

Evolutionary Response to Sexual Selection in Male Genital Morphology

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Summary

Male genital morphology is characterized by two striking and general patterns of morphological variation: rapid evolutionary divergence in shape and complexity, and relatively low scaling relationships with body size. These patterns of variation have been ascribed to the action of sexual selection [1, 2]. Among species, monogamous taxa tend to have relatively less complex male genital morphology than do polygamous taxa [3]. However, although variation in male genital morphology can be associated with variation in mating [4, 5] and fertilization success [6–10], there is no direct evidence that sexual selection generates the evolutionary changes in male genital shape that underlie observed macro-evolutionary patterns. Moreover, the hypothesis that sexual selection acts to reduce the scaling relationship between body and genital size is based entirely on the theoretical argument that male genitalia should be selected to provide an appropriate mechanical and/or stimulatory fit to the most commonly encountered female genitalia [2, 11]. Here, using the dung beetle *Onthophagus taurus*, we combine the power of experimental evolution with multivariate selection and quantitative genetic analyses to provide the most comprehensive evidence available of the form and evolutionary consequences of sexual selection acting on male genital morphology.

Results and Discussion

During courtship, male dung beetles mount the female and tap her dorsum and flanks with their forelimbs, while reaching with their aedeagus to make contact with her genital opening [12]. The distal hooks of the aedeagus engage with pits on the internal surface of the female's terminal segments, following which the endophallus is inflated within the female's genital tract, and five functionally integrated genital sclerites deliver the ejaculate to an opening in the duct that leads to the female's sperm storage organ [13]. We compared morphological variation in the aedeagus and genital sclerites of male beetles from three populations that had been evolving for 19 generations under sexual selection with three populations that had been evolving for 19 generations under enforced monogamy, finding significant evolutionary divergence in the morphology of the aedeagus.

Principal component analysis (PCA) of eight measures of the aedeagus returned three components with eigenvalues greater than 1, which collectively explained 68% of the variance in aedeagus morphology (Figure 1A; see also Supplemental Experimental Procedures and Table S1 available online). We found significant divergence among our lines in aedeagus shape, described by the second principal component (PC2) (Table S2; Figure 1B). This component explained 19% of the variation in aedeagus morphology and contrasted positive values of the linear distances between landmarks B and C, F and G, and G and A with negative values of the linear distance between landmarks A and B (Figure 1A; Table S1). The first component accounted for 35% of the variation and was loaded positively by 7 of 8 aedeagus measurements, thus providing an overall measure of aedeagus size (Table S1). However, there was no significant divergence among our lines in this component of variation (Table S2). Thus, after 19 generations, aedeagus shape, but not size, had diverged in response to sexual selection: males evolving under sexual selection had positive scores on PC2, and thus had an aedeagus with longer and thinner parameres and a longer phallobase than did males evolving under enforced monogamy (Figure 1). We found no significant evolutionary divergence in male body size (Table S3). Neither did we find significant divergence in the PC scores that captured morphological variation in the five genital sclerites of the endophallus (Figure 2; Tables S4 and S5).

To examine the form and strength of selection acting on the aedeagus and genital sclerites, we calculated standardized linear and nonlinear selection gradients [14] acting on these traits within the natural population of beetles used to source our selection lines. We examined selection acting on aedeagus morphology via a male's ability to engage the female in copula. The PCA that described variation in size and shape of the aedeagus in the source population (Table S6) was similar in structure to the PCA conducted on males derived from our lines after 19 generations of selection (Table S1). However, for the source population of beetles, PC3 described overall variation in aedeagus size (Table S6). Selection analysis revealed linear selection acting on the second PC describing variation in aedeagus morphology (Figure 3; Tables S6 and S7); PC2 accounted for 20% of the total variation and contrasted negative values of the linear distances between landmarks A and B, B and C, and G and A with positive values of linear distances between E and F and F and G (Figure 1A). Negative scores on PC2 were thus associated with long, thin parameres and a long phallobase, an aedeagus shape that was favored by directional selection (Figure 3). Aedeagus size (PC3) was not subject to significant selection (Table S7).

