

EVOLUTION OF MALE AND FEMALE GENITALIA FOLLOWING RELEASE FROM SEXUAL SELECTION

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Despite the key functions of the genitalia in sexual interactions and fertilization, the role of sexual selection and conflict in shaping genital traits remains poorly understood. Seed beetle (*Callosobruchus maculatus*) males possess spines on the intromittent organ, and females possess a thickened reproductive tract wall that also bears spines. We investigated the role of sexual selection and conflict by imposing monogamous mating on eight replicate populations of this naturally polygamous insect, while maintaining eight other populations under polygamy. To establish whether responses to mating system manipulation were robust to ecological context, we simultaneously manipulated life-history selection (early/late reproduction). Over 18–21 generations, male genital spines evolved relatively reduced length in large males (i.e., shallower static allometry) in monogamous populations. Two nonintromittent male genital appendages also evolved in response to the interaction of mating system and ecology. In contrast, no detectable evolution occurred in female genitalia, consistent with the expectation of a delayed response in defensive traits. Our results support a sexually antagonistic role for the male genital spines, and demonstrate the evolution of static allometry in response to variation in sexual selection opportunity. We argue that further advances in the study of genital coevolution will require a much more detailed understanding of the functions of male and female genital traits.

KEY WORDS: Adaptation, allometry, experimental evolution, genitalia, sexual selection.

In insects, as in many other animal groups, the genitalia are highly complex, integrated suites of morphological structures whose function often remains obscure (Scudder 1971; Eberhard 1985, 2010). The important influence of genital structures on interactions between sexes prior to and during copulation and sperm transfer suggests an important role for sexual selection and sexual conflict in genital evolution (Hosken and Stockley 2004; Edvardsson and Tregenza 2005). Consistent with this expect-

tation, comparative evidence shows that the genitalia diversify much more rapidly in insect clades characterized by polyandrous mating systems than in clades characterized by monandry (Arnqvist 1998). Yet, the role of sexual selection and conflict in genital evolution remains poorly understood.

Sexual selection often drives the evolution of exaggerated mean trait size and/or steeper scaling of trait size with body size (i.e., elevated static allometry) in signals and weapons (Andersson 1994; Emlen and Nijhout 2000; Bonduriansky 2007). Genital traits could function as sexual signals involved in “copulatory courtship” and cryptic female mate choice (Eberhard 1991), or as sexually antagonistic traits involved in coercion and manipulation (Arnqvist and Rowe 2002), and thereby evolve exaggerated

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expression and/or elevated static allometry, as do other secondary sexual traits (Arnqvist 1998; Eberhard 2010). However, genital traits appear to be constrained to a shallow static allometry (e.g., Eberhard et al. 1998; but see Green 1999), which has been attributed to strong stabilizing selection on genital traits (and, consequently, greatly reduced variation among individuals), reflecting the need for male genitalia to fit like a “key” into the species-specific “lock” of the female genitalia, thereby preventing interspecies mating, and/or for male genitalia to be compatible with the greatest range of conspecific females (one size fits all) (Eberhard et al. 1998; Eberhard 2010). Shallow static allometries may be characteristic even of genital traits subject to directional sexual selection (Bertin and Fairbairn 2007). It therefore remains unclear whether sexual selection can drive the evolution of steep static allometry in genital traits.

The role of sexual conflict in genital evolution also remains contentious. In some species of insects and spiders, males possess genital structures that appear to injure females (Crudginton and Siva-Jothy 2000; Stutt and Siva-Jothy 2001; Tatarnic et al. 2006; Kamimura 2007; Rezac 2009). These male traits have been interpreted as sexually antagonistic adaptations that enhance male performance in intrasexual competition, while inflicting collateral harm on females (Hosken and Stockley 2004; Arnqvist and Rowe 2005). However, the interpretation of such traits as sexually antagonistic has been challenged on the grounds that females could derive a net benefit from mating with damage-inflicting males if such males sire sons that achieve high mating success (Eberhard and Cordero 2003; Eberhard 2010). Although theory suggests that indirect benefits are unlikely to balance direct harm (Kirkpatrick 1996; Cameron et al. 2003) and empirical evidence from seed beetles supports this conclusion (Maklakov and Arnqvist 2009), this question remains challenging because indirect benefits are notoriously difficult to estimate (Hosken and Tregenza 2005).

In the seed beetle *Callosobruchus maculatus*, the tip of the male intromittent organ (aedeagus) is densely covered with spines (Mukerji and Bhuya 1937; Crudginton and Siva-Jothy 2000). The spines may function in male–male competition by acting as an anchor that enhances males’ ability to cling to females (Edvardsson and Tregenza 2005), and thereby influencing male performance in sperm competition (Hotzy and Arnqvist 2009). Male spines cause genital scarring in females (Crudginton and Siva-Jothy 2000; Rönn et al. 2007; Hotzy and Arnqvist 2009), which may reduce female longevity (Crudginton and Siva-Jothy 2000; Gay et al. 2011) and lifetime fecundity (Edvardsson and Tregenza 2005; Gay et al. 2011). In species where males have longer spines, females have evolved a thicker copulatory tract wall (Rönn et al. 2007). Like many other bruchids (Southgate 1971; Kingsolver 1979; Anton 2000; Mergen 2004), *C. maculatus* females also possess blade-like spines on the copulatory tract wall near the entrance to the bursa copulatrix (Southgate et al. 1957).

To our knowledge, the function of these female spines has never been investigated before.

Evidence that sexually antagonistic selection drives the evolution of genital traits in seed beetles, and other species where sexual conflict is manifested as struggle over copulation (Arnqvist and Rowe 2002; Brennan et al. 2007), comes primarily from correlational interspecies comparisons and interpopulation crosses. Only one previous study has investigated genital evolution in response to experimental manipulation of the mating system (Simmons et al. 2009), but no putatively harmful male traits are known in that system, and female traits were not examined. Although male-limited evolution experiments (in which constraints on the evolution of antagonistic male traits are reduced) have shown that *Drosophila melanogaster* males evolve to become more male-like, and more harmful to females (Rice 1996, 1998; Holland and Rice 1999; Prasad et al. 2007; Bedhomme et al. 2008), it remains unclear which male traits mediate the increased harm.

