

# Phenotypic Engineering Unveils the Function of Genital Morphology

Cosima Hotzy,<sup>1</sup> Michal Polak,<sup>2</sup> Johanna L. Rönn,<sup>3</sup> and Göran Arnqvist<sup>3,\*</sup>

<sup>1</sup>Evolutionary Biology, Department of Ecology and Genetics, Uppsala University, Norbyvägen 18D, 752 36 Uppsala, Sweden

<sup>2</sup>Department of Biological Sciences, University of Cincinnati, Cincinnati, OH 45221, USA

<sup>3</sup>Animal Ecology, Department of Ecology and Genetics, Uppsala University, Norbyvägen 18D, 752 36 Uppsala, Sweden

## Summary

The rapidly evolving and often extraordinarily complex appearance of male genital morphology of internally fertilizing animals has been recognized for centuries [1]. Postcopulatory sexual selection is regarded as the likely evolutionary engine of this diversity [2], but direct support for this hypothesis is limited. We used two complementary approaches, evolution through artificial selection and microscale laser surgery, to experimentally manipulate genital morphology in an insect model system. We then assessed the competitive fertilization success of these phenotypically manipulated males and studied the fate of their ejaculate in females using high-resolution radioisotopic labeling of ejaculates. Males with longer genital spines were more successful in gaining fertilizations, providing experimental evidence that male genital morphology influences success in postcopulatory reproductive competition. Furthermore, a larger proportion of the ejaculate moved from the reproductive tract into the female body following mating with males with longer spines, suggesting that genital spines increase the rate at which seminal fluid passes into the female hemolymph. Our results show that genital morphology affects male competitive fertilization success and imply that sexual selection on genital morphology may be mediated in part through seminal fluid [3].

## Results and Discussion

In the vast majority of animal taxa where females mate with more than one mate, reproductive competition between males continues beyond mating [4]. Within the female, ejaculates from several males will compete for fertilization of her eggs, either directly through sperm competition [4] or indirectly through cryptic female choice [5]. Male traits that increase fertilization success in this competition are favored by postcopulatory sexual selection. A range of male traits, including sperm morphology and number, mate guarding, copulatory courtship behavior, copulatory plugs, and seminal fluid substances with hormonal effects in females, have evolved by postcopulatory sexual selection [4–6]. The morphology of male intromittent genitalia shows an almost explosive diversification in many groups, and genital shape is often the only trait that allows closely related species to be distinguished [1, 2, 7]. Postcopulatory sexual selection is currently regarded as the

likely evolutionary engine of this diversity [2]. Yet, support for this longstanding hypothesis is limited to comparative and correlational studies [7–12], despite the fact that genital diversification is a major evolutionary trend in animals.

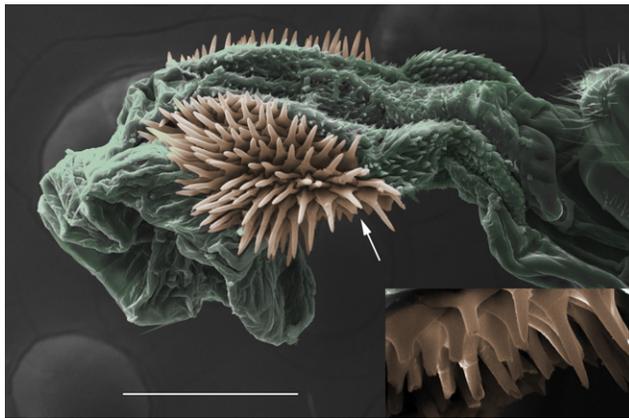
Seed beetles (Coleoptera, Bruchidae) are a model system in the study of postcopulatory sexual selection (e.g., [12–17]). They are typical of many internally fertilizing animals: both males and females mate with multiple mates, and the key taxonomic character is the morphology of the male genitalia. In many seed beetles, male genitalia are equipped with species-specific sclerotized spines (Figure 1) that partly penetrate the wall of the female reproductive tract during copulation [12, 18, 19]. Various spiny structures are a prominent feature of male genital morphology in many groups, ranging from nematodes, mollusks, and insects to lizards and mammals [1]. Here, we experimentally manipulated genital spine length in the seed beetle *Callosobruchus maculatus* (see Supplemental Experimental Procedures available online) both (1) genetically, by artificial selection for relatively long and short genital spines for several consecutive generations, and (2) phenotypically, by shortening genital spines using microscale laser surgery [20] (Figure 1). We then performed experimental assays of the reproductive performance of males with relatively short versus long spines and, in addition, studied the fate of their ejaculate within females using high-resolution radioisotopic labeling of ejaculates. Our main objective was to assess the effects of genital morphology on competitive fertilization success in males using two independent and complementary experiments.

