

Genital Evolution in Beetles

1 Male density and rapid evolution of genital morphology in the seed beetle *Callosobruchus*

2 *maculatus*

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Genital Evolution in Beetles

24 **Summary**

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26 Male reproductive structures are known to be extremely diverse, particularly in insect taxa.

27 Male genital structures are thought to be some of the fastest evolving traits, but the

28 processes responsible for this pattern remain unclear. In the present study we

29 manipulated the mating regimes of *Callosobruchus maculatus*, a seed beetle, to determine if

30 male genital structures would be altered under forced monogamy and polyandry. Males in

31 this species have an intromittent organ that contains spines that are known to puncture the

32 female reproductive tract. We measured both testes size and genital spine length in

33 monogamous and polyandrous treatments over seven generations. We found that testes

34 size was not significantly different between treatments, but that genital spine length was

35 significantly longer in the polyandrous treatment within seven generations. These results

36 highlight the fact that evolution can occur rapidly when under strong sexual selection, a

37 process that has been implicated in leading to morphological differences in male genitalia.

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45 **Keywords:** genital evolution, experimental evolution, sexual selection

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Genital Evolution in Beetles

47 **Introduction**

48 Male genitalia of internally fertilizing species are extremely diverse and thought to evolve
49 at a rapid pace based on phylogenetic species comparisons [1]. In fact, insect male genitalia
50 are so diverse compared to other morphological characters that taxonomists have used the
51 structure for species identification and classification [2]. Several studies have attempted to
52 determine the cause for such a plethora of diversity; current evidence appears to
53 overwhelmingly support hypotheses under the umbrella of sexual selection [3, 4].

54
55 Mating system of a species can influence the strength of sexual selection [5]. For example,
56 polyandry may strengthen sexual selection because males compete with one another at the
57 cellular level through sperm competition [6], at the organismal level through direct combat
58 [7], and/or at the organ level, whereby genital shape may influence sperm transfer and the
59 number of fertilization events [2]. Although current evidence supports the idea that sexual
60 selection is responsible for the widespread diversity of male genitalia and suggests that
61 genital evolution can occur rapidly [4], direct observation of morphological evolution
62 remains scant.

63
64 Here we track the evolution of male genitalia in *Callosobruchus maculatus*. In this sexually
65 dimorphic seed beetle, males have testes and a seminal vesicle that leads to an intromittent
66 organ with sclerotized spines (Fig. 1). Spines are known to puncture the reproductive tract
67 of the female, possibly as a way to inject antiaphrodisiacs and shorten female life span [8].
68 Using experimental evolution, we measured male genital spines, testicle size, and body size
69 over seven generations under two mating regimes of monogamy and polyandry. We

Genital Evolution in Beetles

70 predicted that increased spine length and testes size would occur in the polyandry
71 treatments due to increased sperm competition.

72 **Materials and Methods**

73 **Study System**

74 *Callosobruchus maculatus*, a cosmopolitan pest, was used as the study system because they
75 are sexually dimorphic, easy to care for, and have a generation time of 3-4 weeks. Eggs are
76 laid on bean hosts and after 4-8 days the larvae hatch and burrow into the beans where the
77 beetles develop into adults. Adult beetles live approximately two weeks without the need
78 for food or water.

79

80 **Treatments**

81 This experiment used two levels of male-male mate competition as treatments.
82 Monandrous treatments had no male competition because they consisted of one male and
83 one female. Polyandrous treatments had intense male competition because they consisted
84 of six males and one female. Carolina Biological (Burlington, NC) supplied the initial
85 generation of beetles. Mating groups were created by randomly assigning individuals to a
86 mating pairings within its treatment, and half-sib inbreeding was avoided.

87

88 Mating pairs were placed in 35mm Petri dishes, and were observed for 20 minutes before
89 dried mung beans were added for the females to use for egg laying. This observation
90 period allowed for behavioral data collection that will be published independently. Beetles
91 remained in their dishes for 7 to 8 days before being removed. The beans with eggs were
92 isolated before emergence to yield virgin beetles for the next generation. Once the new

Genital Evolution in Beetles

93 beetles emerged (approximately every 4 weeks), they matured 48 h before being placed
94 into mating groups. This procedure was repeated for a total of seven generations of
95 offspring from the initial populations.

96

97 **Data Collection**

98 Starting at generation three, 12 males from each generation were randomly selected from
99 each treatment for data collection. Body size, testicular size, and average intromittent
100 organ spine length was collected from each individual. Dissection, body images, and
101 testicle images were all generated with a Leica EZ4HD dissecting microscope. Intromittent
102 organ spine images were captured with a JEOL JSM-5900LV scanning electron microscope,
103 and images were then analyzed using Image J v. 1.48 [9].