Experimental evolution permits powerful tests of theory because the evolutionary response over multiple generations integrates the net effects of both direct and indirect selection on the trait of interest. Theory yields several testable predictions. First, release from sexual conflict through experimentally imposed random, lifetime monogamy is expected to select for de-escalation of sexual antagonism because this mating system eliminates intrasexual competition and leads to a convergence in the reproductive interests of mates (Arnqvist and Rowe 2005; Edward et al. 2010). Any harm imposed by a male on his mate reduces his own fitness by the same amount. Under monogamy, the size of males’ harm-inflicting traits should therefore be reduced. Moreover, a disproportionate reduction in trait size in large males is predicted for two reasons. First, the largest males are likely to impose the greatest harm on females because their larger genitalia will tend to cause greater damage to the female reproductive tract. Large males may be particularly harmful if they express disproportionately longer genital spines (i.e., if spine static allometry is positive). Second, it is likely that some minimum spine length is necessary to achieve successful copulation in this species, at least without major structural and behavioral modifications. Those conditions may result in directional selection for reduced sizes of traits such as genital spines in large males but stabilizing selection on trait size in small males, favoring a reduction in static allometry slope in populations released from sexual conflict. Third, the evolutionary response to release from sexual conflict is expected to be more immediate in males than in females. A delayed response is expected in females because expression of females’ defensive traits only becomes costly after male traits have evolved to become substantially less harmful (Parker 1979).

Although theory and empirical research on sexual coevolution have focused on sexual selection, net selection on secondary

sexual traits also reflects their effects on viability and fecundity (Chenoweth et al. 2008), suggesting that sexually antagonistic coevolution can be influenced by ecological context (Maklakov et al. 2010). Experimental studies that vary both mating system and ecology simultaneously can therefore establish whether evolutionary responses to sexual selection and conflict are robust to ecological context, and permit broader generalizations from observed patterns of sexual coevolution (Bonduriansky et al. 2008; Edward et al. 2010).

We released eight populations of *C. maculatus* from sexual selection and conflict by imposing random, lifetime monogamy (each male paired with one female for life), while maintaining eight other populations under polygamy (57 males with 57 females) comparable to the mating system of the ancestral stock. To determine whether responses to a monogamous mating system are robust to ecological context, populations within each experimental mating system were also subjected to contrasting regimes of life-history selection: four populations selected for early reproduction and four populations selected for late reproduction. We have shown elsewhere that these life-history regimes influence the evolution of mating behavior (Maklakov et al. 2010). After 18–21 generations under these experimental treatments, we tested for evolution of body size and genitalia of both sexes.

Methods

STUDY ANIMALS

Seed beetles (Bruchidae) are valuable model organisms for research on sexual selection and genital evolution because they possess putatively sexually antagonistic morphological and behavioral traits (Crudginton and Siva-Jothy 2000; Rönn et al. 2007; Hotzy and Arnqvist 2009; Gay et al. 2011), are adapted to laboratory-like conditions in their natural environment (grain stocks) (Fox et al. 2003; Messina and Karren 2003), and have rapid generation time. We used the seed beetle *C. maculatus*—a world-wide pest of dry legumes that develops from egg to adult in 24–26 days under 30°C. Both sexes can mate multiply: males persistently attempt to mount females, which rebuff most mating attempts by kicking males with their hind legs or by moving away. Both sexes possess elaborate genitalia endowed with sclerotized spines (Fig. 1).

We used an Australian population of *C. maculatus*, which originates from Kingaroy, Queensland (Maklakov et al. 2009). Briefly, this population was initiated with 357 beetles from mung beans (*Vigna radiata*) and propagated at 250–300 beetles per generation since 2003. From this source population, we obtained a sample of 600 beetles in 2006 and propagated the population in our laboratory with ~500 beetles per 200 g of organic mung beans per generation at 30°C, 70% relative humidity, and 14:10 light:dark cycle (Maklakov et al. 2009).

EXPERIMENTAL EVOLUTION

We established 16 replicate populations, half of which were released from sexual selection by imposing random monogamy through pairing virgin beetles for life (Monogamy lines), whereas the other half of the experimental populations were maintained as panmictic polygamous populations (Polygamy lines) (Maklakov et al. 2009). We further manipulated the ecological context by imposing divergent life-history selection on age at reproduction: in half of the populations, only eggs laid during the first 24 h after hatching contributed to the next generation (Young lines), whereas in the other half only eggs produced after 72 h were collected (Old lines). This resulted in a 2 × 2 factorial design, where mating system and resulting opportunity for sexual selection (SS), as well as life-history selection (LH), were manipulated simultaneously.

We addressed potential confounding effects of differential larval competition by ensuring that each egg was laid on a separate mung bean. We also reduced inadvertent selection on maturation time by randomly selecting among virgin beetles three days after the start of hatching when most of them had eclosed (Maklakov et al. 2009). Lines were propagated by either 50 (Monogamy) or 57 (Polygamy) pairs of beetles to compensate for the anticipated differences in effective population size between the two mating system regimes (Maklakov et al. 2009; Edward et al. 2010). Because nearly all beetles survived beyond 72 h, there was no need to adjust population size for LH regimes (Maklakov et al. 2009). The lines were propagated for 18 (Old) and 21 (Young) generations, after which samples of virgin beetles were frozen at –20°C for analysis of genital trait evolution.

MORPHOMETRIC DATA

From each replicate population, seven males and five females were dissected and imaged (actual sample sizes vary because some traits could not be measured on some individuals). Elytron length (mean of the right and left elytra) was used as an index of body size because this trait loaded most strongly in each sex on the first principal component from a principal component analysis on the correlation matrix for elytron length and the genital traits (Cayetano 2010), and could be measured with very high repeatability (Table 1). The genital traits examined in males were genital spine length (mean of the longest three spines from the spine base to the tip; Fig. 1E), the total area (square-root transformed) of the bases of the genital spines (Fig. 1B, C), aedeagus length and width (Fig. 1C), paramere length (mean of right and left arms from the base; Fig. 1A), and the length of the flap on the aedeagus from the base to the tip (Fig. 1D). For females, we examined the combined length of the copulatory tract and bursa copulatrix (henceforth, reproductive tract; Fig. 1H), the maximum thickness of the connective tissue lining the copulatory tract (Fig. 1F; see below for details), and the number and mean length of bursal spines (Fig. 1G).

