate (proportion of offspring produced within the first 24
7 hours following mating, $F_{1,15} = 1.6$ p = 0.224) and males from more damaging
8 populations were not more successful at sperm competition ($\chi^2_{13} = 0.32$ p = 0.571).

9
10 **EFFECT OF POPULATION SIZE AND GENETIC VARIABILITY ON MALE**
11 **MANIPULATIVE ABILITY (RE-MATING, OVIPOSITION RATE AND SPERM**
12 **COMPETITION)**

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

20
21 There was no effect of population size or standing genetic variability on the index of
22 male manipulation of female re-mating, which implies that all males were equally good at

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

3
4 Both population size and initial genetic variability influenced male success in sperm
5 competition. Males from small populations with basal initial genetic variability were the
6 best competitors (Fig. 7c, large population: $P_2 = 0.73 \pm 0.03$; small pop. $P_2 = 0.82 \pm$
7 0.02 , $F_{29,1} = 9.9$ $p = 0.004$; enriched variability population: $P_2 = 0.75 \pm 0.03$; basal
8 variability: $P_2 = 0.81 \pm 0.02$, $F_{29,1} = 4.8$ $p = 0.037$; Table 5).

11 *Discussion*

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

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

7
8 Mean damage levels were not associated with female oviposition rate or propensity to
9 re-mate. Our results thus provide no support for the adaptive harm hypothesis. This is in
10 agreement with previous work: Edvardsson and Tregenza (2005) manipulated copulation
11 duration to elevate female damage (Crudginton 2001) and also found no benefits to
12 harming males via delayed re-mating or increased rate of offspring production.
13 Consequently, and despite theoretical support, there is still no empirical evidence for the
14 adaptive harm hypothesis, whether the mechanism involved is terminal investment or
15 delayed re-mating (Edvardsson and Tregenza 2005; Hosken et al. 2003; Morrow et al.
16 2003), and our results serve to reinforce this. Males from populations with basal genetic
17 variability were better at stimulating female oviposition in the first 24 hours. This could
18 be because favourable gene combinations were broken up by mixing of the two
19 monogamous lines to create the populations with enriched genetic variability, although
20 more work is needed to determine whether epistatic interactions can explain this finding.

21
22 If harm does not benefit males directly, it could be a side-effect of some other male
23 adaptation to male-male competition (the collateral harm hypothesis), with the obvious

1 candidate being sperm competitive ability. However, we found no evidence supporting
2 the idea that males from more damaging populations are more successful in sperm
3 competition. P2 is a composite trait that is likely to be influenced by an unknown number
4 of male derived chemicals and behaviours, so that the prediction of the effect of
5 population size might be less straightforward than for simpler traits such as genital
6 damage. Nevertheless, in the dung fly *S. cynipsea* more damaging males were not more
7 competitive (Teuschl et al. 2007) and our findings are in agreement with results from
8 Edvardsson and Tregenza (2005) who failed to find an effect of damage on P2. In
9 contrast, Hotzy and Arnqvist (2009) found that across 13 geographically distinct
10 populations of *C. maculatus*, male genital armature and the harm males inflict upon
11 females were positively correlated with male success in sperm competition. This
12 discrepancy between *C. maculatus* studies could result from the fact that the balance
13 between the advantage in sperm competition and the cost of harming females is
14 “contingent upon mating system, female life histories and sperm competition regime”
15 (Hotzy and Arnqvist 2009), which may differ when looking within rather than across
16 populations, and certainly could differ across studies. Our results, in conjunction with
17 Edvardsson’s (2005), suggest that the damage inflicted by the spines is not associated
18 with male success in sperm competition, but the damage they inflict did evolve after only
19 30 generations of restored polygamy. Perhaps a direct measure of spininess would be
20 more revealing (e.g. Hotzy & Arnqvist, 2009), but perhaps the spines serve other
21 purposes too, such as anchoring males firmly during copulation (Edvardsson and
22 Tregenza 2005). Using spines as an anchor could be beneficial for males if female

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

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10 Like the damage inflicted by males which evolved after 30 generations in our
11 polygamous lines, females have also evolved resistance to harm. It is interesting that the
12 number of scars inflicted by males did not differ in females evolving under polygamy or
13 monogamy, but the effects did. Damage inflicted by males could increase female
14 investment in immunocapacity, as has been suggested in other insects (Reinhardt and
15 Siva-Jothy 2007). As a result, the LRS and longevity of females evolving under
16 polygamy were on average higher. Our longevity results are straightforward: increased
17 damage leads to reduced longevity and females from polygamous populations always live
18 longer than monogamous females at any given level of damage. Similarly, LRS of
19 females from monogamous populations always tended to be lower across damage levels.
20 Nevertheless, LRS results are somewhat more complicated in that the damage effect only
21 shows up in an interaction with the population size of the male. When males are from
22 larger populations, more damage equates to lower LRS, but when males are from smaller
23 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

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

14
15 Despite manipulating population size for 30 generations, we found no evidence for
16 inbreeding depression in smaller populations. This could result from purging of
17 deleterious mutations over the 90 generations of monogamy when population size was
18 relatively small (between 100 and 150 individuals for each of the two monogamous
19 lines), assuming that inbreeding depression is primarily due to the expression of
20 deleterious recessives and not to loss of heterozygosity in *C. maculatus*. Alternatively,
21 population sizes of this order may escape serious inbreeding over this time frame. Recent
22 results suggest that the spectrum of deleterious mutations contains a high proportion of
23 very small effect mutation ($\ll 1\%$) (Estes et al. 2004) such that even large finite

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

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

14
15 Our experimental design manipulated population size and standing genetic variability
16 simultaneously and independently. It thus contributes empirical data relevant to debates
17 on the effect of population size and inbreeding in experimental evolution, in particular
18 experimental sexual selection. Effective population size is a key parameter in these
19 experimental evolution studies, firstly because the experimental manipulation of mating
20 systems or sex ratio can lead to different effective population sizes between treatments
21 and confound effects (Snook et al. 2009). Secondly, small populations may lack the
22 influx of new beneficial mutations, but slightly deleterious mutations are more likely to
23 get fixed. Finally, small populations suffer less intense conflicts. Consequently, effective

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

21
22 In conclusion, this study is the first attempt at reversing experimental evolution under
23 sexual conflicts. Reintroducing sexual selection and sexual conflict for 30 generations