The five genital sclerites are housed within the phallobase and function only once genital contact has been established and the endophallus has been inflated within the bursa copulatrix [13]. Previously, variation in the morphology of the genital sclerites was found to predict variation in paternity when males compete for fertilizations [9]. However, the form of post-copulatory sexual selection acting on the sclerites is unknown. The five genital sclerites form a single functional unit [13], so we used multivariate selection analysis to examine the form of selection operating on this functional unit. We estimated

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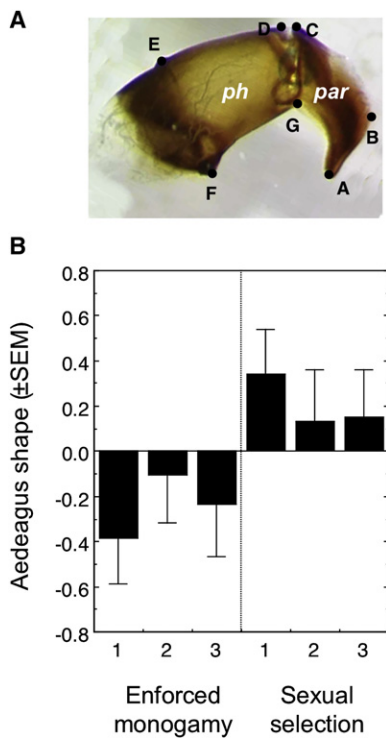


Figure 1. Evolutionary Divergence in the Shape of the *Onthophagus taurus* Aedeagus after Nineteen Generations of Experimental Evolution (A) Landmarks A–G used to measure the dimensions of the two components of the aedeagus, the phallobase (*ph*) and the parameres (*par*), which are delineated by the landmarks D–E–F–G and A–B–C–G, respectively. (B) Variation in the second principal component describing variation in aedeagus morphology among divergent lines. Positive scores characterize a long, thin aedeagus; negative scores characterize a short, thick aedeagus (see Supplemental Data). Data are presented as mean \pm SEM.

male fitness in two contexts: when a male was first to mate (defensive role in sperm competition), and when a male was second to mate (offensive role in sperm competition) with a doubly mated female. In the defensive role, multivariate selection analysis revealed significant nonlinear selection along 4 of 5 eigenvectors from a canonical analysis of the matrix of standardized nonlinear selection gradients (Tables S8–S10). The eigenvalues illustrate how selection acts on the configuration of the genital sclerites within the functional unit of the endophallus: for example, along the eigenvector m_5 in

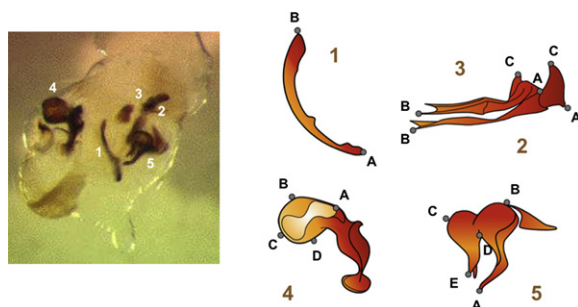


Figure 2. The Five Genital Sclerites of the *Onthophagus taurus* Endophallus. The sclerites are shown in situ on the left; the positions of landmarks used to measure their dimensions are shown on the right.

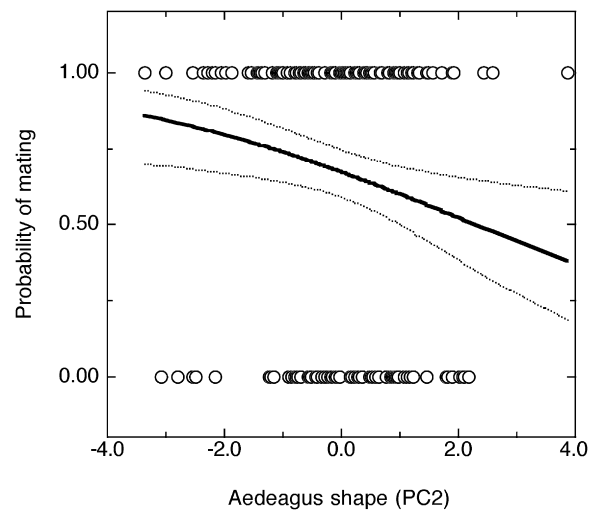


Figure 3. Cubic Spline Visualization of Sexual Selection Operating on the Shape of the Aedeagus of *Onthophagus taurus*. The spline corrects for variation in fitness resulting from pronotum width and was fitted by minimizing the generalized cross-validation score with Schluter’s generalized linear modeling software (GLMS) 4.0. The standard error of the spline was calculated from 50 bootstrap replications.

