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Sexual conflict and correlated evolution between male persistence and female resistance traits in the seed beetle *Callosobruchus maculatus*

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Traumatic mating (or copulatory wounding) is an extreme form of sexual conflict whereby male genitalia physically harm females during mating. In such species females are expected to evolve counter-adaptations to reduce male-induced harm. Importantly, female counter-adaptations may include both genital and non-genital traits. In this study, we examine evolutionary associations between harmful male genital morphology and female reproductive tract morphology and immune function across 13 populations of the seed beetle *Callosobruchus maculatus*. We detected positive correlated evolution between the injuriousness of male genitalia and putative female resistance adaptations across populations. Moreover, we found evidence for a negative relationship between female immunity and population productivity, which suggests that investment in female resistance may be costly due to the resource trade-offs that are predicted between immunity and reproduction. Finally, the degree of female tract scarring (harm to females) was greater in those populations with both longer aedeagal spines and a thinner female tract lining. Our results are thus consistent with a sexual arms race, which is only apparent when both male and female traits are taken into account. Importantly, our study provides rare evidence for sexually antagonistic coevolution of male and female traits at the within-species level.

1. Introduction

Males and females may differ in their evolutionary interests, leading to sexual conflict over the optimum expression of phenotypic or genotypic traits [1,2]. One of the most extreme examples of sexual conflict is traumatic mating (also referred to as copulatory wounding), whereby the male reproductive anatomy damages the female during mating [3]. This is evidenced in many species by visible scarring of the female tract following mating (e.g. [4–7]). The evolutionary advantage of such male harm has been the subject of considerable debate. Males could benefit from harming females directly (the adaptive harm hypothesis) if injury causes females to increase their short-term investment in reproduction [8], or reduces their likelihood of remating [9]. However, empirical studies have revealed little support for this theory (e.g. [10–12]), and it is now thought that trauma during mating is a pleiotropic by-product of selection on genital traits that increase a male's mating or fertilization success [10,13,14].

Regardless of its evolutionary advantage to males, traumatic mating may negatively impact female fitness (e.g. [4,5,15]). Thus, as with other forms of sexual conflict, the evolution of harmful male traits is expected to drive the coevolution of defensive female traits which minimize harm [2]. The result of this

process is a positive correlation between the degree of elaboration of harmful male traits and defensive female traits. Such a correlation has been frequently demonstrated using interspecific comparisons (e.g. [16–20]), but has only rarely been unveiled at the intraspecific level (e.g. [21–24]). Detection of correlated evolution at the species level is important for two reasons. First, different processes may influence the outcome of sexually antagonistic coevolution at the within-species and between-species levels [22]. Second, micro-evolution occurs at the population level, and so intraspecific studies are needed in order to link micro-evolutionary processes to species-wide outcomes [22]. It is important to note that female resistance should generally not be limited to single traits. Theory instead suggests that resistance in most cases should be built by a suite of morphological, physiological and behavioural adaptations acting together to reduce harm [2]. In these cases multivariate analyses, taking multiple male and female traits into account, are most appropriate if we are to detect signs of correlated evolution. This approach may be especially important in intraspecific studies, for which the phenotypic differences in any single trait are typically smaller than in interspecific comparisons.

The seed beetle *Callosobruchus maculatus* (Chrysomelidae; Bruchinae) is a model species for the study of sexual conflict [25]. The male intromittent organ (aedeagus) is covered with hundreds of sharp spines that penetrate and damage the walls of the female reproductive tract during mating [4]. Males with longer aedeagal spines have increased competitive fertilization success [13], an effect which seems to be mediated via the passage of male seminal fluid compounds into the female haemolymph, though it remains unclear whether such passage occurs via wound sites [14]. There is some evidence that multiple mating reduces female fitness in *C. maculatus* ([4,26,27], but see [25]), and one potential female counter-adaptation to traumatic mating is a thickened reproductive tract lining [19]. This is supported by the fact that there is a strong correlation between the degree of elaboration of aedeagal spines and the thickness of the reproductive tract lining across seed beetle species [19]. However, this relationship between male and female traits has not been shown within any seed beetle species, nor has it been shown that variation in female tract thickness influences the outcome of traumatic mating in *C. maculatus*. Females may need physiological as well as morphological defences against copulatory wounding, if this wounding for example increases the likelihood of microbial infection (e.g. [7,28]). In *C. maculatus*, copulatory damage induces a rapid immune response by females to prevent infection, resulting in the melanisation and plugging of damaged areas within 24 h of mating [4,26]. However, it is not clear how important female immunity is in mitigating male harm in this species.