104

105 *Body and Testicle Size*

106 Beetles were placed on ice for approximately 1 minute to anesthetize the organisms to
107 allow for microscopy and dissection. If the beetle regained activity prior to dissection, the
108 organism was placed back on ice for an additional minute. Body size was measured from
109 images taken prior to dissection and calculated as the area created by multiplying the
110 width of the elytra by the length of the elytra at their widest points. Dissection for removal
111 of the reproductive organs occurred by submerging the beetle in 50 μ l of buffer solution
112 that consisted of 1.4 M NaCl, 0.03M KCl, 0.1 M Na₂HPO₄, and 0.02 M KH₂PO₄ (Sigma-
113 Aldrich, St. Louis, MO). Testicle images (Fig. 1A) captured after the removal of the seminal
114 vesicle were measured to obtain a testicle area for each male.

115

Genital Evolution in Beetles

116 *Intromittent Organ Spine Length*

117 Prior to imaging, gentle pressure was applied to the base of the intromittent organs so that
118 spines, which are folded inward when stimulation is not present, were exposed. The organs
119 were then dried and mounted for imaging. Images of the extended intromittent organ were
120 taken on a scanning electron microscope for enhanced magnification. Mean individual
121 spine length was calculated with 5-10 randomly selected tip spines from each individual.

122

123 **Statistical Analysis**

124 Body size was first checked for correlation with both testicle size and intromittent organ
125 spine length to determine whether larger beetles had larger testes or longer spines. After
126 data was analyzed for normality, both testicle size and intromittent organ spine length
127 were analyzed with an Analysis of Variance with interaction between generation and
128 treatment. ANOVAs were used to determine if there was a significant difference in the
129 means of our treatments across the seven generations. All statistical analysis was
130 conducted with JMP Statistical Discovery, from SAS (Cary, NC).

131

132 **Results**

133 Mating regime did not affect testicle size ($F=0.016$, $p = 0.9181$). There was also no
134 correlation of body size to testicle area ($R^2=0.006$, $p = 0.955$) or genital spine length ($R^2 =$
135 0.069 , $p=0.694$). We did find evidence for an evolved relationship between mating regime
136 and genital spine length: When averaged across generations, polyandrous males had
137 significantly longer spines than monandrous males (Fig. 2A, Polyandrous mean = $1.2 \mu\text{m}$,
138 Monandrous mean = $1.0 \mu\text{m}$, $F = 4.27$, $p = 0.01$). Both treatment (Fig. 2B, $F = 4.87$, $p = 0.03$)

Genital Evolution in Beetles

139 and generations (Fig. 2B, $F = 6.93$, $p = 0.01$) significantly interacted with genital spine
140 length, driven by the increase in polyandrous male spine length over the seven generations
141 of the experiment. By generation seven, egg production declined in females in the
142 polyandrous treatment to an extent that led to the termination of the experiment due to the
143 lack of ability to maintain six males to one female without inbreeding. This phenomenon
144 did not occur in the forced monogamy treatment.

145

146 **Discussion**

147 After seven generations, we found that males experiencing intense male competition had
148 significantly longer sclerotized spines on genital intromission organs than males that
149 experienced no competition from conspecifics. Body size did not correlate with either
150 spine length or testes size in either mating regime. These results suggest that mating
151 regime, which itself might be driven by population sex ratio, could influence the evolution
152 of genital morphologies and influence the fitness of individuals within populations. The
153 polyandrous treatment experienced a decline in the number of viable offspring until
154 generation seven, when numbers were reduced to the point of forcing the termination of
155 the study.

156

157 The genital spines of *Callosobruchus maculatus* are known to produce extensive genital
158 damage in females, which can lead to reduction in fitness [10], but also are necessary to
159 alter the female's physiology to increase the mating male's chance of fertilization [11].
160 *Callosobruchus maculatus* males with long spines are more successful at fertilization and
161 inject significantly more seminal fluid into female hemolymph [11], which can positively

Genital Evolution in Beetles

162 influence fertilization success but also may lead to negative fitness effects particularly for
163 females that mate multiply due to genital damage and reduced female longevity [8, 12]. It
164 is possible that the increase in length observed for the polyandrous treatment may have
165 contributed to extensive damage and reduction in female fecundity. Moreover, female life
166 span may have been reduced leading to fewer eggs being produced.

