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Males evolve to be more harmful under increased sexual conflict intensity in a seed beetle.

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Abbreviated title: Evolution of male copulatory damage

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Abstract

One conspicuous manifestation of sexual conflict is traumatic mating, in which male genitalia damage the female during copulation. The penis of the seed beetle, *Callosobruchus maculatus,* is covered in spines that damage the female reproductive tract. Females kick males ostensibly to shorten these harmful copulations. How these iconic conflict behaviours coevolve in response to sexual conflict intensity can provide insight into the economics of these traits. We examined whether male harm and female resistance coevolved in response to elevated sexual conflict. We quantified copulation behaviour and female reproductive tract damage of individuals from replicated populations evolving for 32 generations under low or high sexual conflict (female- and male-biased treatments, respectively). First, we permitted females ad libitum matings with males from either sex-ratio treatment, recording her tract damage and longevity. Second, we performed a full-factorial cross of matings by males and females from each of the replicate populations, recording mating and kicking duration and reproductive output. We found manipulation of sexual conflict intensity led to the evolution of male harmfulness, but not female resistance to harm. We also demonstrate that female kicking does not respond to sexual conflict intensity, suggesting it does not function to mitigate male harm in this species. Our findings demonstrate the complexities of behavioural and morphological co-evolutionary responses to sexual conflict intensity in an important model species.

Keywords: experimental evolution; sexual selection; genital evolution

Introduction

Reproduction is rife with conflict (Parker 1979, Arnqvist et al. 2005). Sexual traits that enhance male fitness can have harmful side effects for females (Hotzy et al. 2009). Such sexual conflicts favour female responses that reduce male-imposed costs, leading to co-evolutionary arms races between harm and resistance traits (Parker 1979, Arnqvist et al. 2005) that can profoundly affect an individual's fitness and the course of a species' evolution (Arnqvist et al. 2000).

Importantly, due to the predicted coevolution of male harm and female resistance traits, the apparent fitness costs of mating under sexual conflict may be 'weak and transitory' (Rowe et al. 2006). Sexually antagonistic coevolution (SAC) is predicted to be hidden, as any increases in male persistence traits are predicted to be quickly balanced by female resistance traits (Chapman et al. 1996, Arnqvist et al. 2002, Arnqvist et al. 2005). Thus, SAC is typically revealed in interspecific comparisons of male and female sexual traits (Arnqvist et al. 2002, Bergsten et al. 2007, Tatarnic et al. 2010). Costs may also be revealed, within species, when populations are pushed from their equilibria, typically through manipulation of sexual conflict intensity via experimental evolution. A number of such studies have explored female fitness parameters under high and low sexual conflict (typically by manipulating population sex ratio or by enforcing monandry) (Rice 1996, Holland et al. 1998, Crudgington et al. 2005). These studies demonstrate female-borne costs under high sexual conflict environments. For example, Wigby and Chapman (2004) used sex-ratio bias to explore costs of mating in *Drosophila melanogaster*. Females from male-biased (high sexual conflict) populations had greater longevity when housed continuously with wild-type males than did females from female-biased (low sexual conflict) populations. Similarly, in the seed beetle *Callosobruchus maculatus,* males evolved to be more harmful in populations that were released from imposed monandry (Gay et al. 2010). Experimental evolution studies such as these can be an important tool for revealing intraspecific patterns of sexual conflict.