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

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3 **Figure 1.** Diagram of the experimental design. 90 generations of relaxed sexual selection
4 and sexual conflicts (monogamy, in grey) was followed by 30 generations of restored
5 polygamy (in black). In parallel, the two monogamous lines were maintained to be used
6 as testers. At generation 90, the two monogamous lines were crossed. Generations 91 and
7 92 were population expansion. At generation 92, the four treatments were set up by
8 manipulating population size (large or small) and using the enhanced genetic variability
9 of the crossed line to form four treatments: large population size enriched genetic
10 variability, large population size basal genetic variability, small population size enriched
11 genetic variability and small population size basal genetic variability, with four replicates
12 for each treatment (16 lines in total). All lines were standardized for mating rate and
13 larval density at generation 122 and 123.
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32 **Figure 2.** Test of the effect of inbreeding in the experimental lines with low genetic
33 variability, small or large population size. Inbreeding depression was assessed in terms of
34 (a) fecundity (number of eggs laid in the first 24 hours), (b) longevity (days) or (c)
35 lifetime reproductive success (total number of offspring that emerged). Bars and error
36 bars stand for means and standard errors respectively.
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46 **Figure 3.** Genital damage (measured as the mean number of scars in the female genital
47 tract) suffered by females from monogamous or polygamous lines mated to males from
48 monogamous or polygamous lines. White bars indicate polygamous line males and
49 standard errors are shown.
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4 **Figure 4.** Effect of male population size (large or small) and initial genetic variability
5 (basal or enriched) on genital damage (mean number of scars) inflicted by polygamous
6 males to females (monogamous tester $\sigma_P \times \text{♀}_M$ or line females $\sigma_P \times \text{♀}_P$) with standard errors.
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12 **Figure 5.** Effect of genital damage (measured as the number of scars in the female genital
13 tract) on female longevity (in days). Damage is inflicted by polygamous males on
14 females from monogamous (crosses and dotted line) or polygamous lines (circles and
15 solid line) ($\sigma_P \times \text{♀}_M$ or ♀_P).
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24 **Figure 6.** Effect of genital damage (number of scars) on female lifetime reproductive
25 success (total number of offspring that emerged) in lines of small (triangles and solid
26 line) or large (crosses and dotted line) population size, when males from polygamous
27 lines are mated to females from either monogamous (tester) or polygamous lines ($\sigma_P \times$
28 ♀_M or ♀_P).
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39 **Figure 7.** Effect of male population size (large or small) and initial genetic variability
40 (basal or enriched) on (a) oviposition speed measured as the mean percentage of
41 offspring produced by a female that hatched from eggs laid in the first 24 hours following
42 mating, (b) the mean index of male manipulation of female re-mating (see text) and (c)
43 the success of a male in sperm competition P2, measured as the mean proportion of
44 offspring sired by that male when he was the 2nd male to mate. Error bars stand for
45 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 ($\sigma_P \times \text{♀}_M$ 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 ($\sigma_P \times \text{♀}_M$ 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.

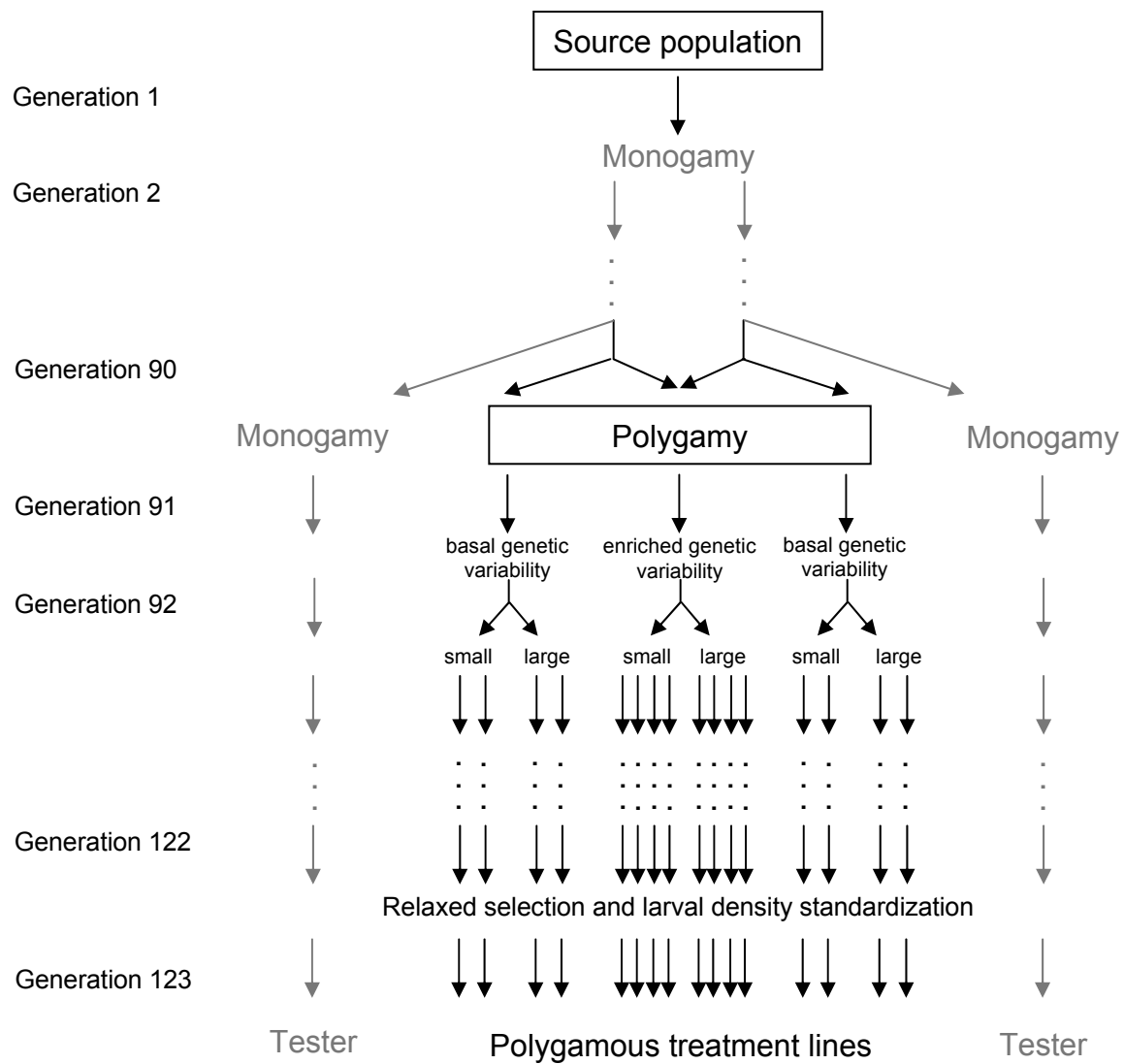
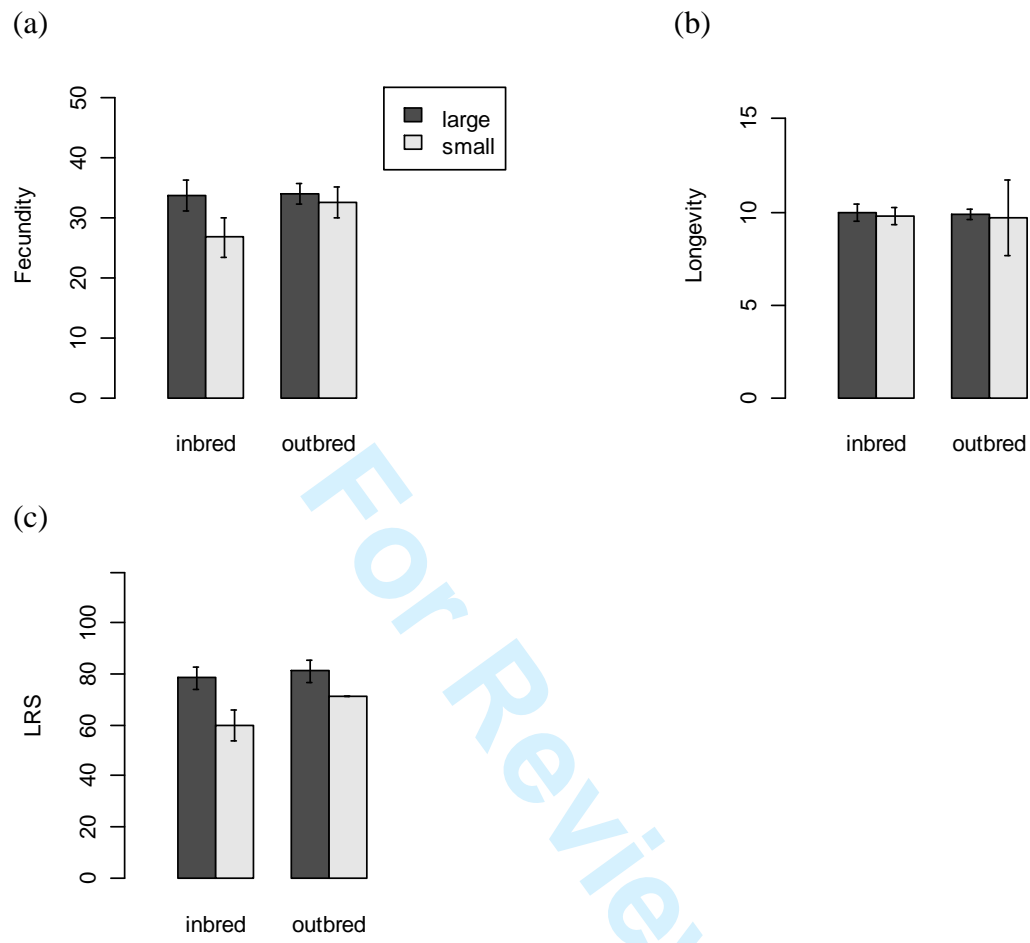