167

168 A similar study also using *C. maculatus* found that forced monogamy resulted in shorter
169 male genital spines, while a polygamous (multiple males and multiple females) male genital
170 spine length was maintained [13]. Although the present study also had a forced monogamy
171 treatment, here we found that spine length was maintained in monogamy and under
172 polyandry (one female and multiple males) spine length increased. This difference
173 between study outcomes may have occurred because Cayetano et al. (2011) was carried
174 out to 18-21 generations, while the present study was performed to generation 7.

175 Therefore, it is possible the same result for the monogamous treatment would have been
176 achieved if the study were continued for more generations. Spine length increase may not
177 have been observed under polygamous mating because males had the opportunity to mate
178 with multiple females. Whereas in the present study, under polyandry, a male was
179 required to outcompete other males in order to be successful at fertilization. This increase
180 in sexual selection pressure could have been responsible for the increase in spine length.

181

182 Another reproductive trait that is commonly positively correlated with increased male
183 competition is testicle size [14, 15] because typically larger testes results in higher sperm
184 counts. Counter to this expectation, we found that testicle size did not differ between

Genital Evolution in Beetles

185 monogamous and polyandrous treatments. This may be due to the fact that *C. maculatus*
186 males produce and inseminate more sperm than can be possibly stored in the female
187 spermathecae [16], and thus, *C. maculatus* males already produce the maximum amount of
188 sperm that is possible regardless of the level of male competition that is present. Excess
189 sperm deposition in this species is thought to occur because large numbers of sperm
190 deposited in the female spermathecae increases the time with which she will re-mate,
191 which allows more time for fertilization to occur [16].

192

193 Male reproductive morphologies are known to be some of the fastest evolving characters
194 due to their direct effect on fitness [1]. In insects, this has led to a wide diversity of genital
195 morphologies including a breathtaking array of intromittent organs [17] that have even
196 been used for species identification. From lock-and-key mechanisms in damselflies [18] to
197 twin claw-like genital structures in *Drosophila* [19], genital intromittent organs occur in a
198 variety of structures for seemingly various reasons. Sexual selection has been the primary
199 force suggested as to how these structures can evolve such diversity, but observation of
200 rapid genital change has been rare. Lack of direct evidence that male competition can lead
201 to genital morphological alterations means that if and/or how this process occurs remains
202 obscure. The present study shows that genital intromittent organ evolution can occur
203 rapidly, under intense male competition.

204

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Genital Evolution in Beetles

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208 department for assistance and training in use of the Scanning Electron Microscope.

209

210 **Data Accessibility**

211 Data to support figure 2A & 2B will be deposited to Dryad upon acceptance.

212

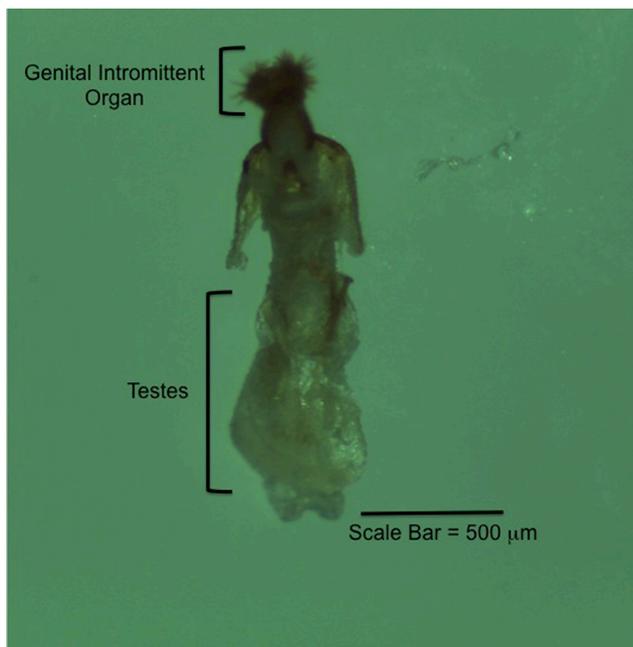
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Genital Evolution in Beetles

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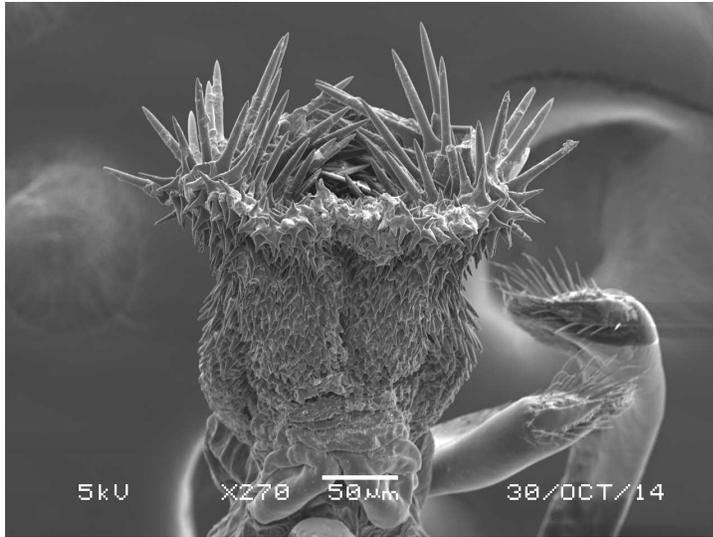
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258 **Figure 1A**