Male genital spine length showed a highly significant evolutionary response to the divergent artificial selection imposed (Figure S1; lateral spines:  $F_{1,7} = 7.75$ ,  $p = 0.027$ ; ventral spines:  $F_{1,7} = 48.12$ ,  $p < 0.001$ ), but male body size did not show any correlated response to selection ( $F_{1,7} = 0.23$ ,  $p = 0.649$ ; Table S1). On average, males in the “long” selection lines evolved ventral and lateral spines that were 0.9 and 0.6 standard deviations longer, respectively, than those of males in the “short” lines. Given the strength and duration of selection, this yields a conservative estimate of the average heritability of spine length of  $h^2 = 0.24$  and 0.16, respectively.

Sperm competition assays showed that males from “long” selection lines enjoyed a higher postcopulatory competitive fertilization success compared to males from “short” lines ( $t_4 = 5.92$ ,  $p = 0.004$ ; Table S2). This effect was mirrored in the experiments using laser-ablated males (Figure 2), although the effect of spine length here was marginally nonsignificant (Table 1). However, these two independent experiments test a common hypothesis. We thus employed a combined probability test, which showed that males with shorter genital spines indeed suffered a reduced competitive fertilization success (Fisher’s combined probability test,  $\chi^2_4 = 16.33$ ,  $p = 0.002$ ). Our experiments hence provide evidence for a direct and causal effect of genital morphology on competitive fertilization success.

Although our main objective was to assess the ultimate effect of genital morphology on male competitive fertilization success, our results also shed some light upon the proximate mechanisms by which genital spines affect male fertilization

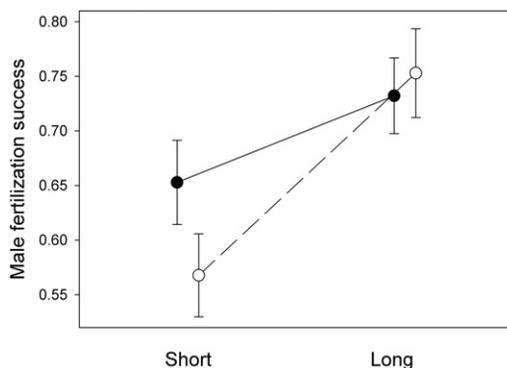
\*Correspondence: [goran.arnqvist@ebc.uu.se](mailto:goran.arnqvist@ebc.uu.se)



**Figure 1. Male Genitalia of *Callosobruchus maculatus***

Various spine-like structures are a prominent feature of the male genitalia in many animals with internal fertilization. In the seed beetle *Callosobruchus maculatus*, the male genitalia is equipped with elaborated spines on the lateral and ventral sides. We manipulated the length of these spines by high-precision microscale laser ablation (arrow indicates area with ablated ventral spines). Inset at lower right shows a magnification of an area with several ablated spines. Scale bar represents 200  $\mu\text{m}$ . (Scanning electron micrographs by N. Kaval.)

success. It has been hypothesized that genital spines can be favored by postcopulatory sexual selection through either of two non-mutually exclusive routes. First, spines may serve as a hold-fast device that allows males to prolong copulation duration and thus increase the amount of ejaculate or the number of sperm transferred [21]. Our assays showed that matings involving males with long versus short spines did not differ significantly in copulation duration (selection lines:  $t_4 = 1.35$ ,  $p = 0.247$ ; spine-ablated males:  $F_{1,40} = 0.227$ ,  $p = 0.629$ ; Fisher's combined probability test,  $\chi^2_4 = 5.76$ ,  $p = 0.218$ ). Neither did they differ in the amount of ejaculate transferred per copulation, whether assessed by ejaculate