Figure 4, selection acts on the size of sclerite 2 relative to the sizes of sclerites 3–5 (Table S10). Visualization of the fitness surface for the strongest positive and negative eigenvectors suggests that nonlinear selection is stabilizing along the m_5 axis and disruptive along the m_1 axis (Figure 4). Qualitatively similar patterns were found from the canonical analysis of the genital sclerite morphology of males mating in the role of second male (Tables S11–S13). These broadly similar patterns

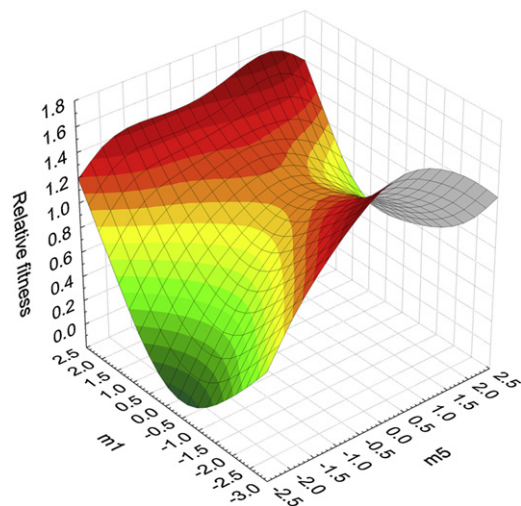


Figure 4. Visualization of Postcopulatory Sexual Selection Operating on the Genital Sclerites of Male *Onthophagus taurus* Mating in the Defensive Role of First Male. The surface is a thin-plate spline of the strongest positive and negative eigenvectors from a canonical analysis of the matrix of standardized quadratic and correlational selection gradients for individual sclerites and was fitted with a stiffness parameter of 0.25 using the statistical software package Statistica 6.0. Surface colors correspond to the highest (red) and lowest (green) fitness.

Table 1. Descriptive Phenotypic and Genetic Statistics for Measures of the Aedeagus

Measure	Mean ± SD	Sire h^2 ± SE	Dam h^2 ± SE	CV _P	CV _A	CV _R
A-B	0.55 ± 0.03	0.37 ± 0.13**	0.52 ± 0.18	6.03	1.84	5.74
B-C	0.80 ± 0.03	0.21 ± 0.14*	0.44 ± 0.16	4.24	0.98	4.12
C-G	0.47 ± 0.02	0.40 ± 0.16**	0.28 ± 0.21	5.21	1.66	4.93
D-E	0.78 ± 0.03	0.21 ± 0.14*	0.44 ± 0.16	4.34	1.00	4.23
E-F	0.74 ± 0.03	0.56 ± 0.19***	0.53 ± 0.19	6.19	1.55	5.99
F-G	0.71 ± 0.04	0.68 ± 0.16***	0.18 ± 0.16	6.34	2.61	5.78
G-D	0.46 ± 0.02	0.37 ± 0.15***	0.41 ± 0.20	4.59	1.40	4.37
G-A	0.60 ± 0.04	0.57 ± 0.14***	0.42 ± 0.16	6.52	2.45	6.04

The following abbreviations are used: SD, standard deviation; SE, standard error; CV_P, coefficient of phenotypic variation; CV_A, coefficient of additive genetic variation; CV_R, coefficient of residual variation. *p < 0.05, **p < 0.01, ***p < 0.001.

of selection for first and second males are consistent with the previous finding that males successful in the offensive role of sperm competition are also successful in the defensive role [15]. However, the strength of the nonlinear selection on the genital sclerites was greater when males were first to mate ($F_{5, 204} = 2.289$, $p = 0.047$; contrast Table S10 and Table S13).

The different forms of selection that we have observed acting on the aedeagus and genital sclerites can account for the different patterns of evolutionary divergence that we have documented in our evolving lines. Directional selection on aedeagus shape was associated with an evolutionary divergence in the direction expected from the observed selection gradients. Interestingly, evolutionary divergence in the shape of the aedeagus has been found among natural populations of *O. taurus* [16] that appear to differ in the strength of sexual selection [17], and this divergence is qualitatively similar to that seen among closely related species [16]. In contrast, the genital sclerites were subject to patterns of nonlinear selection characteristic of stabilizing and disruptive selection, and accordingly, we found no directional response to sexual selection. Our data are thus consistent with the notion that different male genital traits may be subject to different tempos and modes of evolution [18, 19].