We examine covariation between three putative aspects of female counter-adaptation to male-induced harm (one measure of female reproductive tract morphology and two measures of female immune function) and male genital morphology and harmfulness across 13 laboratory populations of *C. maculatus*. These populations were collected in different parts of the distributional range and have since been evolving independently in the laboratory for more than a decade (which corresponds to more than 100 generations). Males vary across populations in their average aedeagal spine length, and also in the amount of copulatory damage their genitalia inflict on common standard reference females [13]. Previous work with these populations has also demonstrated covariation among populations in aedeagal spine length and male competitive fertilization success [13]. Therefore, given that there is substantial variation in harmful male traits present across these populations, we expect to see significant between-population variation in female resistance traits as well.

We use micro-CT X-Ray scanning to measure the amount of tissue in the female reproductive tract in three dimensions along the entire region contacted by the male aedeagal spines. This approach allows us to control for any differences in the shape of the tract which may be missed when using a small number of histological slices. If the lining of the reproductive tract protects against traumatic mating, then we expect to see a positive correlation between tract thickness and male persistence. We took two measures of female immune function: phenoloxidase (PO) level and lytic activity. Phenoloxidase is an important component of the insect immune system, performing a key role in wound repair and the encapsulation and melanisation of foreign objects such as microbial cells [29]. The lytic activity measures the efficacy of antibacterial peptides in the haemolymph. Both of these immune traits are predicted to increase as the level of copulatory damage increases.

We use a multivariate statistical approach to test for a positive correlation between these three female resistance traits (female tract volume, female PO level and female lytic activity) and three male traits that collectively describe male persistence (see below). We then use multivariate models to test whether the relative level of female resistance and male persistence [30] in a population influences the degree of harm females receive during mating. Showing such an effect would support the hypothesis that sexual conflict, rather than some other process, has driven correlated evolution between males and females. Here, we use the area of melanised scar tissue in the female reproductive tract lining, following mating with males from their own population, as a proxy for female harm [4,26]. Finally, by using previously measured estimates of population-level growth rate (which is to a large extent determined by female lifetime fecundity) for the 13 populations, we examine whether investment in resistance traits significantly influences this measure of population fitness.

2. Methods

(a) Populations

Beetles from 13 established laboratory populations were used: Benin, Brazil/USA, California, Mali, Nigeria/Lossa, Nigeria/OYO, Nigeria/Zaire, Oman, Uganda, Upper Volta, IITA, South India and Yemen. These populations were sourced from the wild and were brought into the laboratory at different times. They are all laboratory-adapted, having been kept in controlled conditions for at least 10 years, and have been used in several intraspecific studies (e.g. [13,31–35]). All beetles used were reared on black-eyed beans (*Vigna unguiculata*) and maintained under constant conditions at $30 \pm 0.5^{\circ}$ and $60 \pm 10\%$ RH with a 12 : 12 h L : D cycle. We stress that all data presented here were gathered under common garden conditions, such that significant difference between populations must represent genetic differences. Further, previous studies have demonstrated a general lack of phylogenetic signal in variation in reproductive phenotypes across these populations [32,35]. Thus, we interpret phenotypic correlations across populations as representing correlated evolution.

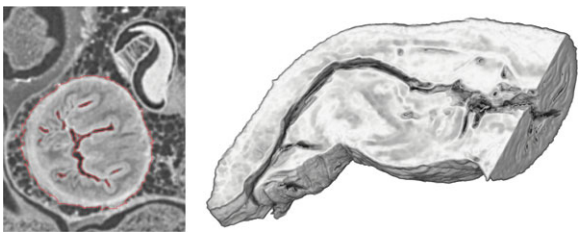

Figure 1. Female reproductive tract morphology in *Callosobruchus maculatus*. Panel (*a*) shows a representative CT slice image of a female tract outlined in red, showing the thick walls and dark lumen. Panel (*b*) shows a three-dimensional volume rendering of a female tract viewed laterally, created by combining multiple two-dimensional slices (note that a 3d slice has been used to virtually cut the tract in half). In both cases brightness represents the degree of tissue staining.