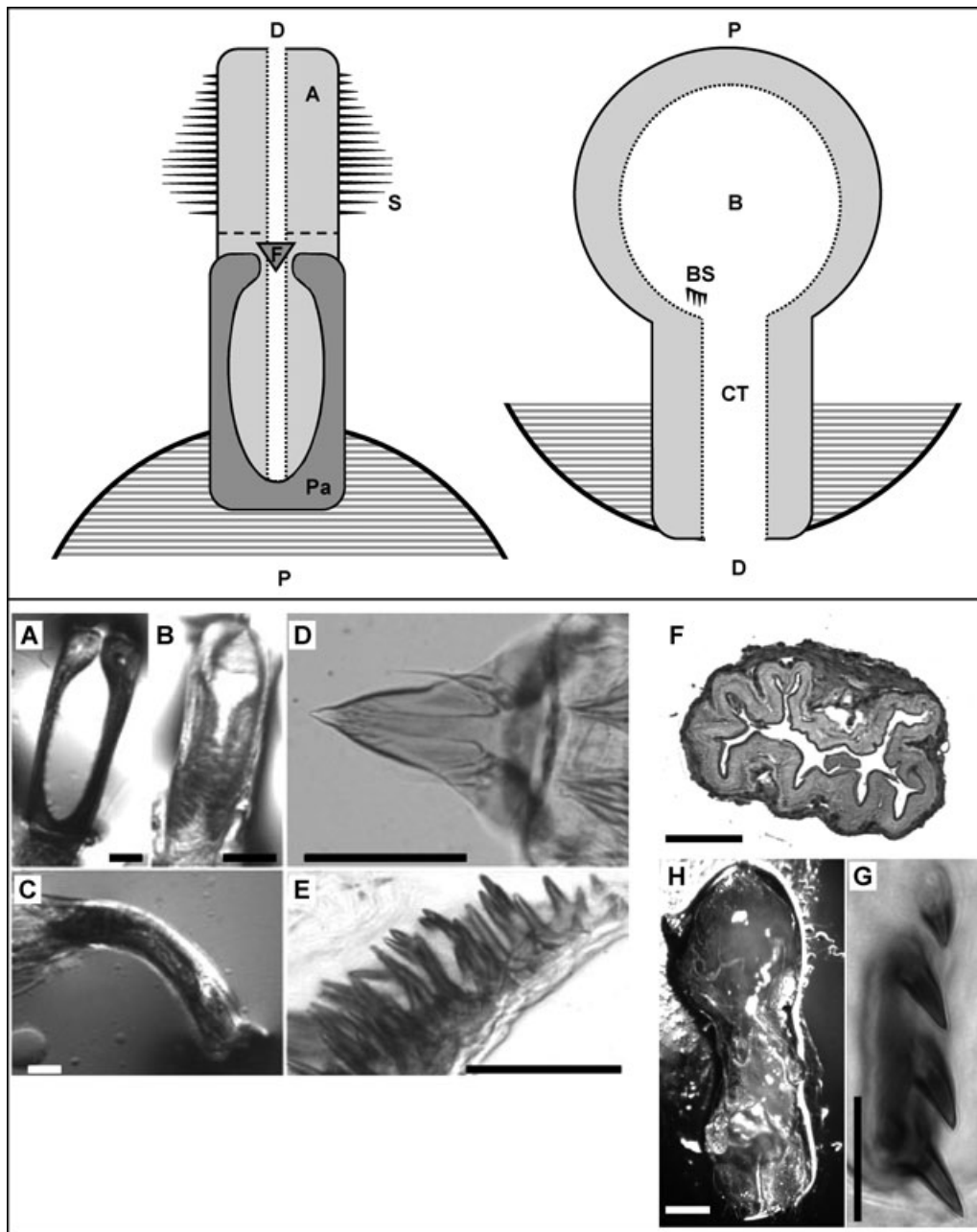


Figure 1. Genitalia of *Callosobruchus maculatus*. Top panel: A diagrammatic representation of the male (left) and female (right) genitalia. Structures are labeled according to contemporary convention, with older terms (see Mukerji and Bhuya 1937; Southgate et al. 1957) shown below in quotation marks. A transverse section of the male aedeagus (A) in everted state is depicted, with the dashed horizontal line representing the approximate extent of the intromittent portion (“endophallus” or “phallosome”) bearing genital spines (S) (denticles) that is everted inside the female copulatory tract. The basally fused parameres (Pa) and flap (F) (“valve” or “end plate”), which remain external to the female, are depicted in dark gray. The female copulatory tract (CT) and bursa copulatrix (B) containing bursal spines (BS) (teeth) are also shown in transverse section. For each sex, the posterior abdomen is depicted as a striped semi-circle, and the proximal (P) and distal (D) ends are shown. Bottom panel: Images of male (A–E) and female (F–G) genital traits. Male genital traits are (A) parameres; (B) sclerotized bases of genital spines; (C) lateral view of the aedeagus (for measurement of aedeagus length and width); (D) flap; (E) lateral view of the genital spines. In images B and C, the aedeagus tip (endophallus) (with spines) is retracted. Bars represent approximately 100 μm . Female genital traits are (F) transverse section of the copulatory tract; (G) bursal spines; (H) entire reproductive tract. Bars represent approximately 100 μm in panels F and H, and 50 μm in panel G. See Methods for a precise description of the measurements used to quantify variation in the sizes of these structures.

Table 1. Mean size (μm) or number, standard deviation, number of individuals measured, and measurement repeatability for each trait measured.

| Trait | Mean | Standard deviation | <i>N</i> | Measurement repeatability |
|---|--------|--------------------|----------|---------------------------|
| Males | | | | |
| Elytron length | 1777 | 53.41 | 112 | 0.97 |
| Aedeagus width | 200 | 10.50 | 111 | 0.94 |
| Aedeagus length | 569 | 28.70 | 101 | 0.93 |
| Genital (aedeagal) spine length | 120 | 9.45 | 112 | 0.80 |
| Area of genital spine bases | 7491 | 669.26 | 100 | 0.85 |
| Paramere length | 563 | 31.22 | 112 | 0.95 |
| Flap length | 127 | 7.09 | 112 | 0.59 |
| Females | | | | |
| Elytron length | 1974 | 59.02 | 80 | 0.97 |
| Reproductive tract length | 1540 | 207.44 | 80 | 0.85 |
| Copulatory tract connective tissue area | 31,452 | 6673.82 | 73 | 0.93 |
| Bursal spine length | 43.65 | 7.63 | 80 | 0.80 |
| Bursal spine number | 3.09 | 1.06 | 80 | 1.00 |

Dissections were performed on glass slides with a pair of fine forceps under a Leica MZ16A stereoscope (Wetzlar, Germany). The abdomen was separated from the thorax and the elytra removed. For males, the aedeagus was then removed by pulling away tergites and gently coaxing it away from them. Once separated, the fused parameres were removed by pulling them away from the aedeagus. The female reproductive tract was removed in a similar way. Using a fine graphite blade, the reproductive tract was then bisected just posterior to the bursal spines. The bursal spines were imaged under a compound microscope while the neck of the copulatory tract was sectioned (see below).