One particularly conspicuous manifestation of sexual conflict is traumatic mating, in which male genitalia damage the female during copulation (for a review, see Lange et al. 2013). Copulatory wounding in insects is now thought to be a pleiotropic effect of selection on male genital traits that increase copulation success or paternity share (Morrow et al. 2003, Edvardsson et al. 2005), rather than male harm being adaptive, per se. Copulatory wounding is typically evidenced by the scarring present on the female's reproductive tract following mating, and has been demonstrated in several taxa (Crudgington et al. 2000, Blanckenhorn et al. 2002, Kamimura 2012, Dougherty et al. 2017). Traumatic mating is expected to be costly to females, due to the direct costs of the damage (wound infection, immunological responses, and enhanced risk of acquiring a sexually transmitted disease), and the indirect costs of investment into behavioural, physiological and morphological counteradaptations to mitigate these direct costs (Arnqvist et al. 2002, Arnqvist et al. 2005, Tatarnic et al. 2010, Dougherty et al. 2017). However, because of the expected co-evolution of male and female traits, the costs to females of traumatic mating may be hidden, and thus difficult to quantify (Arnqvist et al. 1995). Evidence of potential costs of traumatic mating have been demonstrated in a number of comparative phylogenetic studies (Rönn et al. 2007, Tatarnic et al. 2010). For example Rönn et al (2007) showed coevolution between damaging penile spine traits and female reproductive tract thickness among species of seed beetles. Intraspecific studies of sexually antagonistic genital coevolution are somewhat less common (Morrow et al. 2003, Dougherty et al. 2017). Using experimental evolution, the costs of male-female interactions can be quantified in systems that have been manipulated to evolve away from current equilibria, revealing the potential hidden costs of traumatic mating, and sexual conflict, more broadly. Relatively few experimental evolution studies, however, have used population sex-ratio bias to explore the evolution of sexual conflict traits (Wigby et al. 2004, van Lieshout et al. 2014, McNamara et al. 2019), despite it providing an alternative mechanism (compared to artificially-imposing monandry) for altering sexual conflict intensity. Exploring alternative methods for altering sexual conflict intensity is important as it provides insight into the generality of the patterns previously observed regarding the evolution of traumatic mating (Crudgington et al. 2010, Cayetano et al. 2011, Gay et al. 2011).

The polyandrous cowpea seed beetle, *Callosobruchus maculatus,* is an ideal model species to explore the costs of traumatic mating. The male penis (aedeagus) is covered in sharp spines that penetrate the female reproductive tract during mating (Crudgington et al. 2000). A comparison of different populations of *C. maculatus* demonstrated that males with longer spines inflict more damage on females, yet are more successful in sperm competition (Hotzy et al. 2009). This relationship may be due to the transfer of seminal proteins (which affect receptivity and reproductive output in other species) directly into the haemolymph through the wound sites (Hotzy et al. 2012). Mating duration increases the degree of damage incurred by the female (Crudgington et al. 2000), creating a potential conflict over optimal mating duration in this species. Females begin to kick males approximately halfway through copulation, a behaviour that has been interpreted as an attempt to dislodge mating males. However, recent evidence demonstrates that the onset of male-imposed genital damage and female kicking is temporally separated (Dougherty et al. 2017). Despite the damage caused during mating, evidence to suggest that multiple mating or copulation duration negatively impact female fitness, or that the female kicking behaviour is an adaptive behaviour to reduce male harm is absent. For example, when females are prevented from kicking (via leg ablation) copulations increase in duration and more reproductive tract damage is incurred (Crudgington et al. 2000). However, longer matings are also associated with the transfer of larger ejaculates (van Lieshout et al. 2014), which confer direct benefits to females, increasing female fitness (Edvardsson et al. 2006). Furthermore, there is no clear advantage to males when females are unable to kick; non-kicking females do not increase oviposition, or delay re-mating (Edvardsson et al. 2005). Thus, despite the conspicuous presence of kicking, there appears to be no apparent conflict over mating duration (Edvardsson et al. 2006), nor any clear fitness benefit to females of kicking. Despite this, female kicking responds plastically to male quality and socio-sexual environment: females kick less when mating with previously-mated males, and kick more in the presence of multiple rival males (Wilson et al. 2014). The costs and benefits of female kicking are certainly complex, and an understanding of how the behaviour evolves in response to sexual conflict intensity may provide insight into this trait. Although there have been multiple phenotypic studies on the costs and benefits of female kicking behaviour, there is very limited evidence of how this apparently sexually antagonistic trait evolves in response to sexual conflict intensity (but, see van Lieshout et al. 2014).