Here, we test for a multivariate association between three traits in females (reproductive tract volume, phenoloxidase activity, lytic activity) and three traits in males (length of ventral genital spines, length of dorsal genital spines, genital injuriousness). In addition, we assess whether these traits relate to copulatory wounding and population fitness.

(b) Female reproductive tract volume

One day old virgin females from each of the 13 populations were euthanized and then weighed to the nearest 0.01 mg using an electronic balance (Sartorius Genius ME 235P-OCE). The abdomen was then removed and stored in phosphate-buffered formalin in order to fix tissues. Samples were stained in 1% Iodine in 100% ethanol (I2E: [36]) for 24 h. After staining samples were stored in 100% ethanol at room temperature, and scanned between 1 and 12 weeks after staining. The order of scanning was randomized with respect to the population of origin. Samples were scanned using a ZEISS Xradia Versa 520 X-Ray microscope located at the University of Western Australia Centre for Microscopy, Characterisation and Analysis. All samples were scanned using identical parameters (for more detail see the electronic supplementary material, methods), resulting in a voxel size of 2.35 µm. Scan data were reconstructed using the XRADIA reconstructor package (XRADIA Inc). A total of 60 females were scanned across the 13 populations (4–5 females per population).

The micro-CT data were analysed in two and three dimensions using Avizo 6 (FEI software). All analyses were performed blind to the population origin of each sample. We manually selected the area contacted by the spines of the aedeagus during mating ([19] figure 1; electronic supplementary material, figure S1). For full details see the electronic supplementary material, methods. Once the entire region of interest was selected, the number of voxels selected across all slices was then determined, excluding the tract lumen, and converted into μm^3 to give a measure of the total volume of tract tissue (figure 1). We note that tract volume is thus a measure of overall investment in reproductive tract tissue, taking into account both the thickness of the tract but also the number of folds. A single observer performed the manual selection of the micro-CT data for all females. To determine the repeatability of this manual selection, the tracts of ten females were selected a second time using the same scan data but blind to the original selections. Repeatability was determined using analysis of variance [37] and found to be very high ($r = 0.993$).

(c) Female immune function

At least 24 females from each of the populations ($N = 323$) were used for immune function assays. Females were first weighed to the nearest 0.01 mg using an electronic balance (Sartorius Genius ME 235P-OCE). Mean female weight per population was then used as our measure of female size. Females were then gently crushed in a microtube in 20 µl of phosphate buffered solution (Amresco E404) (PBS). Samples were centrifuged at 0°C for 10 min at 17G, the supernatant was removed and then frozen at −80°C.

Phenoloxidase (hence, PO) level was measured using a method modified from [38]. For each sample, 100 µl PBS was added to 10 µl of thawed haemolymph sample, and 100 µl was then pipetted into a 96-well microtitre plate. After adding 90 µl 8 mM dopamine hydrochloride (Sigma-Aldrich H8502), plates were loaded into a Tecan Infinite M200 plate reader (Tecan Trading AG, Switzerland), where absorbance at 492 nm was measured every 5 min for 30 min. This period was determined previously to be in the linear phase of the reaction. PO activity (Vmax) was measured as the maximum linear rate of substrate conversion.

To assay antibacterial activity, lytic zone assays were conducted. Agar plates were made with 9 ml of 1% agar in which 5 mg ml^{-1} of *Micrococcus luteus* (Sigma-Aldrich M3770) and 15 µg ml^{-1} streptomycin sulfate (Sigma-Aldrich S6501) was suspended [39]. Using a sterilized Pasteur pipette, wells were punched in the agar. Into these wells, 2 µl of undiluted, thawed haemolymph sample were pipetted and incubated at 33°C for 24 h. Zones of inhibition around each well were imaged under 10× magnification and measured using ImageJ (v. 1.48), with the area measured in pixels.