Each trait was imaged under a standardized magnification using a Leica DFC420 digital camera fitted to either a Leica MZ16A stereoscope or a Zeiss Axioskop 40 (Göttingen, Germany) compound microscope. Measurements were made from the images using *ImageJ* software (Rasband 1997–2009). Male genital spines, parameres, and triangular flaps were measured by finding the distance from the distal tip to the proximal boundary of sclerotization (Fig. 1). Aedeagus length was measured as the curve-length from the base (distal end) of the aedeagus (not including the length of the flap) to the proximal margin of the spines (Fig. 1C). The area covered by spines was quantified by tracing an enclosing line around the sclerotized spine bases (Fig. 1B, C).

Prior to dissection, females were placed into glass shell vials containing Bouin's solution for 4 h to prepare the copulatory tract wall for sectioning. Females were then removed and passed through two consecutive baths of 2 mL 70% ethanol, each bath lasting 1 h. After this, the bursa was removed and left in 2 mL 70% ethanol overnight. The tissue was then embedded in a hot agarose solution over an ice tray. Samples were placed in a labeled cassette and transferred to 70% ethanol and then into a Thermo-Scientific Excelsior ES Tissue Processor for overnight processing at the UNSW Histology and Microscopy Unit. They were then embedded in molten paraffin wax using the Thermo Shandon Histocentre 3 and cut on a Leica RM 2135 microtome at 4- μm thickness and floated onto a water bath set at 45°C. Every fifth section, representing an interval of 20 μm , was collected onto a glass slide. Slides were dried in a 57°C oven and then placed on a Leica XL Autostainer (programme 1) for haematoxylin and eosin staining. Stained slides were coverslipped on a Leica CV5000 Coverslipper using Ultramount glue and allowed to dry overnight. The connective-tissue area from each copulatory tract cross section was obtained by tracing a line around the copulatory tract wall and subtracting the area of the lumen. For each individual female, a quadratic curve was fitted to the sample of connective tissue areas, and the maximum thickness was estimated from this curve.

Measurement repeatability, estimated as the intraclass correlation coefficient (Lessells and Boag 1987), was obtained by taking two measurements for each trait on two individuals of each sex from each replicate population. To include as many sources of measurement error as possible, structures were re-mounted either by moving them to a different position on the microscope slide, or lifting and reapplying the cover slip. For each specimen, each of the two preparations was photographed and measured to obtain two replicate measurements. To estimate repeatability for copulatory tract wall thickness, the mounted copulatory tract sections from two individuals per population were remeasured and individual maxima re-estimated. Repeatabilities were > 0.8 for all traits except male flap length (Table 1).

To examine trait role (intromittent/nonintromittent), male–female pairs were placed into mesh containers and, once mating was underway, were frozen in copula by placing the container into a -20°C freezer. Their conjoined genitalia were removed (taking care to avoid altering the relative positions of the male and female structures) using fine forceps. The genitalia ($N = 4$ pairs) were then photographed under the MZ16A microscope, and one preparation was sectioned at intervals of 20 μm . Examination of these specimens confirmed previous observations (Mukerji and Bhuya 1937) that the parameres and flap remain outside the female genital tract during copulation (Cayetano 2010), and these traits were therefore considered to be nonintromittent appendages.

STATISTICAL ANALYSIS

Effects of selection treatments on male and female body size (mean elytron length) were tested by analysis of variance (ANOVA) on male and female population-means, with sexual selection (SS) and life-history selection (LH) as categorical, fixed factors and sex as repeated-measures factor. Effects on sexual size dimorphism were tested as sex \times selection treatment interactions.

Static allometry was estimated using both ordinary least squares (OLS) regression and reduced major axis (RMA) regression of log-transformed trait size on log-transformed elytron length (body size). Because neither model is clearly more appropriate for our data, we interpret with greatest confidence those results that are qualitatively consistent across both regression models. OLS regression provides better estimates of scaling slope than RMA regression when measurement error in Y substantially exceeds measurement error in X (McArdle 1988; Smith 2009). By this criterion, OLS regression is more appropriate for our data, because the body size index (elytron length) exhibited higher measurement repeatability than all genital traits except genital spine number (Table 1). OLS regression can also be considered more appropriate because the relationship between the genital traits and body size is inherently asymmetrical: genital size varies as a function of body size (Smith 2009). The analysis of covariance (ANCOVA) models described below test for selection treatment effects on OLS slopes. However, because RMA is commonly used to describe static allometry, and is the preferred model for comparing slope estimates to isometry (Warton et al. 2006; Smith 2009), we also report RMA slopes for traits whose OLS slopes responded to selection. RMA analysis was performed using tools developed by David Warton (<http://web.maths.unsw.edu.au/~dwardon/programs.html>).

Variation in each genital trait was analyzed separately because principal components analysis failed to reveal any useful multivariate shape factors (Cayetano 2010). For each genital trait, treatment effects on static allometry slopes were first tested by ANCOVAs on log-transformed trait size data. Following log-transformation, each trait (including elytron length, which was used as the index of body size) was centred at a mean of zero to allow testing the main effects of selection treatments (Quinn and Keough 2002; Schielzeth 2010). Selection treatments (sexual selection, life-history selection, and their interaction) were modeled as categorical, fixed factors, and replicate population (nested within selection treatments) as a random factor. Log-transformed elytron length (Log EL) was included as a covariate, and its interactions with the selection treatments were used to test for selection treatment effects on static allometry slope. Because the unit of replication is the population, main effects of selection treatments and their interactions with Log EL were tested using variation among populations (nested within selection treatments) in intercept and slope, respectively, as the *F*-ratio denominator

(Quinn and Keough 2002). For traits that exhibited selection treatment effects on allometric slope, we used individual data to test for deviations from isometry (i.e., slope = 1).