**Figure 2. Fertilization Success of Males with Relatively Short versus Long Genital Spines**

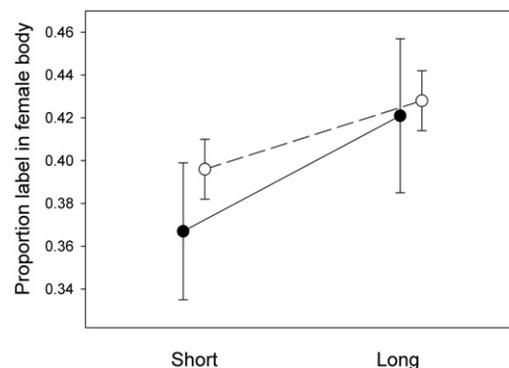
Male genital spines were either shortened by microscale laser ablation (black circles and solid lines) or evolved by divergent selection for spine length for several generations (white circles and dashed lines). Least-squares means ( $\pm$ SEM) are shown; male fertilization success refers to the proportion of twice-mated females' offspring fathered by the second and focal male (i.e., competitive fertilization success or P2). For males subjected to laser ablation, relatively "short" spines refers to those males that had 30 spines ablated, and relatively "long" spines refers to those males that had only 10 spines ablated.

**Table 1. The Effects of Spine Length on Male Competitive Fertilization Success**

Source	df	$\chi^2$	p
Spine ablation	1	3.24	0.071
Mating duration (second mating)	1	2.15	0.142
Male size (second male)	1	0.15	0.695
Number of eggs laid after first mating	1	4.41	0.036

The table shows results of a generalized linear model of variation in sperm precedence (P2) across females mated to spine-ablated males ( $n = 39$ ). df, degrees of freedom.

weight (selection lines:  $t_4 = 1.03$ ,  $p = 0.359$ ) or using radiolabeling data (selection lines:  $t_4 = 0.14$ ,  $p = 0.896$ ; spine-ablated males:  $F_{1,40} = 1.037$ ,  $p = 0.313$ ; Fisher's combined probability test,  $\chi^2_4 = 2.54$ ,  $p = 0.637$ ). Thus, our results do not support the first hypothesis. Although it remains possible that our manipulation of genital morphology may have affected the number of sperm transferred to females, this is made less likely by the fact that the amount of ejaculate transferred per copulation covaries with the number of sperm transferred per copulation in this species [22, 23]. Second, genital spines may favor males by allowing a more rapid passage of seminal fluid substances into the female body from her reproductive tract by rupturing, abrading, or perforating its wall [24]. Male seminal fluid is a complex mixture of organic and inorganic compounds, many of which have hormonal effects in females [3, 25] that could be facilitated either through uptake by blood vessels in the wall of the reproductive tract (in vertebrates) or through direct leakage into the body cavity [24]. We found that a large proportion ( $\approx 40\%$ ) of male-derived ejaculate material was found in the female body, outside of her reproductive tract, 18 hr after mating. Importantly, a larger amount of material derived from the male ejaculate was found in the female body following matings with males with relatively long genital spines (Figure 3). This was true both for spine-ablated males (Table 2) and selection-line males ( $t_4 = 3.13$ ,  $p = 0.035$ ; Fisher's combined probability test,  $\chi^2_4 = 13.24$ ,  $p = 0.010$ ;



**Figure 3. Dispersal of Radiolabeled Male Ejaculate Substances from the Reproductive Tract into the Body of Females**

Relative content of material derived from the male ejaculate in the female body 18 hr after mating with males having either relatively short or long genital spines. Males were either laser ablated (black circles and solid lines) or derived from the selection lines (white circles and dashed lines). Least-squares means ( $\pm$ SEM) are shown; the ordinate refers to the amount of radioisotopic label deriving from the male ejaculate that was found in the female body outside the reproductive tract, divided by the total radioisotopic label in the female body and reproductive tract.