To examine male and female evolutionary responses to sexual conflict intensity, we examined the mating behaviours and resulting female reproductive tract damage of individuals from populations evolving under either low or high conflict (female-biased and male-biased treatments, respectively). After 32 generations of experimental evolution, we performed a full-factorial cross of matings by males and females from each of 5 replicate populations from two sex ratio treatments. We recorded mating behaviour and female reproductive output. In a separate experiment, we assessed the amount of reproductive tract damage incurred by females from both sex-ratio treatments when they were provided with ad libitum mating opportunities with males from either the same or opposite sex-ratio treatments. Previously we reported evolutionary divergence in immune function and mating behaviour among these populations (van Lieshout et al. 2014). In this study, we predicted that co-evolution in male persistence and female resistance would generate differences in mating behaviour, reproductive output and female reproductive tract damage between females mated to males with which they had co-evolved, compared to females mated to males that had evolved under a different sex-ratio environment. Specifically, we predicted that males from high sexual conflict treatments should inflict greater damage on females during mating, and that females from these high-conflict treatments should show evidence of counter-adaptation, demonstrated by a reduced susceptibility to reproductive tract damage.

Materials and methods

Experimental evolution populations

Experimental evolution populations were founded using beetles sourced from a large outbred population (hereafter referred to as the stock population) (van Lieshout et al. 2013) that originated from a stock culture held by CSIRO (Canberra, Australia). Evolution treatments manipulated the sex ratio of the population (80:40 or 40:80 males:females). Virgin individuals were haphazardly assigned to one of five replicate female-biased or five replicate male-biased populations. Female-biased populations received 200g of mung beans (*Vigna radiata*) whereas male-biased populations received 100g. We did this to avoid differences in larval competition between treatments, based on the assumption that 80 females should produce approximately twice as many offspring as 40 females. Populations were maintained at 30˚C under 12h:12h light:dark. Offspring were obtained by isolating 300 beans into 1.5mL microtubes 24h following the first observed adult emergences in each population. Once sufficient virgin adults had emerged, typically after two days, new sex-biased populations were composed (as above). The five replicate populations within each evolution treatment were maintained for 32 generations. All populations were kept under relaxed selection (equal sex ratio) for one generation prior to experimentation to reduce non-genetic parental effects (the common garden populations).

To obtain virgins for mating trials, beans from the common garden populations were isolated into pinhole-ventilated 1.5 mL microtubes and checked twice daily for emerged adults. Following emergence, virgin beetles were isolated individually into 1.5 mL microtubes. All focal individuals were weighed prior to experimentation, and their post-emergence age recorded.

Does sexual conflict intensity affect female genital damage, longevity and reproductive output?

To examine the effect that evolution under sex-ratio bias has on the harm imposed and received by females and their subsequent fitness, single females from both sex-ratio treatments were mated to multiple males derived from either male- or female-biased treatments. Specifically, approximately 10 virgin common garden females from each of the 5 replicate populations of both male- and female-biased treatments were individually housed and provided with 4 virgin males (and allowed ad libitum matings). Approximately half of the 10 females in each replicate population were mated to males derived from male-biased treatments, and half with those derived from female-biased treatments. These male groups were haphazardly chosen from a pool of all the female-biased replicate populations or all the male-biased replicate populations (ie. they were not common-garden). Females and the four males were placed in 60ml vials with 9g of mung beans and were inspected each day for male and female deaths. Dead males were replaced with a male from the same pool of male-biased or female-biased replicate populations. Females were either 1 or 2 days old at the commencement of the trial, and this variation was accounted for in statistical analyses. Dead females were recorded and immediately frozen at -20°C for later dissection. The number of eggs laid by the female (hereafter referred to as 'fecundity') were counted immediately following the female's death, and the proportion of offspring that emerged counted 40 days later (hereafter referred to as 'reproductive success') were recorded. The number of days survived in the mating chamber was recorded as 'longevity'. Frozen females were dissected in a small quantity of insect ringer. The female's bursa copulatrix was removed, cut along the midline and spread onto a glass slide. The tract was then photographed at x400 and a digital image recorded. The areas of melanisation (sites of wound repair) were measured using ImageJ (version 1.48).