Next, for traits that exhibited no significant interactions between selection treatment and Log EL (i.e., the homogeneity-of-slopes assumption was not violated), we simplified the ANCOVA models by removing interactions with elytron length. The resulting models thus included selection treatments and their interaction as fixed effects, population (nested within selection treatments) as random effect, and elytron length as covariate. Results based on log-transformed, centred data were qualitatively identical to those based on standardized raw data, so only the former are shown.

All morphometric data were approximately normally distributed. Area data (i.e., areas of male spines and female connective tissue cross-sections) were square-root transformed prior to log-transformation. Although sample sizes were unequal for the sexes ($N = 5$ females and seven males per population), similar results (not shown) are obtained if only the first five males measured are included in the analysis. Because distinct predictions were made for different traits (e.g., male aedeagal spines vs. other male traits, male traits vs. female traits), correction for multiple testing was not appropriate (Perneger 1998; Moran 2003). Means, standard deviations, and sample sizes for each trait are shown in Table 1.

Results

BODY SIZE

We did not detect evolution of body size in either sex, or of sexual size dimorphism, in response to manipulation of the mating system (i.e., sexual selection), ecological context (i.e., life-history selection), or their interaction (all $F_{1,12} < 2.5$, $P > 0.14$).

MALE GENITAL TRAITS

We detected evolution of allometric slope in two male genital traits in response to manipulation of the mating system, and evolution of mean size (allometric intercept) of a third male genital trait in response to the interaction of mating system and ecology (Table 2).

For male genital spine length, static allometry OLS slope was significantly shallower in monogamous populations than in polygamous populations ($F_{1,13} = 6.62$, $P = 0.0231$; Fig. 2), but the ecological context did not have a significant effect on the allometric slope for this trait ($F_{1,13} = 4.43$, $P = 0.0553$). The OLS slope estimate for male genital spine length was negatively allometric (slope < 1) in monogamous populations (slope \pm standard error = 0.29 ± 0.32 ; $t_{55} = -2.18$, $P = 0.0168$), but did not differ significantly from isometry (slope = 1) in polygamous populations (1.30 ± 0.32 ; $t_{55} = 0.85$, $P > 0.1$). Likewise, the RMA slope

Table 2. *F*-ratios from general linear mixed models of male and female genital trait sizes, including sexual selection (SS) and life-history selection (LH) as fixed factors, population nested within selection treatments (Pop(SS × LH)) as random factor, and elytron length (EL) as covariate. Models including SS × EL and LH × EL were used to test selection treatment effects on allometric slope (df = 1, 13). For traits that did not exhibit significant treatment effects on slope, the *F*-ratios shown for SS, LH and SS × LH (df = 1, 12) are based on a simplified model lacking interactions with EL. For random factors Pop(SS × LH) and Pop(SS × LH) × EL, tests are based on among-individual variation (males: df = 1, 68–80; females: df = 1, 48). Data were log-transformed and centred at a mean of zero prior to analysis. *F*-ratios significant at $\alpha < 0.05$ are highlighted in bold.

| Source | Male genital traits | | | | | | Female genital traits | | | |
|-------------------|---------------------|-----------------|-----------------|-----------------|-----------------|------------------|-----------------------|------------------------|---------------|--------------|
| | Aedeagus length | Aedeagus width | Spine length | Spine base area | Paramere length | Flap length | Rep. tract length | Conn. tissue thickness | Spine length | Spine number |
| LH | 0.583 | 0.269 | 4.329 | 0.371 | 1.972 | 8.592* | 0.080 | 0.192 | 0.013 | 0.040 |
| SS | 0.083 | 0.209 | 0.499 | 1.275 | 5.624* | 0.089 | 0.052 | 1.122 | 1.356 | 1.458 |
| LH × SS | 1.497 | 0.076 | 0.025 | 0.672 | 8.025* | 3.694 | 0.874 | 0.029 | 0.694 | 0.220 |
| EL | 0.083 | 14.720** | 12.064** | 8.189* | 3.169 | 35.549*** | 0.547 | 4.031 | 2.772 | 0.008 |
| LH × EL | 0.667 | 1.835 | 4.429 | 0.097 | 0.007 | 0.400 | 1.664 | 0.011 | 0.015 | 0.531 |
| SS × EL | 0.056 | 0.093 | 6.624* | 0.910 | 0.236 | 7.786* | 3.188 | 0.030 | 0.339 | 0.337 |
| Pop(LH × SS) | 0.661 | 1.858 | 1.011 | 2.375** | 1.228 | 1.267 | 1.298 | 0.703 | 1.681 | 1.859 |
| Pop(LH × SS) × EL | 0.640 | 1.305 | 0.587 | 1.547 | 0.720 | 0.449 | 0.836 | 1.035 | 2.096* | 1.457 |

* $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

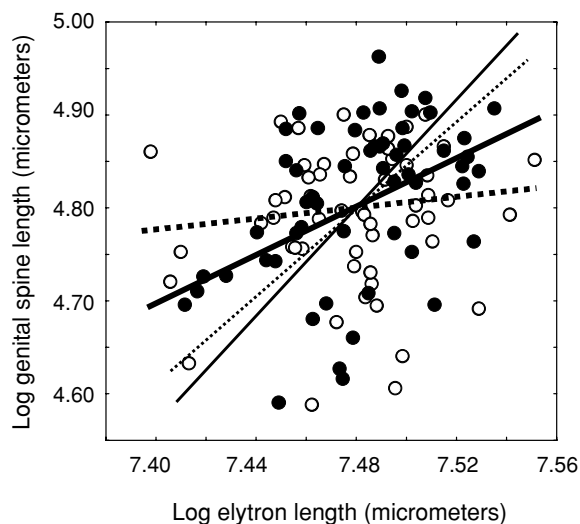


Figure 2. Static allometry of male genital spine length in monogamous (open points and dotted lines) and polygamous (closed points and solid lines) populations. Thick lines represent OLS slopes and thin lines represent RMA slopes.

estimate for male genital spine length was shallower in monogamous populations (slope = 2.41, 95% CL: 1.84–3.14) than in polygamous populations (slope = 2.91, 95% CL: 2.28–3.70), although this difference was not significant (Chi-square = 1.07, df = 1, $P > 0.3$). RMA slope estimates for both monogamous and polygamous populations were positively allometric (Chi-square > 54.24, df = 54, $P < 0.0001$).