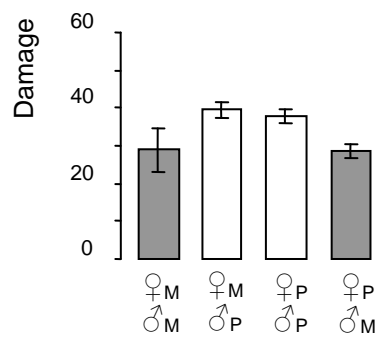
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Figure 3.

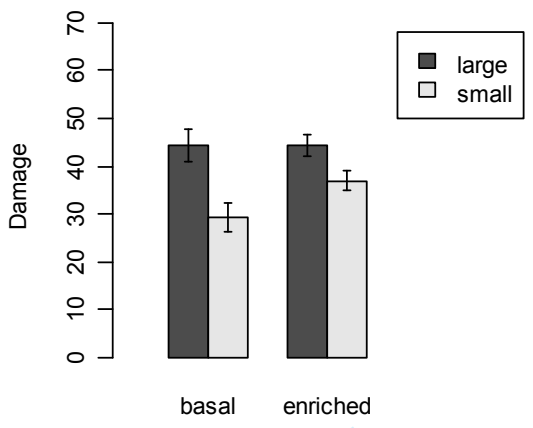


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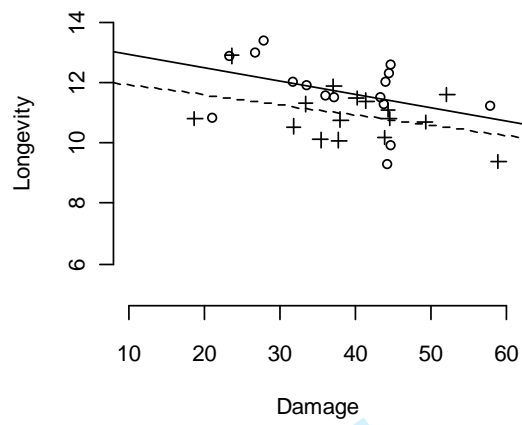
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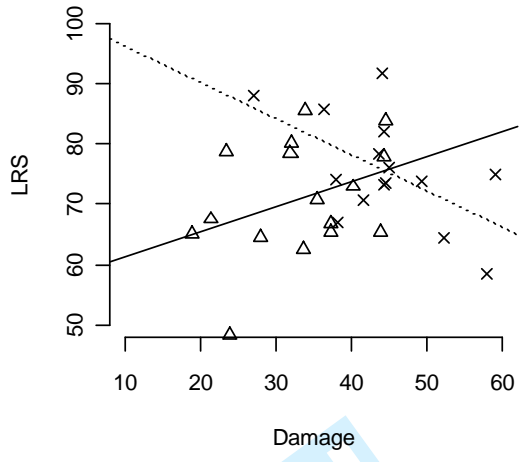