Does sexual conflict intensity affect kicking behaviour and reproductive output?

To assess the effect of evolution under sex-ratio bias on mating behaviour and female reproductive output, a fully factorial mating design was used. Here, virgin common garden males and females from every population replicate of both male- and female-biased treatments were mated to a male or a female from every replicate of both male- and female-biased populations, creating 100 population replicate crosses. Three male and female pairs from each population replicate cross were mated, for a total of 300 matings. All matings took place in 1.5mL microtubes. For each mating, the duration of female kicking and the duration of the total copulation were recorded. Mated females were provided with 9g of mung beans and placed in a 60ml vial. The number of eggs laid by the female over her lifespan (counted immediately following her death) and the proportion of offspring that emerged, 'reproductive success' (counted 40 days later) were recorded.

Statistics

All analyses were conducted in JMP (v 13.0)(SAS Institute Inc. 2016). For the experiment examining the effect of evolution under male and female sex-ratio bias on female scarring and reproductive output, we first summarized variation in potentially correlated dependent variables of female scarring damage (number of wounds and area of damage), reproductive success and longevity with a Principal Components Analysis (PCA). We expressed female harm received as rates, to account for the different ages (1 or 2 days old) at which females entered the trials. For both the number of wound sites and the area of damage, we divided these measures by the number of days the female survived in the trial. The PCA was based on a correlation matrix (given that our variables had different units of measurements). The analysis returned two axes of variation (PCs) with eigenvalues >1.0. These two principal components were then used as the dependent variable in two different mixed effect models. Here, female sex-ratio population replicate was nested within female sex-ratio treatment as a random effect. Male sex-ratio treatment, female body weight and an interaction between male and female sex-ratio treatments were also included as fixed effects. However, non-significant interactions were removed from final models (Engqvist 2005).

For the experiment examining the effect of male and female sex-ratio bias on female genital damage and female reproductive output, one of the 91 females assayed (a female from a male-biased population mated to males from a male-biased treatment) did not lay any eggs; this female was excluded from analyses. Due to an error, for one female-biased population replicate, data on scarring was collected for females that mated to male-biased males only. Thus, these data are also not included in our final analysis. Final samples sizes are shown in Table 1.

For the experiment examining the effect of male and female sex-ratio bias on kicking and copulation duration and female reproductive output, we obtained kicking and copulation data for 296 females. However, due to logistical constraints, we obtained copulation data *and* female reproductive output data for a subset of these females (n = 211). We chose to analyse data only for females with complete data sets. We did this so that we could employ a more powerful multivariate analysis (PCA), and because separate analysis of the larger copulation and kicking datasets revealed patterns identical in direction and significance to our multivariate analysis of the subset of data. We first summarized variation in four potentially correlated dependent variables (copulation duration, female kicking duration, and female fecundity and reproductive success) with a PCA. The analysis returned two axes of variation (PCs) with eigenvalues >1.0. These two principal components were then used as the dependent variable in two different mixed effect models. Here, female sex-ratio population replicate was nested within female sex-ratio treatment as a random effect. A second random effect was generated for male sex-ratio population replicate, nested with male sex-ratio treatment. Male and female body weight and an interaction between male and female sex-ratio treatment were also included as fixed effects. However, non-significant interactions were removed from final models (Engqvist 2005).

Results

Does sexual conflict intensity affect female genital damage, longevity and reproductive output?

The PCA returned two axes of variation (PCs) with eigenvalues >1.0, that collectively explained 68.78 per cent of the variation in the recorded traits (Table I). PC1 was positively weighted by variables describing the extent of reproductive tract damage incurred by the female and negatively by female longevity. PC2 was weighted negatively by reproductive success, and to a lesser extent, positively by fecundity (Table I).