(d) Male traits

Data on the average size of male aedeagal spines across the 13 populations were taken from [13]. The spines are positioned on both the ventral and dorsal surface of the aedeagus. Spine length was defined as the average length of the five longest spines for each male. Average spine length was then calculated for each population ($N = 8–12$ males per population). Hotzy & Arnqvist [13] also mated males from each population to females from a common standard reference population, and the degree of tract scarring was then measured using the same methods as in this study. This represents the degree of copulatory wounding that standard 'yardstick' females receive when they do not share recent co-evolutionary history with their mates and, in this context, represents our third male trait (hence; male injuriousness). For more details, we refer to [13].

(e) Reproductive tract scarring and population fitness

To measure population differences in the amount of genital damage incurred by females from different populations, 284 virgin females (20–24 from each population) were mated to a virgin male each from within the same population. Mated females were then isolated for 24 h to allow wound melanisation before being frozen in 70% ethanol for later dissection. We measured female body weight to the nearest 0.01 mg using an electronic balance (Sartorius Genius ME 235P-OCE).

Preserved females were dissected in a drop of insect ringer (Grace's insect medium; Sigma-Aldrich G81423). The female's bursa copulatrix was removed, cut along the midline and spread onto a glass slide. The tract was then photographed at ×400 and a digital image recorded. Two measures of the damage to the tract were recorded: the total number of differentiated wound sites (regardless of size), and the total combined area of melanisation (sites of wound repair), which was measured using the outline tool of ImageJ (v. 1.48). Some degree of tract scarring was seen in all mated females.

Rankin and Arnqvist [31] quantified population fitness in these populations, as the per generation growth rate in the absence of competition (total offspring produced by 10 males and 10 females in a single generation). This is dictated primarily by female lifetime fecundity, and we hence used this metric to

assess population-level costs of investment in female immunity and resistance adaptations.

(f) Statistical analysis

Statistical analysis were performed using R v. 3.2.2 [40], SYSTAT v. 13.1 (Systat Software, San Jose, CA, USA) and Genstat v. 18.1 [41]. We first used a GLM approach to test whether female traits differed significantly across populations. In all models, female weight was included as a covariate. For the tract scarring data, one pair was removed from the analysis because of a coding error ($N = 283$ pairs in the final analysis).

To ask whether female resistance adaptations (three traits: female tract volume, female PO level and female lytic activity) show correlated evolution with male persistence adaptations (three traits: male dorsal spine length, male ventral spine length and male injuriousness), we used two multivariate methods based on population averages for all traits. First, we employed a canonical correlation analysis (CCA) to assess overall covariance between the two sets of variables. Second, we used partial least-squares modelling (PLS) to achieve much the same goal. Both of these methods assess covariance between a linear combination of one set of variables with a linear combination of the other set of variables (i.e. a pair of latent variables), thus capturing axes of covariation between the two sets of variables. The relative contribution of different original variables to the latent variables can then be gleaned by inspecting the loadings they have upon the latent variables. While CCA and PLS analyses are related, they differ in how well they handle collinearity between original variables within each set. In the population-level analyses, the covariance between traits and size were removed by treating male size (elytra length) and female weight as partials. Following regressions of each trait on size/weight (population means), residuals were retained for analyses. We note that (i) this was deemed preferable to avoid overparameterization of our inferential models but that (ii) analogous models instead using raw trait values with size/weight included were qualitatively identical to the models presented here.

3. Results

(a) Female traits

The 13 populations differed significantly in average female tract volume ($F_{12,46} = 4.16$, $p < 0.001$), PO level ($F_{12,309} = 3.16$, $p < 0.001$), lytic activity ($F_{12,309} = 8.45$, $p < 0.001$), tract scar area ($F_{12,268} = 1.88$, $p = 0.04$) and tract scar number ($F_{12,268} = 4.26$, $p < 0.001$). Across all individuals, heavier females had a larger reproductive tract volume ($F_{1,46} = 15.05$, $p < 0.001$), higher PO level ($F_{1,309} = 36.7$, $p < 0.001$) and higher lytic activity ($F_{1,309} = 7.14$, $p = 0.008$). There was no effect of female weight on tract scar area ($F_{1,268} = 0.46$, $p = 0.5$) or scar number ($F_{1,268} = 3.1$, $p = 0.08$). Females had an average of 17.84 scars (s.d. = 12.77) in the tract wall.