For male flap length, static allometry OLS slope was significantly steeper in monogamous populations (slope = $0.97 \pm$

0.19) than polygamous populations (slope = 0.46 ± 0.27) ($F_{1,13} = 7.79$, $P = 0.0153$; Fig. 3), but ecological context did not affect the allometric slope for this trait ($F_{1,13} = 0.40$, $P > 0.5$). Flap length was negatively allometric (slope < 1) in polygamous populations ($t_{56} = -2.00$, $P = 0.0252$), but did not differ significantly from isometry (slope = 1) in monogamous populations ($t_{56} = -0.16$, $P > 0.5$). However, OLS and RMA regression yielded inconsistent results for this trait: RMA slope was shallower in monogamous populations (slope = 1.72, 95% CL: 1.37–2.15) than in polygamous ones (slope = 1.93, 95% CL: 1.48–2.51), although this difference was nonsignificant (Chi-square = 0.78, df = 1, $P > 0.3$). RMA slope estimates for both monogamous and polygamous populations were positively allometric (Chi-square > 25.47, df = 54, $P < 0.0001$). We also detected a significant effect of ecological context on mean flap length ($F_{1,12} = 8.59$, $P = 0.0126$), with greater mean flap length in populations selected for early reproduction than those selected for delayed reproduction. However, if the inconsistent difference in slopes between selection treatments is ignored, the simplified ANCOVA model yields a significant main effect of ecological context ($F_{1,12} = 6.15$, $P = 0.0290$) as well as a significant mating system × ecology interaction ($F_{1,12} = 7.21$, $P = 0.0199$), with greater mean flap length evolving under selection for early reproduction than under selection for delayed reproduction only under a polygamous mating system (Fig. 3).

Mean male paramere length was greater in polygamous populations than in monogamous ones ($F_{1,12} = 5.62$, $P = 0.0353$), but this difference largely reflected a sexual selection × life-history selection interaction ($F_{1,12} = 8.03$, $P = 0.0151$; Fig. 4). Male

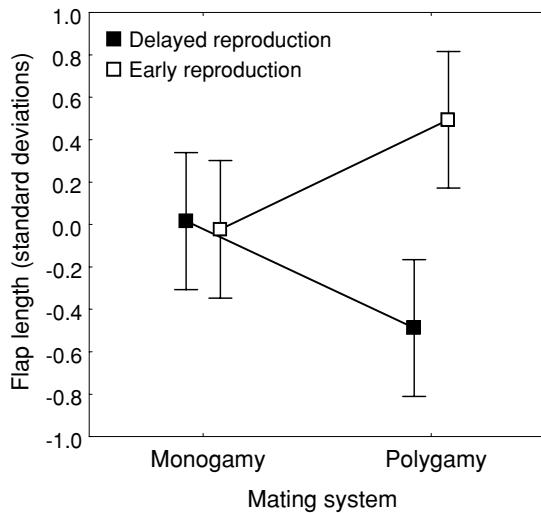
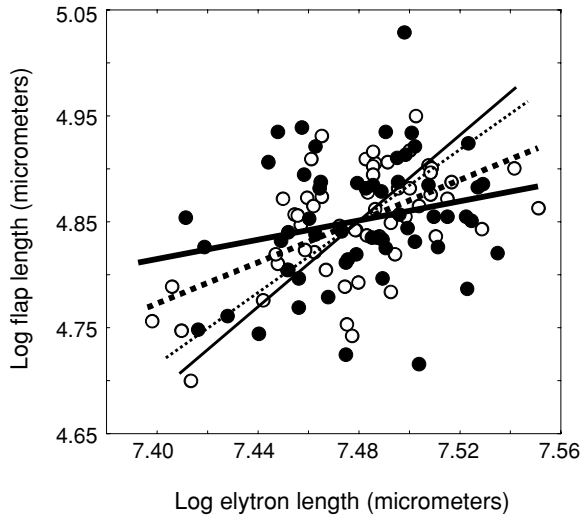


Figure 3. Selection treatment effects on male flap length. Top panel: Static allometry of male flap length in monogamous (open points and dotted lines) and polygamous (closed points and solid lines) populations. Thick lines represent OLS slopes and thin lines represent RMA slopes. Bottom panel: Interaction of mating system (monogamous vs. polygamous) and ecological context (selection for early vs. delayed reproduction) in the evolution of male flap length (bars depict standard errors of least-squares means for standardized data).

aedeagus length, aedeagus width, and spine-base area did not exhibit significant responses to selection treatments in allometric slope or mean size. However, we detected significant interpopulation variation in mean spine-base area (Table 2).

FEMALE GENITAL TRAITS

In contrast with males, there was no evidence of an evolutionary response in allometric slope or mean size of any female trait (Table 2). However, bursal spine length allometric slope exhibited significant interpopulation variation (Table 2).

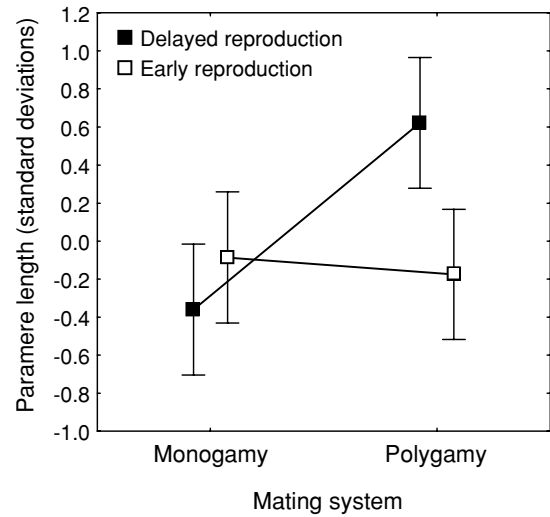


Figure 4. Interaction of mating system (monogamous vs. polygamous) and ecological context (selection for early vs. delayed reproduction) in the evolution of male paramere length (bars depict standard errors of least-squares means for standardized data).

Discussion

We manipulated the mating system within contrasting ecological contexts in 16 replicate populations of *C. maculatus*, and observed rapid evolution of some genital traits in males, but no evidence of genital evolution in females. Notably, male genital spines evolved a shallower static allometry in populations released from sexual selection and conflict through experimentally imposed monogamy, relative to populations maintained under polygamous mating. In addition, two nonintromittent genital traits (the parameres and flap) responded to manipulation of the mating system, as well as ecological context.