PC1, which describes female reproductive tract damage and survival, was affected by the sex-ratio treatment of the males with which she mated ($F_{1,84}$ = 4.53, β = -0.32 (standard error = 0.15), P = 0.04; Fig. 1); the principal component loadings suggest that this was due to males from male-biased sex-ratio treatments being more harmful to females, increasing both the number and area of wounds incurred by females, and also reducing female longevity. PC1 was not, however, affected by the sex ratio treatment of the female herself ($F_{1,8}$ = 0.60, β = 0.14 (0.18), P = 0.46), suggesting that female susceptibility to harm has not coevolved with male harmfulness. Finally, PC1 was negatively affected by female weight ($F_{1,83}$ = 1.67, β = -0.95 (0.30); P = 0.002), suggesting that heavier females incur less damage and have greater longevity. A non-significant interaction between female and male sex-ratio treatment was removed from the final model ($F_{1,82}$ = 0.12, P = 0.73).

PC2, which describes female reproductive output, was not affected by the sex ratio population of the female ($F_{1,7}$ = 3.21, β = 0.27 (0.14), P = 0.11), or male ($F_{1,83}$ = 1.48, β = -0.14 (0.11), P = 0.23), but was positively correlated with female weight ($F_{1,84}$ = 4.19, β = 0.46 (0.23); P = 0.04). A non-significant interaction between female and male sex-ratio treatment was removed from the final model ($F_{1,81}$ = 1.69, P = 0.20).

Does sexual conflict intensity affect kicking behaviour and reproductive output?

In total, 211 females were assayed for mating behaviour and female reproductive output. Seventeen females did not lay eggs and were excluded from analyses. Final samples sizes for each treatment are shown in Table 2.

The PCA returned two axes of variation (PCs) with eigenvalues >1.0, that collectively explained 79.24 per cent of the variation in the recorded traits (Table 2). PC1 was weighted most strongly by variables describing mating (male kicking and copulation duration), and PC2 was weighted most strongly by variables describing female reproductive output (fecundity and reproductive success) (Table 2).

PC1, which describes copulation traits, was not affected by the sex ratio treatment from which the female was drawn ($F_{1,8}$ = 0.41, β = 0.08 (0.93), P = 0.54), the sex ratio treatment from which the male was drawn ($F_{1,8}$ = 0.06, β = 0.03 (0.11), P = 0.81), or by female weight ($F_{1,186}$ = 0.36, β = -0.09 (0.15), P = 0.55), and male weight ($F_{1,171}$ = 0.99, β = -0.17 (0.17), P = 0.32). A non-significant interaction between female and male sex-ratio treatment was removed from the final model ($F_{1,76}$ = 2.81, P = 0.09).

PC2, which describes female reproductive output, was not affected by the sex ratio treatment of the female ($F_{1,8}$ = 0.38, β = 0.08 (0.19), P = 0.55), or male ($F_{1,8}$ = 0.37, β = 0.09 (0.14), P= 0.56), or by female weight ($F_{1,85}$ = 0.24, β = 0.05 (0.11), P = 0.63). PC2, however, was affected by male weight ($F_{1,84}$ = 10.99, β = 0.41 (0.12); P = 0.001). A non-significant interaction between female and male sex-ratio treatment was removed from the final model ($F_{1,73}$ = 1.48, P = 0.22).

Discussion

Evidence of the costs of sexual conflict can be hidden by coevolution between male and female physiology, behaviour and morphology. We allowed males and females that had evolved under high and low intensity of sexual conflict to mate within and between their populations. We demonstrate rapid evolution in male harmfulness in response to elevated sexual conflict intensity under male-biased treatments. Despite predictions, female resistance to harm did not appear to coevolve with male harmfulness; we found no divergence in female susceptibility to reproductive tract damage when mating with males from within or between their sex-ratio treatments. Yet, despite incurring greater harm and a reduced survival, females mated to males from elevated sexual conflict backgrounds had a comparable fitness to females mated to males from reduced conflict backgrounds. Thus, despite their increased reproductive tract damage, females were able to mitigate the costs of traumatic mating. This was not achieved, however, by female kicking behaviour, as we found no evidence that copulation or kicking duration respond to sexual conflict intensity, casting further doubt on the evolutionary significance of kicking as a conflict trait.