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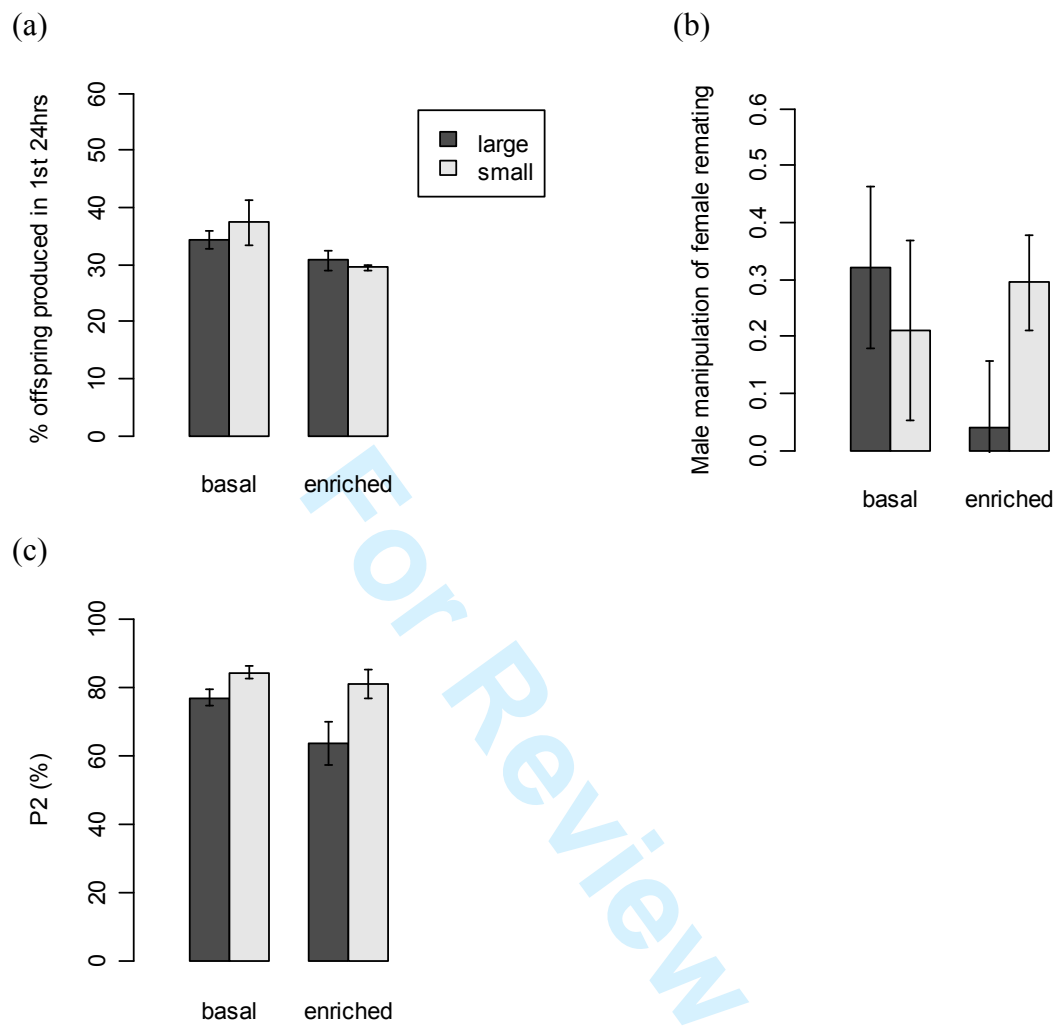
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Figure 6.



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Figure 7.



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1 THE EVOLUTION OF HARM – EFFECT OF SEXUAL CONFLICTS 2 AND POPULATION SIZE

3
4 Laurène Gay,^{1,2} David J. Hosken,¹ Paul Eady,³ Ram Vasudev,³ Tom Tregenza^{1,4}

5 ¹Centre for Ecology and Conservation, University of Exeter, Tremough Campus, Penryn,
6 UK

7 ²Structure and Evolution of Genetic Diversity UMR DIA-PC 1097, Domaine de
8 Melgueil, 34130 Mauguio, France

9 ³Department of Biological Sciences, University of Lincoln, UK

10 ⁴T.Tregenza@exeter.ac.uk

12 ABSTRACT

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

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

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8 **KEYWORDS**

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

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1 Sexual conflict occurs when the evolutionary interests of males and females differ
 2 (Parker 1979), and can result in the evolution of traits beneficial to individuals but
 3 harmful to their mates (Arnqvist and Rowe 2005). Extreme examples of this phenomenon
 4 occur when male reproductive behaviour harms females via traits such as toxic
 5 substances transferred in the ejaculate (Chapman et al. 1995; Eady et al. 2007; Rice 1996)
 6 or damaging intromittent organs (Blanckenhorn et al. 2002; Crudgington and Siva-Jothy
 7 2000; Stutt and Siva-Jothy 2001).

9 Two hypotheses have been proposed to explain the evolution of harm. First, the
 10 collateral harm hypothesis (Hosken et al. 2003; Morrow et al. 2003) suggests that harm is
 11 a side effect of adaptations beneficial in male-male competition (Lessells 2006; Parker
 12 1979). For example, in *Drosophila melanogaster* genotypes that have superior sperm
 13 defence capabilities reduce female longevity (Civetta and Clark 2000). Alternatively, the
 14 adaptive harm hypothesis posits that harm benefits males more directly because of the
 15 reduction of female survival. For example, injuries could deter females from
 16 subsequently re-mating and/or alter female perceptions of their health status resulting in
 17 increased resource reallocation to reproduction. Theoretical treatments support this
 18 “terminal investment” hypothesis (Johnstone and Keller 2000; Lessells 2005), even when
 19 damage decreases the re-mating interval (Lessells 2005). However, empirical support for
 20 these models is lacking (Hosken et al. 2003; Morrow et al. 2003).

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

Deleted: (*Callosobruchus maculatus*)

1 sac of which is covered with spines that puncture the female genital tract during
 2 copulation (Crudginton and Siva-Jothy 2000). Despite comparative evidence supporting
 3 the notion that the spines are involved in male-female antagonistic coevolution at the
 4 interspecific level (Rönn et al. 2007), evidence for an association between sexual
 5 selection and genital damage is scarce at the intraspecific level. Hotzy and Arnqvist
 6 (2009) demonstrated a correlation between spine length and male success in sperm
 7 competition across populations, but no such relationship was found in two other studies
 8 investigating why male bruchid beetles harm their mates (Edvardsson and Tregenza
 9 2005; Morrow et al. 2003). Here we use an experimental evolution approach to further
 10 assess the potential link between harm and sexual selection.