(b) Correlated evolution between male and female traits

The CCA revealed an overall covariation between the male and the female trait sets (canonical $r = 0.93$; Rao's $F_{9,14.7} = 2.86$, $p = 0.035$) of which the first pair of latent variables were significant ($\chi^2_9 = 18.27$, $p = 0.032$). A sizeable fraction of variance in female traits was predicted by variance in male traits (Stewart-Love Canonical Redundancy Index = 0.57) (figure 2). Our PLS analysis also identified a single significant axis of covariation between male and female traits

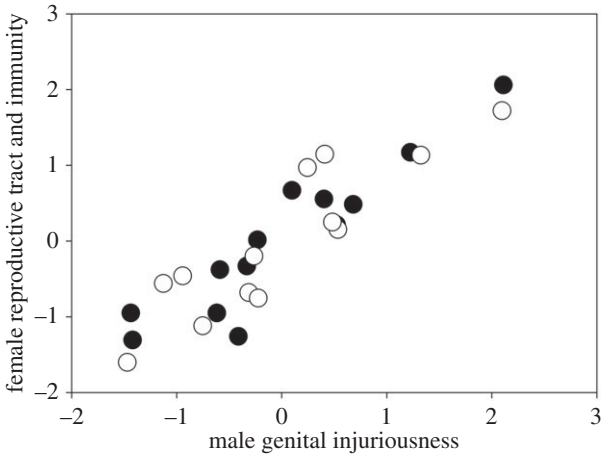

Figure 2. Ordination of the 13 populations along the first pair of latent variables describing covariation between male genital injuriousness and female resistance traits. Closed symbols represent scores from a canonical correlation analysis and open symbols those from a partial least-squares model. See text for statistical details.

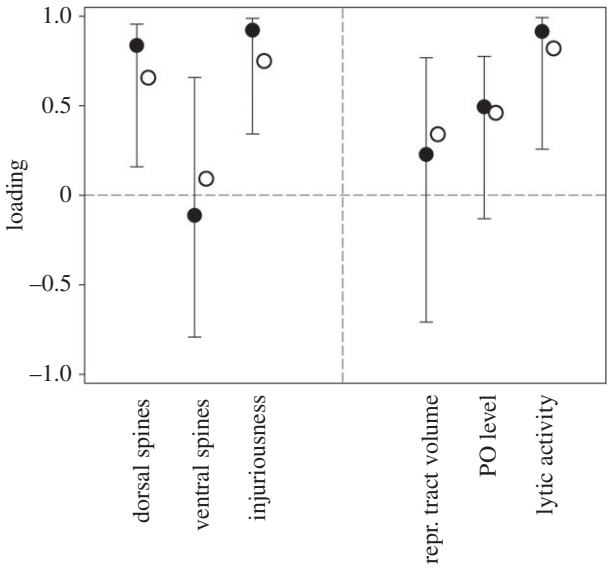

Figure 3. Loadings for the male set (left) and female set (right) of traits upon the two sex-specific latent variables best describing correlated evolution between the sexes. Open circles show loadings from the PLS model. Closed circles represent the CCA. Shown are also bootstrapped 95% CI's for the CCA loadings, based on 10^3 bootstraps corrected for axis reversals.

(Osten's $F_{3,36} = 5.35$, $p = 0.004$), which explained 43.1% of the variance in female traits and 42.9% of the variance in male traits. Inspections of the standardized loadings of the two types of models (figure 3) showed that the CCA and the PLS were highly congruent, in terms of identifying very similar multivariate axes of covariation. In males, the length of the dorsal spines and genital injuriousness contributed to correlated evolution with females. In females, all three traits loaded positively upon the female latent variable, although lytic activity did so most strongly (figure 3). Overall, these analyses support our predictions in terms of the pattern and direction of correlated evolution between these putative male persistence and female resistance traits.