Our findings are consistent with a previous experimental evolution study in *C. maculatus* that manipulated conflict intensity by removing artificially-imposed monandry (Gay et al. 2010). As in our study, elevated sexual conflict resulted in the evolution of more harmful males, as quantified by degree of copulatory damage incurred. Similar to our study, they also found that although male harmfulness evolved, female resistance to male harm did not, as females experienced comparable reproductive tract damage when mating with males from either treatment (Gay et al. 2010). Our findings are also consistent with a number of experimental evolution studies in other species that found direct evidence of increased male harm or indirect evidence of female-borne fitness costs when populations evolve under elevated sexual conflict (Rice 1996, Holland et al. 1999, Martin et al. 2003). We found divergence in the amount of copulatory wounding in females when females were permitted ad libitum matings. As we were not able to measure the number of matings in these experimental trials, it is not clear if the increased damage incurred by females mated to males derived from male-biased populations was due to the increased genital spine length under elevated sexual conflict, as previously demonstrated in *C. maculatus* (Cayetano et al. 2011), or due to a potentially higher copulation frequency of males from male-biased sex-ratio treatments. Although an accurate measure of male and female mating rates within our experimental evolution populations would be largely impossible to acquire, an indirect estimate of male and female mating frequency would be useful in providing a mechanistic explanation for the increased damage incurred by females under elevated sexual conflict.

Nevertheless, the apparent absence of female resistance traits in preventing reproductive tract damage in these experiments is not unexpected in this species; the change in genital spine length observed in *C. maculatus* populations evolving under relaxed sexual conflict was not matched by a change in female reproductive tract thickness (Cayetano et al. 2011), as predicted by a comparative analysis of this genus (Rönn et al. 2007). In general, under sexually antagonistic coevolution, there may be an evolutionary lag in female responses to male adaptations, and the sex currently ‘winning’ the evolutionary contest may change through time (Kokko et al. 2014). Thus, a detectable female response may be yet to evolve, or males may be currently enjoying an advantage. However, it remains possible that the absence of female resistance to reproductive tract damage captured in these trials, reflects a real failure of females to coevolve in preventing male harm using our particular experimental manipulation of sexual conflict intensity, particularly in light of the apparently limited fitness costs incurred by females in these traumatic matings.

One hypothesis regarding the evolution of male traumatic mating is that the costs are a pleiotropic effect of male-male competition, rather than an adaptive mechanism for males to reduce female remating and/or increase immediate reproductive output (Lange et al. 2013). There is support for this in *C. maculatus*: across 13 geographically isolated populations, male genital armature and the male-imposed damage received by females was positively correlated with male sperm competitiveness (Hotzy et al. 2009). Given its rapid evolution, reproductive tract damage must benefit males. Yet a previous study, using the same experimental evolution populations, found no effect of sexual conflict intensity on male success in sperm competition (McNamara et al. 2016). Whilst this is certainly not evidence to reject the ‘collateral harm,’ hypothesis, we find it noteworthy that there is no clear relationship between harmfulness and sperm competitiveness in the same experimental populations. Thus, the benefits to males of inflicting harm are not established in this instance. It is possible that, as females produce eggs at a higher rate when mated to males from an elevated conflict background (given females had reduced survival, but comparable reproductive output), males may benefit by maximising their paternity share before females remate or die.