Genital Evolution in Beetles



259

260 **Figure 1B**

261

262 **Figure 1.** The anatomy of the *Callosobruchus maculatus* male reproductive tract. (a)

263 Testicular structure shown with male intromittent organ in the everted state exposing

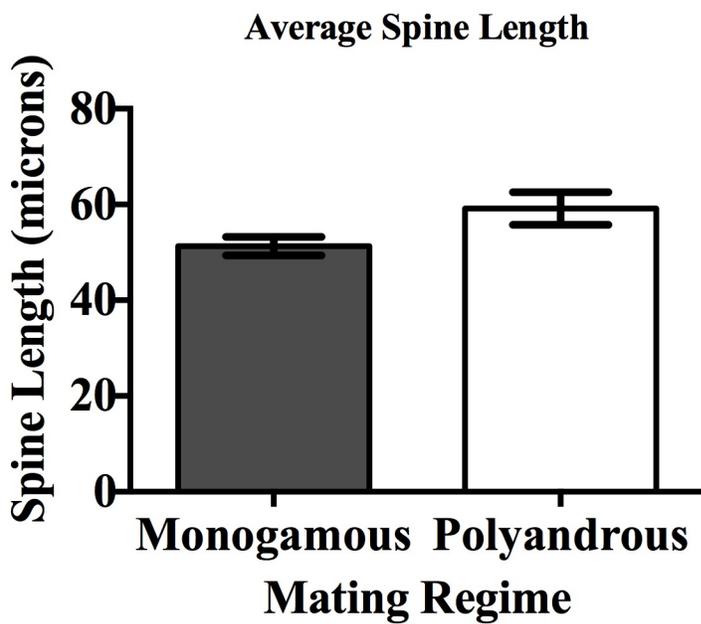
264 spines under a dissecting microscope (b) Male intromittent organ tip showing spines under

265 scanning electron microscope. Scale bar, 500mm in (a), and 50mm in (b).

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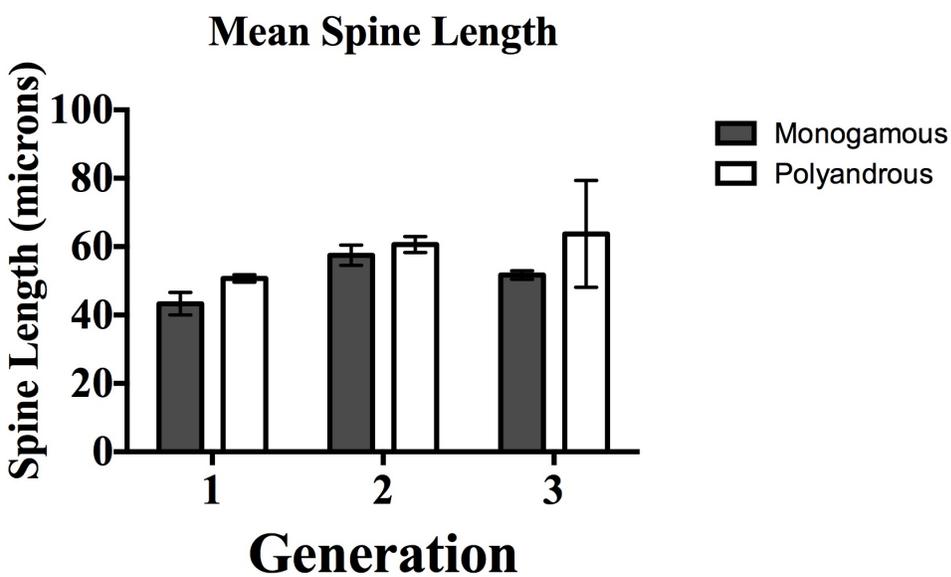
Genital Evolution in Beetles



268

269 Figure 2A

270



271

272 Figure 2B

Genital Evolution in Beetles

273 **Figure 2.** Intromittent spine length (*a*) the effect of treatment (monandrous treatment grey
274 bars, polyandrous treatment white bars) on spine length and (*b*) the effect of generations
275 and treatment on average spine length.

276