The amount of scarring represents an outcome of a male–female interaction and so should not be affected by male persistence or female resistance in isolation, if male–female

Table 1. The results of a multiple regression based on mean trait values across 13 populations, using three male traits (M) and three female traits (F) to predict the number of scars in the female reproductive tract that results from mating. Body size was partialled out from male and female traits prior to analysis. Significant effects are given in italics. See text for discussion.

				bootstrap 95% CI[a]			
variable	β	t	p	lower	upper	ridge β (HKB)	ridge β (LW)
M: dorsal spine length	0.88	1.62	0.157	−3.46	2.52	0.89	0.77
M: ventral spine length	*0.97*	*2.92*	*0.027*	*0.23*	*3.87*	*0.72*	*0.50*
M: male injuriousness	-2.45×10^{-3}	−2.09	0.082	−0.01	0.02	-1.60×10^{-3}	-1.04×10^{-3}
F: reproductive tract volume	*−0.59*	*−4.88*	*0.003*	*−1.74*	*−0.23*	*−0.51*	*−0.42*
F: PO level	479.21	1.64	0.151	−2.05	2869.00	338.39	256.77
F: lytic activity	3.28×10^{-5}	1.12	0.306	-5.40×10^{-5}	2.61×10^{-4}	1.38×10^{-5}	3.61×10^{-6}

[a]Bias corrected.

coevolution is balanced [30]. We tested whether the amount of scarring in females that resulted from within-population matings covaried with either male persistence or female resistance by correlating our population-specific measures of scarring (number and area) with population scores along the latent variables of the CCA and the PLS. As predicted, scarring showed no significant correlation with either of our male or female traits in isolation ($|r| < 0.45$, $p > 0.125$, in all cases). Theory predicts, however, that the outcome could be predicted in a multivariate analysis where male and female traits are used simultaneously [30]. A model using all six male and female original traits to predict scar area was not significant overall ($F_{6,6} = 0.54$, $p = 0.761$) but a model predicting scar number was ($F_{6,6} = 5.70$, $p = 0.026$). Because the model of scar number was potentially overparameterized and suffered from problems with multicollinearity, we also assessed the model using (i) a resampling test involving bootstrapping (10^3 replicates) the regression coefficients and (ii) a ridge regression using both the Hoerl–Kennard–Baldwin (HKB) estimator and the Lawless & Wang (LW) estimator of lambda. These assessments (table 1) showed that the initial model was robust against the above potential problems and that two variables showed independent effects on the number of female scars: injury to females was higher in populations where males had size-corrected ventral genital spines that were long relative to the reproductive tract volume of females (figure 4).

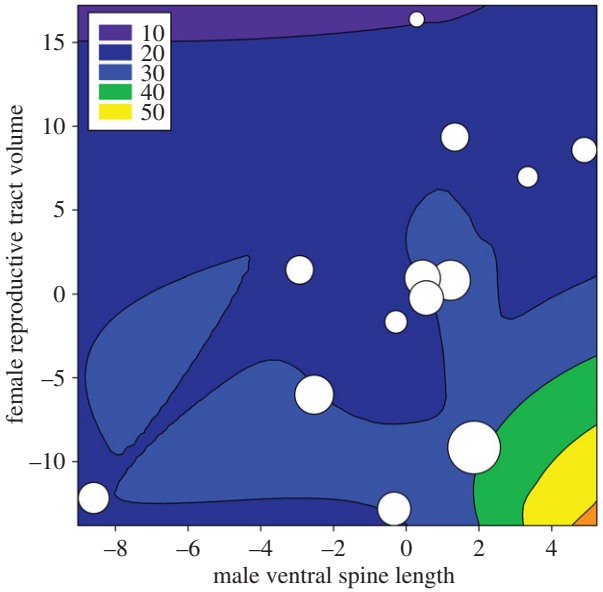

Figure 4. Heat map of the amount of genital damage incurred by females during mating (number of scars in reproductive tract) as a result of variation in size-corrected male genital spine length and volume of the female reproductive tract, across the 13 populations (circles). Circle size is also proportional to the number of scars.