Our manipulation of the mating system altered both the opportunity for sexual selection, and the intensity of sexual conflict. Random, monogamous pairing eliminates male–male sexual competition, rendering redundant any trait that functions in such competition. If the expression of such traits imposes developmental costs, selection should favor their reduction in monogamous populations. Thus, for example, male genital traits that function in “copulatory courtship” and cryptic female mate choice (Eberhard 1991, 1994, 1996), or other processes that influence the outcome of sperm competition (Nessler et al. 2007; Simmons and Garcia-Gonzalez 2008), should evolve reduced expression in monogamous populations because sperm competition and female mate choice are precluded. If male secondary sexual traits cause direct harm to females, however, then selection should favor their reduction in monogamous populations for the additional reason that harmful traits reduce a male’s own fitness to the same extent that they harm his mate. Both classic sexual selection models and sexual conflict theory therefore predict the evolution of

reduced secondary sexual traits under random, lifetime monogamy (Hosken and Stockley 2004; Arnqvist and Rowe 2005).

Nonetheless, classic sexual selection and sexual conflict models yield contradictory predictions in relation to female mean fitness. Classic good genes and condition-dependent sexual selection theory predict that male–male competition will favor sires in high phenotypic condition, and carrying alleles that confer high viability (good genes). For example, *C. maculatus* males transfer large ejaculates containing nutrients and water that could enhance female fitness (Savalli and Fox 1999; Fox et al. 2006; Ursprung et al. 2009); ejaculate size and quality are expected to reflect male phenotypic condition which, in turn, is expected to reflect both environmental and genetic quality (Rowe and Houle 1996; Agrawal 2002). Under classic models, polyandry will thus confer benefits (or at least impose no net costs) on females (Zahavi 1975; Borgia 1979; Nur and Hasson 1984; Rowe and Houle 1996), and evolution under experimentally imposed random, lifetime monogamy should lead to reduced mean fitness in females because such a mating system will promote the accumulation of deleterious mutations, and fail to promote the spread of “good genes” (Agrawal 2001; Lorch et al. 2003). In contrast, sexual conflict theory suggests that male–male competition can favor male adaptations that confer a fertilization advantage while imposing collateral harm on females (Parker 1979; Arnqvist and Rowe 2005), or even select for harmfulness per se, because injured females may elevate their reproductive rate as a form of “terminal investment” (Johnstone and Keller 2000). Because release from sexual conflict should cause such harmful male traits to be opposed by selection, evolution under random, lifetime monogamy is expected to lead to enhanced mean female fitness (Kokko and Brooks 2003; Rowe and Day 2006).

In this light, our findings suggest that sexual conflict plays a key role in *C. maculatus*, and is likely to have driven the evolution of genital spine allometry. Female lifetime productivity in within-population crosses evolved to be greater in our monogamous populations than in the polygamous ones (Maklakov et al. 2009), supporting the conclusion from short-term studies on seed beetles (den Hollander and Gwynne 2009; Gay et al. 2009; Maklakov and Arnqvist 2009; Ursprung et al. 2009) that the potential benefits of polyandry do not generally compensate for direct harm. The evolution of reduced harm under random monogamy is also generally consistent with results of evolution experiments on other insects (e.g., Holland and Rice 1999; Martin and Hosken 2003; Tilszer et al. 2006; reviewed in Edward et al. 2010). We cannot eliminate the possibility that intertreatment differences in mating rate contributed to selection on the genitalia in our experiment (see Reuter et al. 2008), although we note that behavioral assays yielded no evidence of evolved intertreatment differences in propensity to mate (Maklakov et al. 2010).

The evolution of shallower allometry of the male genital spines under monogamy (Fig. 2) may have contributed to the fitness advantage of females in monogamous lines. The largest males are likely to be the most harmful for a variety of reasons (Pitnick and Garcia-Gonzalez 2002; Friberg and Arnqvist 2003). In *C. maculatus*, even if spine length did not covary with male body size, their wider (i.e., thicker) aedeagus would tend to drive the spines deeper into the female reproductive tract wall. If genital spine length is also disproportionately greater in the largest males, as suggested by both OLS and RMA slope estimates for our polygamous populations, then the level of harm to females may escalate very steeply with increasing male body size. Under monogamy, selection should therefore favor reduced spine length most strongly in the largest males, resulting in a reduced static allometry slope for this trait. An alternative potential target of selection for reduced harmfulness—aedeagus width—may be constrained by strong stabilizing selection on the diameter of the aedeagus lumen, reflecting the biomechanics of sperm transfer. The observed reduction in genital spine allometry furnishes experimental support for the view that the genital spines impose direct, net harm on females (Crudginton and Siva-Jothy 2000; Eady et al. 2007; Rönn et al. 2007; Hotzy and Arnqvist 2009), although male ejaculate fluids and harassment behaviors may also contribute to direct harm (den Hollander and Gwynne 2009; Gay et al. 2009). Importantly, the reduction in male genital spine length under monogamy was robust to ecological context, suggesting that this key prediction is valid against diverse backgrounds of adaptive evolution.

Our results provide the first experimental demonstration (to our knowledge) of the evolution of secondary sexual trait static allometry in response to a change in the intensity of sexual selection. Some secondary sexual traits exhibit unusually high allometric slopes (Emlen and Nijhout 2000; Kodric-Brown et al. 2006), but many secondary sexual traits exhibit shallow static allometries (Bonduriansky 2007), and the causal link between sexual selection and elevated static allometry remains poorly understood (Bonduriansky and Day 2003). A direct role for sexual selection in maintaining elevated static allometry has never before (to our knowledge) been demonstrated experimentally.

The role of sexual selection in the evolution of static allometry is particularly unclear for genital traits, which often exhibit shallow static allometries (Eberhard et al. 1998; Eberhard 2010), even when they experience strong directional selection (Bertin and Fairbairn 2007). Our results demonstrate that sexual selection maintains elevated static allometry of the male genital spines in *C. maculatus*, because removal of sexual selection resulted in the rapid evolution of reduced static allometry for this trait. This finding also shows that sexual selection can drive the evolution of positive static allometry for some genital traits: the RMA allometric slope for the male genital spines (especially in our

polygamy lines) is comparable to RMA slopes for many nongenital signal and weapon traits (see Kodric-Brown et al. 2006), and exceeds RMA slopes for many nongenital traits in *C. maculatus* (Colgoni and Vamosi 2006). Positive static allometry of genital components reflects a high degree of phenotypic variation, and thus conflicts with the lock-and-key and one-size-fits-all hypotheses, which predict strong stabilizing selection on genital size (Eberhard et al. 1998; Eberhard 2010). Rather, our findings suggest that, like nongenital signal and weapon traits (Bonduriansky 2007), some genital traits evolve elevated allometric slopes (e.g., the male genital spines) or intercepts (e.g., the male parameres) in response to intense sexual selection and conflict, whereas others do not respond in this way, for reasons that remain unknown.