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

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

8
9 | Following the publication of the first experimental evolution studies [aimed at](#)
10 | [understanding the role of sexual selection by manipulating the mating regime](#), Snook
11 | (2001) and then Wigby and Chapman (2004) argued that altering the sex ratio or
12 | population density can result in differences in effective population size, so that different
13 | treatments experience different levels of drift and inbreeding. Additionally, because
14 | monogamous lines often have a smaller population size, differences in population sizes
15 | can be confounded with treatment. However, while these criticisms are in principle
16 | sound, they were refuted for the specific studies initially criticized (Rice et al. 2005; and
17 | see Reuter et al. 2008). More recently, Snook et al. (2009) raised additional concerns
18 | about inbreeding and genetic variation when population size is manipulated. The authors
19 | stress that a lack of genetic drift and higher genetic variability could result in more
20 | efficient selection in large populations. Beyond the effect of drift and genetic variability,
21 | theoretical models also suggest that sexually antagonistic coevolution is more likely in
22 | large populations (Gavrilets 2000). Higher densities might favour more intense sexual
23 | conflicts, due for example to interference from other males, through physical harm to

1 females, seminal fluid toxicity or polyspermy (Arnqvist 1997; Arnqvist and Nilsson
 2 2000; Gavrilets et al. 2001). Population size could therefore affect evolution via sexual
 3 conflict in two ways: either because sexually antagonistic coevolution is more likely in
 4 large populations, or because selection is more efficient in large populations (Robertson
 5 1970). The later could result from the fact that large populations harbour greater levels of
 6 standing genetic variation and experience more mutations and little drift (Schultz and
 7 Lynch 1997; Willi et al. 2006). While there is evidence consistent with population size
 8 effects on sexually antagonistic evolution (Gay et al. 2009; Hosken et al. 2009; Martin
 9 and Hosken 2003b), there have been few attempts to document the relative effects of the
 10 potential causal factors involved (but see Ödeen and Florin (2000) regarding selection
 11 efficiency). Here we use a fully factorial experimental design where both population size
 12 and standing genetic variability are manipulated to disentangle the effect of intensified
 13 sexual conflicts from the effect of increased genetic diversity, in a context of reintroduced
 14 conflicts.

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Deleted: We also tested for an effect of inbreeding in small and low variability populations.

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

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1 | [\(relaxed conflict\) lines from which they had been established 30 generations previously.](#)

2 | [Then we examined](#) whether sexual conflict resulted in more rapid evolution in larger

3 | populations or those with greater initial genetic variation [\(2\)](#), by comparing the evolution

4 | of adaptations to polygamy across our lines. Additionally, we assessed the costs of

5 | damage [\(3\)](#) by evaluating associations between level of damage and female longevity and

6 | lifetime reproductive success. [Finally](#), we [tested](#) the two hypotheses about why males

7 | harm females [\(4\)](#): Are damaging males better at accelerating female oviposition or

8 | deterring females to re-mate (adaptive harm hypothesis) or are they better at sperm

9 | competition ([collateral](#) harm hypothesis)? [We simultaneously tested for an effect of](#)

10 | [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

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

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17 To retain a constant population size and ratio of resources to beetles, we sieved and
 18 weighed the newly emerging adults each generation and placed another 50 (for the small
 19 populations), or 5000 (for the large ones) individuals on new black-eyed beans. Small
 20 populations were provided with 40g of beans in a cylindrical container 10cm wide and
 21 4cm deep, large populations were provided with 4kg of beans in a rectangular container
 22 30cm x 20cm x 13cm deep. Half of the populations for our genetic variability treatment
 23 are derived from each monogamous line. Comparison between the basal genetic

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2 1 variability populations created from monogamous line 1 and monogamous line 2 revealed
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4 2 male-induced damage, LRS, female re-mating rate, oviposition speed and P2 to be
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6 3 equivalent, although the populations derived from monogamous line 1 lived significantly
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8 4 longer than those derived from monogamous line 2 (12 days versus 11). We accounted
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10 5 for this difference in the analysis of longevity (see below).

11
12 6 To reduce possible maternal and phenotypic effects, we standardized selection one
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14 7 generation prior to the assay (generation 30) for all populations by housing beetles
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16 8 individually under standardised conditions - single mating and one egg per bean (this is in
17
18 9 excess of what a single larva can consume (Cope and Fox 2003)) - for one generation.
19
20 10 Prior to beetle emergence, we isolated these beans in 'virgin chambers' (48-Well cell
21
22 11 culture plates, VWR International Ltd, Lutterworth, UK). Beans were checked every 24h
23
24 12 for emerging virgin adults (generation 31).
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28 29 14 **TEST FOR INBREEDING DEPRESSION**

30
31 15 In our experiment, the small populations are potentially susceptible to inbreeding during
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33 16 experimental evolution. Inbreeding can lead to inbreeding depression affecting life
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35 17 history traits (e.g. fecundity and longevity) (Charlesworth and Charlesworth 1987;
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37 18 DeRose and Roff 1999) and competitive male mating ability (Sharp 1984). These effects
38
39 19 could potentially confound our predictions (see below). We looked for evidence of
40
41 20 inbreeding depression in fecundity, lifetime reproductive success and longevity by
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43 21 crossing males and females between replicate populations and comparing their
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45 22 performance to matings between males and females from within replicate populations
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47 23 (the potentially inbred populations). We assessed those treatments most likely to suffer

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3 1 inbreeding depression, namely the populations of small census size and basal initial
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5 2 standing genetic variation. We also assessed the large populations with basal initial
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7 3 standing genetic variation as this allowed us to determine the potential impact of
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9 4 population size and initial genetic variance on inbreeding depression. We analysed these
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11 5 data using a general linear model including population size, crossing status (within or
12
13 6 between replicate crosses) and their interaction. Elytra length (a measure of body size)
14
15 7 was included as a covariate in the analysis of fecundity and lifetime reproductive success,
16
17 8 whilst fecundity was included as a covariate in the analysis of longevity.
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20 **MALE OFFENCE AND FEMALE RESISTANCE: DAMAGE, LONGEVITY AND** 21 22 **LIFETIME REPRODUCTIVE SUCCESS**

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25 12 Both males and females are likely to influence the amount of damage suffered by females
26
27 13 during copulation. To isolate the damaging effect of males from the susceptibility of
28
29 14 females, we used the two monogamous lines as testers. Four types of crosses were
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31 15 performed: (1) between males from the polygamous populations and tester females (male
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33 16 offence assay - $\text{♀}_M\text{♂}_P$); (2) between males and females from the same polygamous
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35 17 population (female resistance assay - $\text{♀}_P\text{♂}_P$); (3) between females from the polygamous
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37 18 populations and tester males ($\text{♀}_P\text{♂}_M$); (4) a control cross between tester males and
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39 19 females ($\text{♀}_M\text{♂}_M$). For each assay, 20 crosses were performed for each replicate (x4) of
40
41 20 each treatment (x4) (= 1280 crosses).
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43 21