We found that males and females did not display different mating or kicking behaviours when copulating with partners from the same or different sexual conflict backgrounds, providing no evidence that female kicking behaviour evolves to mitigate the potential costs of increased sexual conflict in this species. This lack of evolutionary response is in contrast to phenotypic responses that demonstrate how socio-sexual environments, in particular the risk of sperm competition, alters females kicking behaviour in this species (van Lieshout et al. 2014, Wilson et al. 2014). Ultimately, however, several phenotypic studies have questioned the efficacy of female kicking, as it does not alter copulation duration (Wilson et al. 2014), has an equivocal effect on multiple female fitness parameters (Crudgington et al. 2000, Edvardsson et al. 2005, Edvardsson et al. 2006, van Lieshout et al. 2014) and, most compellingly, micro-CT scanning of couples *in copula* have shown that genital trauma occurs before females commence kicking (Dougherty et al. 2017), demonstrating that kicking is not efficacious in preventing female genital damage in this species. The fact that kicking does not appear to respond to sexual conflict intensity, particularly in light of the concurrent evolution in male harmfulness, further clouds its role in modulating copulation duration and genital damage in traumatic matings.

Our data demonstrate clear costs in terms of longevity for females mating under elevated sexual conflict. Yet we found no evidence of an impact of sexual conflict intensity on female lifetime reproductive success in either of our experiments (in which females were either singly-mated or given ad libitum matings), suggesting a limited net impact of sexual conflict intensity on female fitness. Theoretically, reduction in the intensity of sexual conflict should cause harmful male traits to be opposed by selection, leading to enhanced female, and thereby population fitness (Kokko and Brooks 2003; Rowe and Day 2006). Ultimately, there is mixed empirical support for this idea; experimental evolution studies in a range of taxa, have found both reduced (Holland et al. 1999, Martin et al. 2004) and increased population fitness under elevated sexual selection/conflict (Crudgington et al. 2005). Indeed, our results are in contrast to previous work on *C. maculatus* (Gay et al. 2010) which demonstrated that females from polygamous populations (elevated sexual conflict) lived longer and had greater lifetime reproductive output than monogamous (reduced sexual conflict) populations. This was despite there being no differences in female resistance to damage between populations. The authors suggest that, under polygamy, females may evolve increased immunocompetence to mitigate damage (Gay et al. 2010). We, however, have not found support for this, as immunocompetence of males and females is reduced rather than increased under elevated sexual conflict in these same experimental populations (van Lieshout et al. 2014). Why such different patterns should emerge between studies that differ in the means of manipulating sexual conflict intensity is not clear, although it highlights the importance of examining the impact of sexual conflict in a variety of contexts. While the mechanism by which females limit the impact of male harm on their fitness remains unclear, evidence from these experimental populations suggests that it is not through female kicking behaviour, nor through increased immunocompetence (van Lieshout et al. 2014). One possibility is that adaptive investment in reproductive tract thickness by females may be a means via which females under elevated sexual conflict can incur greater harm without impact on their fitness.

In conclusion, our results provide evidence that sexual conflict can be experimentally manipulated by altering the adult sex-ratio in this species. We find novel evidence of evolution in male harmfulness demonstrated by an increase in female copulatory damage and a reduction in longevity. We find, however, no apparent female coevolution in resistance to reproductive tract damage in these populations. The absence of evolution in female kicking behaviour, especially in light of the evolution of male harmfulness, casts further doubt on its role in mitigating male-imposed copulatory damage. While copulatory damage was costly for females in terms of reduced longevity, the benefits to males remain unclear; females did not increase their reproductive output, while previous research on these populations demonstrates that these more harmful male-biased males are not more sperm competitive. Quantification of the benefits to males of imposing damage and understanding how females who received greater damage were able to mitigate these costs should be the focus of further investigation.

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Data Accessibility Statement

Data will be deposited in Dryad upon acceptance.

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Figure legend

Figure 1. Mean ± standard error Principal Component Scores for PC1 (weighted positively by variables describing female reproductive tract damage and negatively by female longevity) when females from female-biased (FB) and male-biased (MB) sex-ratio treatments were mated to males from female-biased (FB) and male-biased (MB) sex-ratio treatments.