Table 2. The Effects of Spine Length on Female Uptake of Male Seminal Fluid Material

Source	df	MS	F	p
Spine ablation	1	$3.89 \times 10^6$	4.63	0.038
Block	1	$6.31 \times 10^5$	0.75	0.391
Spine ablation $\times$ block	1	$5.03 \times 10^6$	5.99	0.019
Label in reproductive tract	1	$7.72 \times 10^7$	92.04	<0.001
Number of eggs laid after first mating	1	$1.22 \times 10^5$	0.14	0.705
Label in reproductive tract $\times$ number of eggs laid after first mating	1	$8.71 \times 10^6$	10.38	0.003
Error	40	$8.39 \times 10^5$		

The results of an analysis of covariance of variation in the amount of  $^{14}\text{C}$ -radiolabeled ejaculate-derived material in the female body (outside the reproductive tract) after mating to spine-ablated males. The interaction between spine ablation and block was ordinal. df, degrees of freedom; MS, mean squares.

Table S3), providing support for the second hypothesis. This hypothesis is also supported by two additional observations. First, longer spines in seed beetle males are indeed associated with increased abrasion and scarring of the female reproductive tract, and correlational data suggest that the amount of postcopulatory scarring of the female reproductive tract is significantly, though only weakly, related to male competitive fertilization success [12]. Second, male seminal fluid products are known to affect females and to increase male competitive fertilization success in many insects [25–28], and *C. maculatus* is no exception: male seminal fluid in this species contains many different proteins and peptides that are known to affect female reproductive physiology and behavior [29, 30].

Although our study demonstrates that genital morphology directly affects competitive fertilization success in males, it also suggests that the precise proximate mechanisms by which this effect occurs can be complex. In particular, the effect of male genitalia on competitive fertilization success seems to have involved a second set of male postcopulatory adaptations: seminal fluid substances. Because some of these substances undergo enzymatic processing in the female reproductive tract, migrate into the female bloodstream, and are thus transported to their sites of action, a more efficient diffusion of these substances within the female could benefit the donor male by (1) a gonadotrophic effect that elevates the rate of female egg maturation or egg laying, (2) reducing female receptivity to remating, and/or (3) triggering increased uptake or use of the focal male's sperm cells for fertilization [3, 25–28]. Many of these effects are well known in *C. maculatus* [29, 30]. We found that females did not lay significantly more eggs following mating with males with relatively long spines, either when mated to selection-line males ( $t_4 = 0.04$ ,  $p = 0.971$ ) or when mated to spine-ablated males ( $F_{1,38} = 3.015$ ,  $p = 0.091$ ; Fisher's combined probability test,  $\chi^2_4 = 4.85$ ,  $p = 0.303$ ), and remating rate did not differ in control females mated to males from "long" and "short" selection lines ( $F_{1,24} = 0.316$ ,  $p = 0.579$ ). This suggests that the proximate effect of genital morphology on competitive fertilization success may have involved seminal fluid substances that primarily affect the pattern of female sperm usage.

The evolutionary causes of genital evolution have been discussed since pre-Darwinian times [1], and this debate has intensified over the last decade [2]. Previous correlational studies suggesting that postcopulatory sexual selection is involved [7–12, 31] have been deemed inconclusive, simply because covariation between genital morphology and

fertilization success may reflect confounding effects through correlations with other and causal variables [2]. By directly manipulating genital morphology independently of other traits, using two distinct complementary approaches that yielded congruent results, our study overcomes these limitations. Our main findings are that male genital morphology indeed is causally related to male competitive fertilization success and, consequently, that postcopulatory sexual selection operates on male genital morphology. Given the central role that genital evolution has for speciation and morphological diversification [1, 32–34], our results suggest that postcopulatory sexual selection may be at the heart of these key evolutionary processes.

#### Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, Supplemental Results, one figure, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2012.10.009>.

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