In addition to the male genital spines, we detected evolution of the two nonintromittent male genital appendages: the flap and parameres. Importantly, both of these structures appeared to evolve in response to an interaction of mating system and ecological context. Our findings for these traits illustrate the potential for ecological context to influence the response of genital traits to sexual selection and conflict.

The flap is located at the apex of the retracted aedeagus, and endowed with fine bristles (Fig. 1). This small appendage could therefore be a sensory structure that functions in guiding the aedeagus into the female genital opening, or a stimulatory structure (“titillator”: Mukerji and Bhuya 1937) that induces females to allow intromission. We found some evidence of the evolution of OLS allometric slope (steeper in monogamous lines than in polygamous ones) for flap length. However, because RMA regression suggested the opposite pattern (Fig. 3), this result must be regarded as tentative. We also detected a strong effect of ecological context and (in a reduced model) an interaction with mating system: under a polygamous mating system, mean flap length was greater in populations selected for early reproduction than in populations selected for delayed reproduction, whereas monogamous populations exhibited intermediate flap length (Fig. 3).

The male parameres form a large, forked structure (Fig. 1) that may support the aedeagus and guide it into the female genital opening. Mukerji and Bhuya (1937) observed that “males palpate the external genital aperture of the female by the apex of the parameres.” We did not detect any treatment effects on allometric slope for paramere length, but we found an interaction between mating system and ecological context for this trait, with the longest parameres occurring under delayed reproduction and polygamy.

Intriguingly, both flap length and paramere length were similar across ecological contexts under monogamy, but diverged in contrasting ways between ecological contexts under polygamy (Figs. 3 and 4): mean flap length was greatest under selection for early reproduction, whereas paramere length was greatest under selection for delayed reproduction. Populations experiencing

the combination of polygamy and delayed reproduction (wherein only eggs laid > 24 h post-emergence contributed to the next generation) may be subject to intense sexual conflict over the timing of mating: selection on males to mate earlier than their rivals will conflict with selection on females to delay oviposition (Maklakov et al. 2010). Our findings therefore suggest that sexual (perhaps sexually antagonistic) selection in premating interactions acts in opposite ways on the size of the paramere and flap under contrasting regimes of selection on the timing of reproduction. Detailed observations of genital function in precopulatory interactions and during copulation, perhaps combined with experimental manipulation of the sizes of these structures, are needed to illuminate the causes of this result.

Lack of detectable response in female genital traits is consistent with the expectation that, under random monogamy, females’ defensive traits (such as the thickened connective tissue in the reproductive tract wall) impose less-immediate fecundity and viability costs than males’ offensive traits impose. If males were harmful at the start of the experiment, but gradually evolved to become less so when released from sexual conflict, selection would only have favored reduced investment in females’ defensive traits once those defenses were more than sufficient to mitigate male harm—for example, when male genital spines have diminished to the point where a thinner reproductive tract wall is sufficient to mitigate puncture-damage, so that females are over-investing in their defenses. Thus, whereas selection will immediately favor reduced expression of harmful traits in males, reduced expression of females’ defensive traits will commence after a delay of several generations. The 18–21 generations of evolution in our experiment may therefore have provided insufficient time for the evolution of reduced defenses in females. The magnitude of the delay in female response may vary among species. For example, Wigby and Chapman (2004) detected evolution of female defenses after 26 generations of selection under differential intensities of sexual conflict in *D. melanogaster*, a species where male-imposed harm results from ejaculate toxicity (Chapman et al. 1995; Wolfner 1997; Wigby and Chapman 2005) and harassment (Partridge and Fowler 1990). It is, of course, also possible that the lack of detectable evolution in female traits reflects a true failure to respond to mating system and life-history manipulation (for reasons unknown) rather than a delayed response.

Females’ bursal spines offer an intriguing comparison with male genital spines. The blade-like bursal spines point toward the genital opening (Fig. 1), and may limit the extent of intromission and, potentially, damage the male aedeagus. Despite the apparent similarity to the male genital spines, however, our findings suggest that the bursal spines do not harm males in an analogous way to the harm inflicted by male spines on females. Even if males are damaged by the bursal spines, selection will not favor reduction of these spines in monogamous populations unless such damage

impedes ejaculate transfer and reduces female fecundity, or the expression of the spines imposes substantial developmental costs on females. An alternative, nonantagonistic, function for the female spines may be to puncture spermatophores (J. Rönn and G. Arnqvist, pers. Comm.). Like the male genital spines (Rönn et al. 2007), the female bursal spines exhibit considerable interspecific variation in form, size and number (Southgate 1971; Kingsolver 1979; Anton 2000; Mergen 2004).

CONCLUSIONS

Our results address a long-standing controversy (Hosken and Stockley 2004; Bertin and Fairbairn 2007; Bonduriansky 2007; Eberhard 2010) by showing that some genital traits can respond to sexual selection and conflict in the same way as other secondary sexual traits—via evolution of allometric slope and/or mean trait size. Release from sexual selection and conflict drove rapid evolution of static allometry of the male genital spines, corroborating the prediction that this apparently harmful male trait will evolve reduced expression if male–male competition is eliminated. The evolution of a shallower static allometry slope is expected if spine length is subject to negative directional selection in the largest (perhaps most harmful) males, but stabilizing selection in small males. Alongside the increased mean fitness in our monogamous lines (Maklakov et al. 2009), the relative reduction in allometric slope of the male genital spines under monogamy supports the view that the male genital spines impose net fitness costs on females. In addition, two nonintromittent male genital traits evolved in response to the interaction of sexual selection and ecology, illustrating the potential importance of ecological context in shaping evolutionary responses to sexual selection and conflict (Maklakov et al. 2009, 2010; Edward et al. 2010). We also observed asymmetric responses across the sexes, with several male traits but no female traits responding to selection treatments. Lack of response in females may reflect delayed selection for deescalation of defensive traits. Our findings illustrate the complexity of genital coevolution, and highlight the need for observational and experimental studies to illuminate the precise roles of the various components of the genital machinery of each sex.

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