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45 22 Virgin females and males (all <24h post eclosion) were paired and each pair (10 pairs
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47 23 x 4 treatments x 4 replicates x 4 crossings) was placed in a 40mm Petri dish and observed
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1 until copulation had ceased. Mated females were then placed on 10 beans for 24 hours
2 and then moved to another 60 beans for the remainder of their lives. We measured
3 fecundity in the first 24 hours of oviposition by directly counting eggs laid. Longevity
4 was estimated by recording female mortality every 24 hours. After their natural death,
5 females were dissected and the number of damage points (scars) in their genital tracts
6 determined. For 25 females we also measured the area covered by scars and found that it
7 was highly correlated with the number of scars (log-linear regression, $R^2 = 0.68$). Female
8 elytra length was measured as a proxy for body size.

10 **MANIPULATION OF RE-MATING AND OVIPOSITION**

11 We measured the ability of males to deter females from subsequently re-mating (male
12 defence) by mating monogamous tester females with males from the polygamous
13 populations and then exposing them to monogamous tester males ($\text{♀}_M\text{-}\text{♂}_P\text{-}\text{♂}_M$). We also
14 measured male offence - the ability of males to induce previously mated females to re-
15 mate - by mating monogamous tester females with monogamous tester males and then
16 exposing them to males from the polygamous populations ($\text{♀}_M\text{-}\text{♂}_M\text{-}\text{♂}_P$). For each assay,
17 10 females were paired and subsequently offered a chance to re-mate, following 24h of
18 oviposition. Earlier studies revealed that over 80% of females will re-mate 24 h after their
19 initial copulation (Eady et al. 2004; Edvardsson and Tregenza 2005) but in a pilot
20 experiment we found lower re-mating rates in our lines that were maintained
21 monogamous for 90 generations. We thus estimated that 24 h is a time point at which one
22 might be able to distinguish differences in female re-mating propensity between

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

3
4 We measured the ability of males from the polygamous populations to stimulate
5 female fecundity by counting eggs laid during the female refractory period using males
6 from the 16 polygamous lines mated to 10 monogamous tester females. Again, mated
7 females were placed on 10 beans for 24 hours and then moved to another 60 beans for the
8 remainder of their lifespan. We subsequently counted the number of offspring produced
9 during the first 24h after mating and over their entire lifespan, and then used the
10 proportion of offspring produced in 24h relative to the lifetime reproductive success as a
11 measure of male manipulation. Because both female re-mating rate and last male sperm
12 precedence are high in this species (Eady et al. 2004; Edvardsson and Tregenza 2005),
13 the benefits to any additional stimulation [of oviposition](#) beyond the first 24 hours will
14 probably be enjoyed by rival males and as such we did not assess them here.

15 16 **SPERM COMPETITION**

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

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

3
4 Virgin black females and black males were paired in individual 40mm Petri dishes
5 and observed until copulation began. After copulation ceased, males were removed and
6 females were allowed to oviposit for 20 hours on 5 beans. Females were then transferred
7 back to individual 40mm Petri dishes with a virgin brown male from one of the
8 polygamous populations. We repeated this for at least 20 females per replicate (4) per
9 treatment (4). For each pair, we recorded whether copulation occurred successfully within
10 30 minutes. After copulation with the focal (brown) male ceased, each black female was
11 transferred to a 90mm Petri dish containing 80 beans and allowed to oviposit until death.
12 Eggs laid prior to the second mating were counted (first 20 h), as were the total number
13 of offspring after two successive matings, and offspring phenotype (hybrid or black) was
14 recorded. P2 - the proportion of offspring sired by the second (focal = brown) male was
15 calculated as the proportion of intermediate offspring. The experiment was repeated at
16 generation 32 to increase the sample size. We accounted for this by including a
17 generation factor in the analytical models. Additionally, to ascertain confidence in our co-
18 dominant phenotypic marker, we estimated the repeatability of our paternity estimates by
19 re-measuring P2 blind to the first measurement for 20 randomly chosen females. P2
20 repeatability was calculated following Lessells & Boag (1987), and was high ($r = 0.996$).

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22 STATISTICAL ANALYSES

1 Analyses were performed in R. To avoid pseudoreplication, we performed all analyses on
2 population means. We also used mixed effect models adding replicate as a random effect
3 and obtained similar results, but only the results using the population means are presented
4 here. All traits (damage, longevity, fecundity, lifetime reproductive success and elytra
5 length) were normally distributed (Kolmogorov-Smirnov test, all $P > 0.05$). Additionally,
6 residuals did not deviate significantly from normality (Kolmogorov-Smirnov test, all $P >$
7 0.05), and were not autocorrelated (Durbin-Watson test, all $P > 0.05$), and errors were
8 homoscedastic (Breusch-Pagan test, all $P > 0.05$).

Cost of damage

11 We used a general linear model to test the effect of population size, genetic variability
12 and their interaction on genital damage inflicted by polygamous line males. Female type
13 (monogamous or polygamous) was used as a third factor. We examined whether genital
14 damage evolved with the reintroduction of sexual conflict and sexual selection by testing
15 for an effect of male and female type (from a polygamous or monogamous line) on the
16 amount of damage sustained by a female, using data from four assays ($\text{♀}_M\text{♂}_P$, $\text{♀}_P\text{♂}_P$,
17 $\text{♀}_P\text{♂}_M$ and $\text{♀}_M\text{♂}_M$). We also examined the cost of damage by testing for a negative
18 relationship between damage and longevity or damage and LRS using linear models. We
19 included population size, genetic variability and female type in the model, as well as
20 elytra length as a covariate for LRS and 24h fecundity as a covariate for longevity to
21 account for life history trade offs. To account for the difference in longevity between the
22 populations of the low variability treatment derived from the two monogamous lines, we

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

3
4 *Effect of damage on re-mating, oviposition and sperm competition*

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

14
15 *Effect of population size and genetic variability on male manipulative ability*