Patterns of selection acting on male sexual traits have been inferred from the patterns of phenotypic variation in those traits, although the validity of this approach has generated considerable debate [5, 11]. Our data allow us to directly assess the impact of sexual selection on patterns of variation in male genital morphology. Analyses of quantitative genetic variation in the genital sclerites found very low levels of additive genetic variation and low and nonsignificant values of heritability; coefficients of additive genetic variation (CV_A) in 23 measures of the genital sclerites ranged from 0 to 1.46 with a mean of 0.55 ± 0.11 [20]. Here we estimated the levels of additive genetic variation in aedeagus measurements with a half-sibling quantitative genetic design. The linear distances between the eight aedeagal landmarks exhibited significant heritabilities (Table 1), and the mean coefficient of additive genetic variation in aedeagal traits (1.69 ± 0.21) was on average three times greater than that for genital sclerites ($t = 4.99$, $df 29$, $p < 0.001$). Coefficients of phenotypic variation (CV_P) did not differ (CV_P sclerites = 6.46 ± 0.51 , aedeagus = 5.43 ± 0.34 ; $t = 1.14$, $df 29$, $p = 0.262$). This illustrates the importance of estimating additive genetic variation rather than phenotypic variation: only additive genetic variation provides an estimate of the evolvability of traits [21]. Our data show that aedeagus traits subject to directional precopulatory sexual selection harbor greater additive genetic variance than do genital sclerite traits that are subject to nonlinear

postcopulatory selection. As such, aedeagus traits are expected to have a greater evolvability, an expectation confirmed by the response to sexual selection on the aedeagus but not the genital sclerites observed in our experimentally evolving lines.

Sexual selection has also been argued to be responsible for the generally low scaling relationships between male genitalia and body size [2, 11]. As is typically found in arthropods, the genital sclerites and the aedeagus of *O. taurus* do have relatively low allometric slopes (Table S14). However, contrary to expectation [2], the intromittent traits (sclerites) do not have relatively lower allometric slopes than nonintromittent traits (the aedeagus); although subject to different forms of sexual selection, the mean allometric slopes for aedeagus (0.24 ± 0.06) and genital sclerite measures (0.24 ± 0.08) do not differ (Table S14). Neither did we find any evolutionary divergence in the scaling relationships with body size of the aedeagus or the genital sclerites that could be attributed to sexual selection (Tables S15 and S16). Clear patterns of low genital allometry seem to be restricted to arthropods, with evidence for more variable patterns in vertebrates [2]. We suggest therefore that the nutrition-insensitive or canalized patterns of growth that protect genitalia from competition for resources during development [22, 23] may underlie the observed patterns of low genital allometry typically found in arthropods.

In conclusion, we have documented divergence in male genital morphology among populations of dung beetles evolving under sexual selection or enforced monogamy. Genital traits characterized by directional selection exhibited significant divergence, whereas those characterized by nonlinear selection did not. Our empirical data allow a direct assessment of how sexual selection influences patterns of variation in male genital morphology and suggest that allometric scaling of genitalia cannot be used to infer underlying patterns of selection.

Experimental Procedures

Experimental Evolution

Full details of the origin and maintenance of the populations used in this study are provided elsewhere [24]. Briefly, six populations were established from second-generation beetles bred from a population collected from a dairy farm in Byford, Western Australia. The six populations had effective population sizes of ~100 and differed only in their opportunity for pre- and postcopulatory sexual selection. In monogamous populations, females were randomly allocated a single male for a period of 7 days, so that all males had sufficient opportunity to overcome any potential barriers to mating. In sexual selection populations, groups of ten males and ten females were housed together for 7 days. We used molecular markers to estimate that females in these populations mated with an average of 4.5 ± 0.2 males (range 1–8), among whom paternity was on average distributed equally [24, 25]. The standardized slope of male fitness on number of mates

(Bateman's gradient) was 0.83 ± 0.02 , indicative of strong sexual selection in these populations [24, 25]. Populations were allowed to evolve for a period of 4 years, or 19 generations, before being used in this study.