(c) Female resistance and population fitness

Multiple regression suggested that female investment in resistance (i.e. the score along the female latent variable) and female size collectively predicted population fitness when using latent variable scores from the PLS ($F_{2,10} = 4.19$, $p = 0.048$), but not from the CCA ($F_{2,10} = 3.44$, $p = 0.073$). A closer inspection of this pattern showed that the covariation was primarily due to a negative correlation between population fitness and female PO level ($r = -0.59$, $p = 0.032$), rather than reproductive tract volume ($r = -0.38$, $p = 0.199$) or lytic activity ($r = -0.04$, $p = 0.901$). These analyses thus offer support for the hypothesis that female investment in at least some aspects of resistance are costly in terms of reduced population fitness [19,42].

4. Discussion

In this study we examined across-population covariation in male persistence traits and female resistance traits using 13 populations of the seed beetle *C. maculatus*. We found significant across-population differences in all of the female traits measured, indicating that these traits have diverged significantly in isolation. Multivariate analyses revealed

significant positive correlated evolution between male persistence and female resistance adaptations across populations. Our study thus provides a rare example of correlated evolution of male persistence and female resistance traits at the within-species level [22], and illustrates the importance of considering multiple traits given that male and female adaptations to sexual conflict are unlikely to be limited to single traits.

In order to show that the correlated evolution between male and female traits observed here is caused by sexual conflict, we need to demonstrate that an increase in male persistence is associated with a reduction in female fitness [2,43]. Yet, when such a 'sexual arms race' is present we should not expect to find a direct relationship between the level of male persistence and female fitness, as any reduction in female fitness should quickly lead to an increase in female resistance traits to reduce harm [30]. Indeed, when traits were tested in isolation, we found no significant effect of male persistence or female resistance on the degree of tract scarring across populations. This is consistent with a scenario where, within each population, males and females are at an evolutionary equilibrium with respect to the fitness impact of traumatic mating. However, the hallmarks of such an arms race may be detected by considering the levels of both male and female adaptations simultaneously [19,30]. Our multivariate analyses revealed that male ventral spine length and female tract volume significantly influenced the number of scars in the female tract (table 1), although there was no significant effects on tract scar area. Female tract scarring was highest in those populations with relatively long ventral spines and relatively small average female tract volume (figure 4). Further, for most populations the level of investment in ventral spine length is roughly matched by the level of investment in reproductive tract volume. This provides support for the 'arms-race' hypothesis for the evolution of male genital spines and female tract thickness: differences in the absolute level of any male or female trait do not influence the fitness outcomes of mating, whereas differences in the relative level do [19,22,30]. As well as providing evidence for a sexual arms race, this statistical approach has also allowed us to confirm intraspecifically for the first time that both male aedeagal spine length and female tract thickness do indeed influence the outcome of traumatic mating in *C. maculatus*.

It is important to note that we have used a three-dimensional measure of female tract tissue investment in this study, rather than a simple measure of the thickness of the tract in cross-section. Given that the female tract is a three-dimensional structure, we suggest this three-dimensional measurement is the most appropriate when considering the fitness effects of traumatic mating, as it most fully captures differences in total female investment in tract tissue. This method also controls for any confounding effect of tract size or shape across females, which could be overlooked when only taking tract thickness estimates from one or a few transverse slices through the tract (e.g. [19,44]). However, the use of tract volume makes determining the precise proximate mechanisms leading to changes in female fitness more difficult. For example, it has been suggested that a thicker tract lining reduces the cost of mating to females by reducing the amount of male-seminal products that are able to pass into the female body cavity [14]. However, tract volume could be increased in two ways: by increasing the tract thickness, or by increasing the number of folds in the tract lining (as seen in figure 1*a*). The number of folds in the tract lining could also feasibly reduce tract scarring, and thus the fitness costs of mating to females, by giving the tract lining greater flexibility and so making the penetration of spines more difficult. Therefore, we cannot distinguish between the effect of physical distance between the tract lumen and the body cavity, or some other effect such as the number of folds, based on the relationship between tract volume and female fitness alone. Instead, functional studies of the interaction between the male and female genitalia are needed. This is an area in which micro-CT scanning may prove very useful, and indeed this approach has been used effectively to examine the interactions between male and female genitalia during copulation in other arthropod species (e.g. [45–47]).