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

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

7

8

9 *Results*

10 **TEST FOR INBREEDING DEPRESSION**

11 [There was no evidence for inbreeding depression in small and low variability](#)
12 [populations.](#) We found no significant effect of the interaction between population size
13 and crossing status (within or between replicate crosses) (Fecundity: $F_{7,1}=0.5$ $p=0.480$;
14 longevity: $F_{7,1}=0.04$ $p=0.837$; LRS: $F_{7,1}=0.5$ $p=0.517$). Fecundity and longevity were not
15 significantly different in crosses within or between replicate populations (Fig. 2a:
16 $F_{9,1}=0.9$ $p=0.360$ and Fig. 2b: $F_{8,1}=0.01$ $p=0.909$). Population size also had no effect on
17 these fitness measures, suggesting that inbreeding depression was either absent or was
18 similar across experimental populations (Fig. 2a: $F_{10,1} = 2.8$ $p = 0.125$ and Fig. 2b: $F_{9,1} =$
19 0.4 $p = 0.533$). Lifetime reproductive success (LRS) was also equivalent in the within or
20 between replicate crosses (Fig. 2c; $F_{9,1} = 1.6$ $p = 0.234$), but population size had an effect
21 with small populations having lower LRS than large populations (Fig. 2c; $F_{10,1} = 8.6$ $p =$
22 0.015). When the analysis was restricted to small populations only, fecundity, longevity

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

3
4 **GENITAL DAMAGE EVOLVES IN RESPONSE TO THE REINTRODUCTION**
5 **OF SEXUAL CONFLICT**

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

14
15 **DAMAGE EVOLVES FASTER IN LARGER RATHER THAN MORE DIVERSE**
16 **POPULATIONS**

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

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

4 GENITAL DAMAGE IS COSTLY.

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5 The number of damage points in a female's reproductive tract was negatively associated
 6 with female longevity (Fig. 5, slope = -0.04 days/damage point; $F_{30,1} = 5.5$ p = 0.027,
 7 Table 1). Furthermore, females from the polygamous populations tended to outlive
 8 females from monogamous populations (M: 10.9 days \pm 0.2; P: 11.7 \pm 0.3; $F_{30,1} = 4.6$ p =
 9 0.040, Table 1, Fig. 5). This was also reflected in the LRS results, where females from
 10 polygamous populations had greater LRS (M: 69 offspring \pm 2; P: 78 \pm 2; $F_{26,1} = 8.7$ p =
 11 0.006, Table 2). LRS was also influenced by an interaction between the number of scars
 12 in the female tract and polygamous line population size ($F_{26,1} = 7.0$ p = 0.014, Table 2).
 13 More scaring in females from larger populations resulted in lower LRS, but for females
 14 from smaller populations the association between genital damage and LRS was flat or
 15 even positive (Fig. 6). Note that when we removed one outlier from the analysis (the one
 16 small population with very low LRS and damage), the interaction between the number of
 17 scars and population size remained significant (p = 0.028): in large populations, the
 18 relationship between damage and LRS remained negative but was flat in small
 19 populations.

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21 **EFFECT OF DAMAGE ON RE-MATING, OVIPOSITION AND SPERM**

22 **COMPETITION**

1
2 1 We tested three hypotheses relating to the function of male-induced genital damage
3
4 2 (delayed female re-mating, elevation of female oviposition rate and increased success in
5
6 3 sperm competition) using generalized linear models with damage as an explanatory
7
8 4 variable, elytra length and the number of eggs laid in the first 24h as covariates (for re-
9
10 5 mating and P2 only). We found no significant effect of damage on female re-mating (χ^2_{13}
11
12 6 = 0.80 p = 0.37) or oviposition rate (proportion of offspring produced within the first 24
13
14 7 hours following mating, $F_{1,15} = 1.6$ p = 0.224) and males from more damaging
15
16 8 populations were not more successful at sperm competition ($\chi^2_{13} = 0.32$ p = 0.571).
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22 **EFFECT OF POPULATION SIZE AND GENETIC VARIABILITY ON MALE**

23 **MANIPULATIVE ABILITY (RE-MATING, OVIPOSITION RATE AND SPERM** 24 **COMPETITION)** 25 26 27

28 13 We compared oviposition in the 24 hours after mating across the treatments and found no
29
30 14 effect of population size (Table 3, Fig. 7a), but an effect of genetic variability: males
31
32 15 from lines with basal genetic variability seem to accelerate female oviposition (35% of
33
34 16 offspring are produced during the first 24 hours \pm 2%) compared to males from the
35
36 17 enriched genetic variability lines ($30 \pm 1\%$; $F_{30,1} = 6.1$ p = 0.020, Table 3). In this
37
38 18 analysis, there was also a difference between the two monogamous lines used as testers,
39
40 19 with one having significantly elevated oviposition in the 20 hours after mating (Table 3).
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44 21 There was no effect of population size or standing genetic variability on the index of
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46 22 male manipulation of female re-mating, which implies that all males were equally good at
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1 inducing previously mated females to re-mate and at deterring females from subsequently
 2 re-mating (Table 4, Fig. 7b).

3
 4 Both population size and initial genetic variability influenced male success in sperm
 5 competition. Males from small populations with basal initial genetic variability were the
 6 best competitors (Fig. 7c, large population: $P2 = 0.73 \pm 0.03$; small pop. $P2 = 0.82 \pm$
 7 0.02 , $F_{29,1} = 9.9$ $p = 0.004$; enriched variability population: $P2 = 0.75 \pm 0.03$; basal
 8 variability: $P2 = 0.81 \pm 0.02$, $F_{29,1} = 4.8$ $p = 0.037$; Table 5).

11 *Discussion*

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

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

8 Mean damage levels were not associated with female oviposition rate or propensity to
9 re-mate. Our results thus provide no support for the adaptive harm hypothesis. This is in
10 agreement with previous work: Edvardsson and Tregenza (2005) manipulated copulation
11 duration to elevate female damage (Crudginton 2001) and also found no benefits to
12 harming males via delayed re-mating or increased rate of offspring production.
13 Consequently, and despite theoretical support, there is still no empirical evidence for the
14 adaptive harm hypothesis, whether the mechanism involved is terminal investment or
15 delayed re-mating (Edvardsson and Tregenza 2005; Hosken et al. 2003; Morrow et al.
16 2003), and our results serve to reinforce this. Males from populations with basal genetic
17 variability were better at stimulating female oviposition in the first 24 hours. This could
18 be because favourable gene combinations were broken up by mixing of the two
19 monogamous lines to create the populations with enriched genetic variability, although
20 more work is needed to determine whether epistatic interactions can explain this finding.