For our experimentally evolving lines, the independent units for statistical analyses were populations ($n = 3$ per treatment). We thus conducted analyses of variance with replicate line nested within selection history as a random factor to obtain the appropriate error term for the effect of selection history. To examine homogeneity of scaling relationships between genital size and body size across selection histories, we took the Z score for pronotum width to obtain statistical properties identical to the PC scores of the genital traits (see [Supplemental Experimental Procedures](#)) and used this standardized pronotum width as a covariate, testing for homogeneity of slopes as the significance of the interaction term between selection treatment and standardized pronotum width, with the interaction between pronotum width and replicate line nested within selection history as the appropriate error term.

Selection on Aedeagus Morphology

We established each of 187 male beetles within artificial tunnels and allowed them to court and attempt to copulate with a randomly allocated virgin female. Our procedure thus simulated natural matings that occur when a single male encounters a female within her breeding tunnel [26]. Males that successfully engaged in genital coupling within 30 min of the onset of courtship received a fitness score of 1; those that failed to successfully couple received a fitness score of 0. Because there was no direct male contest competition, any selection observed on the aedeagus under this protocol could be attributable only to female choice and/or mechanical constraint. However, we note that in a promiscuous species with intense male-male competition, the ability to rapidly engage in copula would be critical if a male is to gain a share in paternity. There was no significant effect of female size on a male's ability to engage in copula (nominal logistic regression: $\chi^2 = 0.15$, $df = 1$, $p = 0.69$). Following mating trials, the male's aedeagus was removed, photographed, and measured (see [Supplemental Experimental Procedures](#)). A PCA of this data set returned three components that collectively explained 65% of the variation in aedeagus size and shape (Table S6). PC scores have a mean of 0 and a standard deviation of 1, making them ideal for selection analyses [14]. Relative fitness, calculated as individual fitness divided by the population mean fitness, was regressed on the three PCs and standardized male body size. Separate regression models were run to estimate linear and nonlinear selection gradients. Note that univariate selection analyses can underestimate the strength of nonlinear selection [27], so we also conducted multivariate selection analyses on the three PCs, as described below for the five genital sclerites. However, no evidence for nonlinear selection was found with the multivariate approach, so only the univariate analysis is presented here.

Selection on Genital Sclerite Morphology

We used the irradiated male technique to estimate the paternity of a focal male when a female had mated with two males. Detailed methods adopted for the collection of the data analyzed here are provided elsewhere [9, 15, 28]. In summary, virgin females were introduced into artificial tunnels and allowed to mate with two males, one of which had been irradiated by a 10 krad dose of γ radiation. We estimated the paternity success of unirradiated males when they mated either before (103 males) or after (113 males) an irradiated male as the proportion of eggs that hatched. For unirradiated males, measures of each sclerite were subjected to separate PCAs, providing a single component that described variation in the morphology of each sclerite (Table S8). The proportion of offspring sired was converted to relative fitness, and separate regressions were run to extract standardized univariate linear and nonlinear selection gradients acting on the five genital sclerites [27, 29]. We diagonalized the γ matrix through canonical analysis and tested for multivariate linear and nonlinear selection along the major axes. We compared the fitness surfaces for first and second males to mate following the methods outlined in [30].

Heritability of Aedeagus Morphology

Using established protocols [31], we paired each of 67 males with a mean of 3.0 ± 0.1 (range 2–4) virgin females and allowed them to mate for 1 week before establishing females in individual breeding chambers to produce broods. When offspring emerged, we measured the aedeagus of an average of 3.4 ± 0.1 (range 1–6) male offspring per dam family. For hypothesis testing, we used nested analysis of variance, with dams nested within sires as a random factor [32]. Analyses of genetic variation were conducted with sire and dam variance components estimated from the restricted maximum

likelihood procedures in S-Plus [33]. Heritabilities and their standard errors were calculated by jackknifing across paternal half-sibling (sire estimates) families. Coefficients of phenotypic, additive genetic, and residual variation were calculated following [21].

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and sixteen tables and can be found with this article online at [http://www.cell.com/current-biology/supplemental/S0960-9822\(09\)01332-3](http://www.cell.com/current-biology/supplemental/S0960-9822(09)01332-3).

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