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

2   *maculatus*

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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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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

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

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 50μl 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

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 μm,

138   Monandrous mean = 1.0 μm, F = 4.27, p = 0.01). Both treatment (Fig. 2B, F = 4.87, p = 0.03)

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

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

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

205 **Acknowledgements**

206   We would like to thank the Beloit Biology Department, especially Y. Grossman for statistical

207   support and guidance. We also would like to thank the Beloit College Chemistry

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

209

Data Accessibility

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

212

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257

258   **Figure 1A**

259

Figure 1B

261
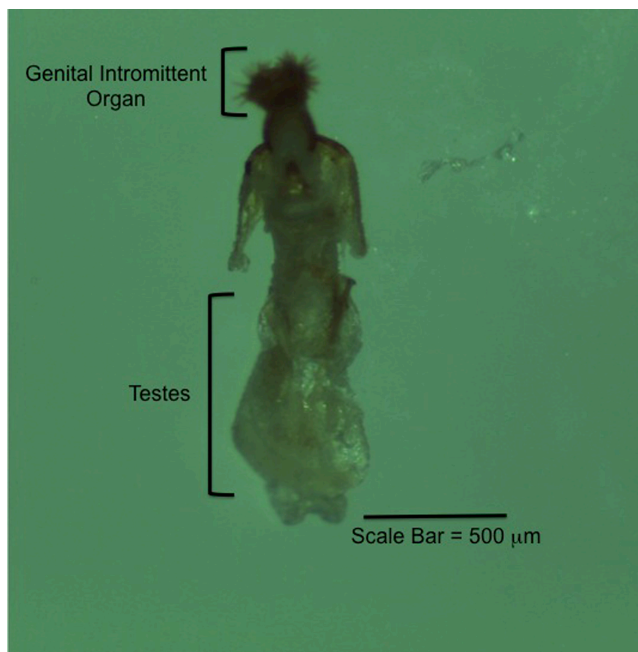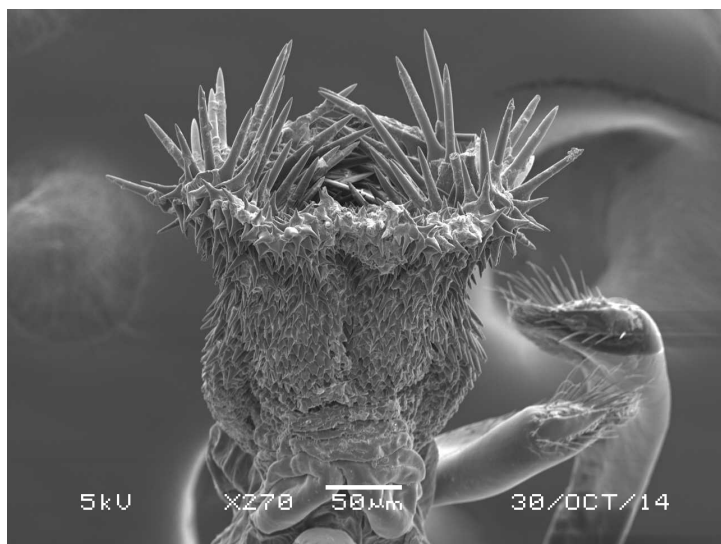
Figure 1. The anatomy of the *Callosobruchus maculatus* male reproductive tract. (*a*)
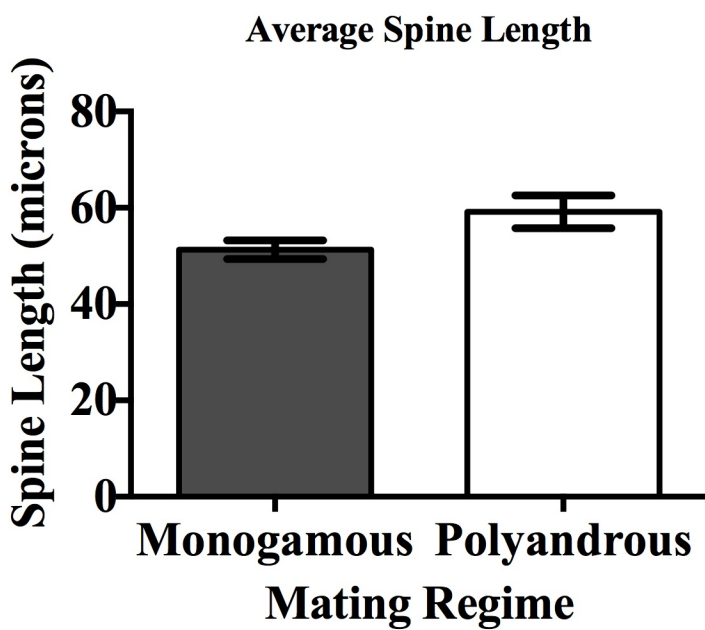Testicular structure shown with male intromittent organ in the everted state exposing
spines under a dissecting microscope (*b*) Male intromittent organ tip showing spines under
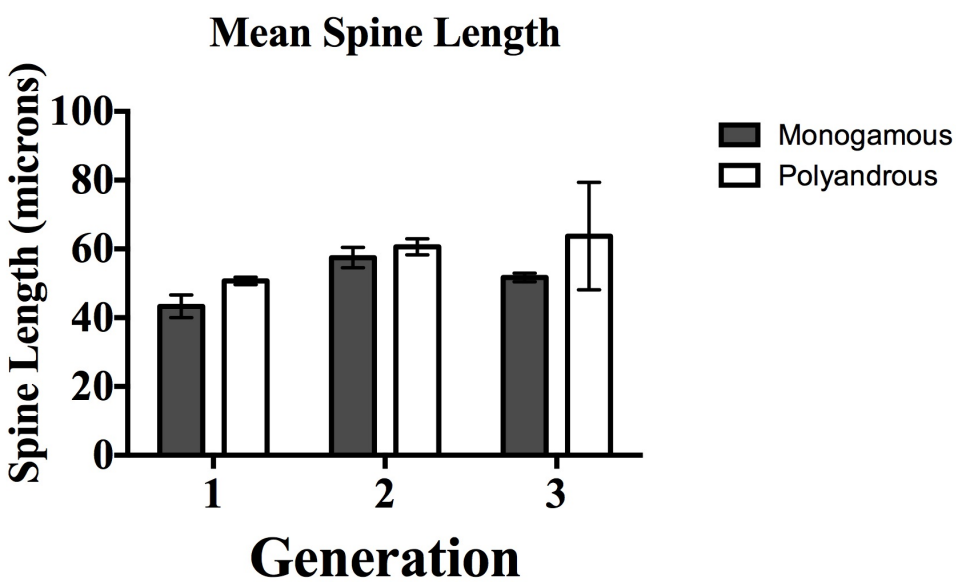scanning electron microscope. Scale bar, 500mm in (*a*), and 50mm in (*b*).

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267

Average Spine Length

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269 **Figure 2A**

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Mean Spine Length

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272 **Figure 2B**

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