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

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1 candidate being sperm competitive ability. However, we found no evidence supporting
2 the idea that males from more damaging populations are more successful in sperm
3 competition. P2 is a composite trait that is likely to be influenced by an unknown number
4 of male derived chemicals and behaviours, so that the prediction of the effect of
5 population size might be less straightforward than for simpler traits such as genital
6 damage. Nevertheless, in the dung fly *S. cynipsea* more damaging males were not more
7 competitive (Teuschl et al. 2007) and our findings are in agreement with results from
8 Edvardsson and Tregenza (2005) who failed to find an effect of damage on P2. In
9 contrast, Hotzy and Arnqvist (2009) found that across 13 geographically distinct
10 populations of *C. maculatus*, male genital armature and the harm males inflict upon
11 females were positively correlated with male success in sperm competition. This
12 discrepancy between *C. maculatus* studies could result from the fact that the balance
13 between the advantage in sperm competition and the cost of harming females is
14 “contingent upon mating system, female life histories and sperm competition regime”
15 (Hotzy and Arnqvist 2009), which may differ when looking within rather than across
16 populations, and certainly could differ across studies. Our results, in conjunction with
17 Edvardsson’s (2005), suggest that the damage inflicted by the spines is not associated
18 with male success in sperm competition, but the damage they inflict did evolve after only
19 30 generations of restored polygamy. Perhaps a direct measure of spininess would be
20 more revealing (e.g. Hotzy & Arnqvist, 2009), but perhaps the spines serve other
21 purposes too, such as anchoring males firmly during copulation (Edvardsson and
22 Tregenza 2005). Using spines as an anchor could be beneficial for males if female

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

3
4 Like the damage inflicted by males which evolved after 30 generations in our
5 polygamous lines, females have also evolved resistance to harm. It is interesting that the
6 number of scars inflicted by males did not differ in females evolving under polygamy or
7 monogamy, but the effects did. Damage inflicted by males could increase female
8 investment in immunocapacity, as has been suggested in other insects (Reinhardt and
9 Siva-Jothy 2007). As a result, the LRS and longevity of females evolving under
10 polygamy were on average higher. Our longevity results are straightforward: increased
11 damage leads to reduced longevity and females from polygamous populations always live
12 longer than monogamous females at any given level of damage. Similarly, LRS of
13 females from monogamous populations always tended to be lower across damage levels.
14 Nevertheless, LRS results are somewhat more complicated in that the damage effect only
15 shows up in an interaction with the population size of the male. When males are from
16 larger populations, more damage equates to lower LRS, but when males are from smaller
17 populations more damage does not reduce LRS. This could reflect a lower cost per scar
18 of male damage in small populations, coupled with lower numbers of scars. Only males
19 from large populations seem to have evolved beyond a threshold where damage becomes
20 costly (in terms of LRS). It is unlikely that the lack of cost in small populations is due to
21 higher female resistance because neither monogamous nor polygamous females suffered
22 reduced LRS when mated to males from small populations. Greater sensitivity to damage
23 in large populations (as suggested by this interaction effect of damage and population size

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

14
15 Despite manipulating population size for 30 generations, we found no evidence for
16 inbreeding depression in smaller populations. This could result from purging of
17 deleterious mutations over the 90 generations of monogamy when population size was
18 relatively small (between 100 and 150 individuals for each of the two monogamous
19 lines), assuming that inbreeding depression is primarily due to the expression of
20 deleterious recessives and not to loss of heterozygosity in *C. maculatus*. Alternatively,
21 population sizes of this order may escape serious inbreeding over this time frame. Recent
22 results suggest that the spectrum of deleterious mutations contains a high proportion of
23 very small effect mutation ($\ll 1\%$) (Estes et al. 2004) such that even large finite

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

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

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

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1 | population size can have a major influence on the outcome of experimental evolution

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2 | (Martin and Hosken 2003a). For example, our experiment suggests that some

3 | evolutionary trajectories might only occur if effective population size is sufficiently large.

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4 | Similarly, Reuter *et al.* (2008) showed that predicted patterns of sexual selection can be

5 | constrained by low effective population size. Ödeen and Florin (2000) further suggested

6 | that low effective population size could constrain the evolution of assortative mating and

7 | thereby limit the power of experimental tests of sympatric or parapatric speciation.

8 | Moreover, sexual selection itself changes effective population size and as the intensity of

9 | selection increases and male mating success becomes more skewed, populations

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10 | experiencing sexual selection will have smaller effective population sizes. Classically,

11 | effective population size is estimated as $(4n_m n_f)/(n_m + n_f)$, where n_m is male number and

12 | n_f is female number (Hartl 2000). If the number of males contributing genes to offspring

13 | is low, then the effective population size is also reduced (assuming that n_f is constant). As

14 | a result, we suggest that attempting to manipulate population size in order to remove this

15 | feature of sexual selection (Snook *et al.* 2009) is only justified where there is an explicit

16 | aim to focus on other effects of selection. Where this is not the case we suggest that

17 | maintaining large census sizes when possible is the best approach, if only because

18 | selection is always more efficient in large populations (Willi *et al.* 2006). In particular, it

19 | can be misleading to focus on maintaining equal effective population sizes if the

20 | increased work load and/or limited space constrain replicates to small census size.

22 | In conclusion, this study is the first attempt at reversing experimental evolution under

23 | sexual conflicts. Reintroducing sexual selection and sexual conflict for 30 generations

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2 1 into previously monogamous populations resulted in the evolution of more harmful
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4 2 males, and female resistance to harm also evolved. Damage was costly for females, in
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6 3 terms of longevity and lifetime reproductive success, but the benefits to males are
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8 4 unclear. It seems unlikely that the aedeagal spines which damage females evolved solely
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10 5 to harm, and further research is needed to assess whether damage is associated with
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12 6 benefits during non-sperm competition forms of male-male competition in these
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14 7 populations. Finally, population size affected the evolutionary responses we detected, but
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16 8 not via an inbreeding effect, suggesting sexual selection was more effective in our larger
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18 9 populations.
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3 **Figure 1.** Diagram of the experimental design. 90 generations of relaxed sexual selection
4 and sexual conflicts (monogamy, in grey) was followed by 30 generations of restored
5 polygamy (in black). In parallel, the two monogamous lines were maintained to be used
6 as testers. At generation 90, the two monogamous lines were crossed. Generations 91 and
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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.

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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 $\sigma_P \times \text{♀}_M$ or line females $\sigma_P \times \text{♀}_P$) with standard errors.

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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) ($\sigma_P \times \text{♀}_M$ 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 ($\sigma_P \times \text{♀}_M$ 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 ($\sigma_P \times \text{♀}_M$ 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 ($\sigma_P \times \text{♀}_M$ 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		

For Review Only

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