We found strong evidence for correlated evolution between male genital morphology and both measures of female immune function, with female lytic activity showing the strongest covariation with male persistence traits. This supports the hypothesis that the female immune response has evolved to reduce the cost of traumatic mating in *C. maculatus*, with microbial infection being a potential target of female resistance. However, neither measure of female immunity was directly related to the degree of tract scarring seen following mating. This is somewhat surprising, given that both lytic activity and phenoloxidase level are predicted to play a role in reducing the costs of female tissue damage. However, the female immune system has to respond to costs of mating other than those arising from copulatory tract damage. For example, females may suffer mating costs via male seminal fluid proteins that are known to vary across populations [35,48,49]. In addition, investment in immunity by females is affected by a suite of other life-history trade-offs in seed beetles (e.g. [50]). Factors such as these are likely to blur the relationship between scarring and immunity.

We also found evidence for a trade-off between one aspect of female immune investment (PO level) and population fitness: populations with high female PO levels tended to have lower population fitness. This is likely due to the fundamental resource trade-offs that are predicted between immunity and reproduction [51,52], given that population fitness primarily reflects differences in female egg production [31]. This trade-off represents an additional, and under-appreciated, potential cost of traumatic mating to females that has been seen in other studies of *C. maculatus* (see also [42,53]). Interestingly, a recent study assessing lytic activity in *C. maculatus* populations subject to an experimentally biased sex-ratio for 11 generations also found evidence for such a trade-off: females from male-biased populations had lower lytic activity than females from female-biased populations [39]. Females in male-biased lines are predicted to experience an increased mating rate (and therefore greater lifetime mating trauma), and so are expected to increase investment in immunity. However, this is the opposite of the pattern observed by van Lieshout *et al.* [39]. Their result could be explained if there is a strong trade-off between investment in reproduction versus immunity, such that females subjected to greater mating costs are adapted to invest in early reproduction at the expense of immune function [39,50].

One outstanding question concerns the extent to which the differences in male and female traits observed among the current laboratory populations reflect differences between the ancestral populations from which they were collected, relative to subsequent divergence among populations since they were introduced into the laboratory. Unfortunately, determining this is not possible without a knowledge of the phenotypes

of the ancestral populations at the time when founder individuals were collected. We suggest that laboratory divergence has been less important than the original population differences, given that (i) all populations have experienced a single common garden environment, and (ii) reproductive traits are not correlated with the time since collection across these populations [31,35]. Indeed, if these populations are adapting to the same common environment the differences we observe now are likely to be reduced compared with ancestral populations. Regardless, the fact remains that there has been significant correlated evolution between males and females across these populations, though the timescale over which such differences have evolved is unclear.

In summary, by combining multiple morphological and physiological measurements we have detected a clear signal of correlated evolution between male persistence traits and female resistance traits involved in sexual conflict in the seed beetle *C. maculatus*. We have also shown that the relative level of male and female 'armament' influences the degree of harm females receive during mating, thus providing support for the hypothesis that this correlated evolution has been driven by sexually antagonistic coevolution. We have thus shown that the process that has resulted in the covariation between male and female phenotypes across seed beetle species is also ongoing within at least one of these species. Finally, we present evidence for a trade-off between investment in female immune function and reproductive function at the population level, thus providing evidence of an additional cost to females of traumatic mating.

Data accessibility. Supporting data have been archived at Dryad (http://dx.doi.org/10.5061/dryad.1b15j) [54].

Authors' contributions. L.R.D. performed the female tract volume measurements, carried out statistical analysis and drafted the manuscript. E.v.L. conceived the study, collected the beetles and performed the immunity measurements. K.B.M. conceived the study and helped draft the manuscript. J.A.M. performed the female tract volume measurements. G.A.S. set up the original populations, performed fitness assays, secured measures of male spines, performed statistical analysis and helped draft the manuscript. L.W.S. conceived the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

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