Figure 1.

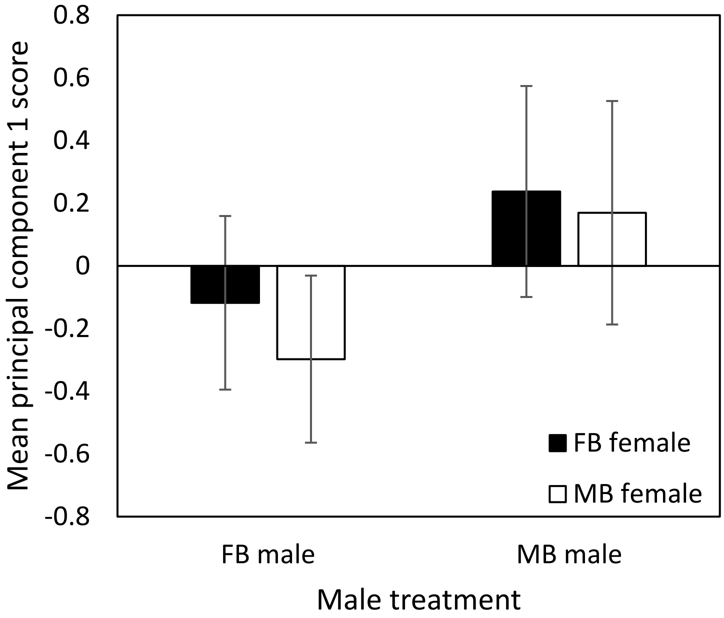
Mean principal component 1 score
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
■ FB female
□ MB female
FB male
MB male
Male treatment

Table I. Summary of fit and loadings of PCA and mean ± standard errors (SE) for reproductive tract damage, reproductive output and longevity for females mated to males from the same or different sex-ratio treatments.

	Mean ± SE					
	MB♀ : MB ♂	MB♀ : FB ♂	FB ♀ : MB ♂	FB ♀ : FB ♂	PC1	PC2
Eigenvalue					2.18	1.26
% variance explained					43.63	25.15
n	21	24	25	20		
Scar number	22.71 ± 3.18	20.95 ± 2.98	29.00 ± 3.21	21.70 ± 2.81	0.85	0.40
Scar area (mm^2) $^{-3}$	5.40 ± 0.91	4.95 ± 0.83	7.07 ± 0.84	5.71 ± 0.96	0.87	0.34
Fecundity	61.19 ± 4.13	60.04 ± 3.56	66.56 ± 2.96	61.65 ± 3.54	-0.52	0.64
Reproductive success	0.70 ± 0.04	0.73 ± 0.02	0.57 ± 0.06	0.62 ± 0.06	0.14	-0.68
Longevity (days)	4.48 ± 0.27	4.96 ± 0.19	4.84 ± 0.22	4.80 ± 0.19	-0.63	0.32

Table 2. Summary of fit and loadings of PCA and mean ± standard errors (SE) for kicking and copulation damage, and reproductive output for females mated to males from the same or different sex-ratio treatments.

	Mean ± SE					
	MB♀ : MB♂	MB♀ : FB♂	FB ♀ : MB♂	FB ♀ : FB♂	PC1	PC2
Eigenvalue					1.94	1.22
% variance explained					48.69	30.56
n	45	46	49	54		
Mating duration (s)	603.09 ± 37.29	691.33 ± 69.16	742.92 ± 78.31	680.48 ± 59.54	0.94	-0.21
Kicking duration (s)	269.53 ± 39.12	402.72 ± 69.90	410.94 ± 77.66	345.94 ± 50.60	0.96	-0.17
Fecundity	61.51 ± 3.58	66.41 ± 2.83	65.71 ± 3.20	67.85 ± 2.81	0.16	0.80
Reproductive success	0.55 ± 0.04	0.63 ± 0.03	0.64 ± 0.03	0.60 ± 0.03	0.33	0.71