



## EVOLUTIONARY CHANGE IN TESTES TISSUE COMPOSITION AMONG EXPERIMENTAL POPULATIONS OF HOUSE MICE

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BRIEF COMMUNICATION

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3 **EVOLUTIONARY CHANGE IN TESTES TISSUE**

4 **COMPOSITION AMONG EXPERIMENTAL**

5 **POPULATIONS OF HOUSE MICE**

6

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21 **Running head:** Evolutionary change in the testes in mice

22

23 Theory assumes that postcopulatory sexual selection favours increased investment in  
24 testes size because greater numbers of sperm within the ejaculate increase the chance  
25 of success in sperm competition, and larger testes are able to produce more sperm.  
26 However, changes in the organization of the testes tissue may also affect sperm  
27 production rates. Indeed, recent comparative analyses suggest that sperm competition  
28 selects for greater proportions of sperm-producing tissue within the testes. Here, we  
29 explicitly test this hypothesis using the powerful technique of experimental evolution.  
30 We allowed house mice (*Mus domesticus*) to evolve via monogamy or polygamy in  
31 six replicate populations across 24 generations. We then used histology and image  
32 analysis to quantify the proportion of sperm-producing tissue (seminiferous tubules)  
33 within the testes of males. Our results show that males that had evolved with sperm  
34 competition had testes with a higher proportion of seminiferous tubules compared  
35 with males that had evolved under monogamy. Previously, it had been shown that  
36 males from the polygamous populations produced greater numbers of sperm in the  
37 absence of changes in testes size. We thus provide unequivocal evidence that sperm  
38 competition selects for an increase in the density of sperm-producing tissue, and  
39 consequently increased testes efficiency.

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45 **KEY WORDS:** Sperm competition, postcopulatory sexual selection, sperm  
46 production, testosterone production, histology

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48 Parker's game theoretic models of ejaculate evolution assume that sperm competition  
49 conforms to a raffle, with the relative number of sperm from each competitor being  
50 the primary determinant of paternity success (Parker and Pizzari 2010). In support of  
51 sperm competition theory, evolutionary and reproductive biologists focusing on *in*  
52 *vivo* sperm competition dynamics have shown that males that produce ejaculates with  
53 high numbers of sperm typically have a fertilization advantage over their rival/s (e.g.  
54 domestic species, Beatty 1957; Martin et al. 1974; wild species, Gage and Morrow  
55 2003; Boschetto et al. 2011; Firman and Simmons 2011). Thus, when male fitness is  
56 contingent on the number of sperm within the ejaculate, it is likely that postcopulatory  
57 selection will target the organ responsible for sperm production, and evolutionary  
58 change will ensue (Parker 1970).

59       A positive correlation between testes size and the level of sperm competition, as  
60 determined by variation in mating system, the frequency of female remating,  
61 differences in population sex ratio, etc, has been observed. A multitude of studies  
62 have implicated intense selection via sperm competition as favoring the evolution of  
63 larger testes both within (Ribble and Millar 1992; Brown and Brown 2003; Long and  
64 Montgomerie 2005; Firman and Simmons 2008; Dzminiski et al. 2009) and between  
65 species (e.g. primates, Harcourt et al. 1981; butterflies, Gage 1994; bats, Hosken  
66 1997; frogs, Byrne et al. 2002; birds, Pitcher et al. 2005; scorpions, Vrech et al. 2014).  
67 To date however, the most convincing evidence for the role of sperm competition in  
68 testes size evolution comes from investigations that have maintained evolving  
69 laboratory populations, and found reductions in testes mass with the removal of sperm  
70 competition (Hosken and Ward 2001; Pitnick et al. 2001; Simmons and Garcia-  
71 Gonzalez 2008). This significant body of literature has resulted in testes size (relative  
72 to body size) being routinely used as an indirect measure of sperm competition risk.

73 Unfortunately, the underlying critical assumption that testes size accurately reflects  
74 sperm production rate has largely been overlooked (discussed by Schärer et al. 2004).  
75 Certainly, variation in testis size alone may not necessarily reflect differences in  
76 sperm production rate, and evidence has emerged to support the idea that sperm  
77 competition selects to maximise testicular efficiency beyond changes in testes size  
78 (reviewed in Ramm and Schärer 2014).

79         The testis consists of spermatogenic tissue, which includes germ cells and  
80 somatic cells (called Sertoli cells in mammals), and non-spermatogenic tissue with  
81 somatic cells only (e.g. blood vessels and Leydig cells). In higher vertebrates the germ  
82 cells are located within the long and convoluted seminiferous tubules (Gier and  
83 Marion 1970; Roosen-Runge 1977; Wistuba et al. 2007). During the process of  
84 spermatogenesis the germ cells develop from spermatogonia into spermatocytes, then  
85 to spermatids, and finally spermatozoa (Roosen-Runge 1977; Wistuba et al. 2007).  
86 Spermatozoa are released from the nourishing Sertoli cells to enter the lumen of the  
87 seminiferous tubule before being passed into the efferent ducts and transported to the  
88 epididymis for maturation. The interstitial tissue that surrounds the seminiferous  
89 tubules supplies blood to the testis and produces endocrine signals via the Leydig  
90 cells (Wistuba et al. 2007).

91         It has long been known that seasonal variation in the amount of sperm-  
92 producing tissue versus non sperm-producing tissue occurs in many different taxa  
93 (e.g. Mayhew and Wright 1970; Goldberg and Parker 1975; Hochereau-de Reviere  
94 and Lincoln 1978; Fuentes et al. 1991). Since Harcourt *et al.* (1981) first indicated  
95 that sperm competition has the potential to select for increased sperm production rates  
96 via alternations in the spatial organization of the testes, a number of comparative  
97 studies have explored the association between the proportion of sperm-producing

98 tissue within the testes and postcopulatory sexual selection (Wistuba et al. 2003;  
99 Lüpold et al. 2009; Rowe and Pruett-Jones 2011; Montoto et al. 2012). Interspecies  
100 comparisons have provided general evolutionary patterns, but fail to demonstrate  
101 cause and effect. In their review on the evolutionary ecology of testicular function,  
102 Ramm and Schärer (2014) called for researchers to explore directly the consequences  
103 of sperm competition for testes machinery by using the powerful technique of  
104 experimental evolution. Here we provide the first account. We quantified the amount  
105 of sperm-producing and interstitial tissue from images of histological preparations of  
106 the testes of male house mice (*Mus domesticus*) from six replicate populations that  
107 had been evolving under a monogamous or polygamous mating regime for 24  
108 generations. Our analyses revealed that the testes of males that had evolved with  
109 sperm competition had greater proportions of seminiferous tubules compared to males  
110 that had evolved via monogamy. Previously, it had been shown that males from the  
111 polygamous populations produced ejaculates with elevated sperm numbers in the  
112 absence of changes in testes size (Firman and Simmons 2010). Thus, our result here  
113 suggests that sperm competition acts directly on the spatial organisation of the testes,  
114 which is likely to select for enhanced efficiency and the production of more sperm.

115

### 116 *Materials and Methods*

#### 117 **EXPERIMENTAL ANIMALS**

118 House mice are actively polygamous in nature, with both males and females  
119 copulating with multiple mates (Dean et al. 2006; Firman and Simmons 2008).  
120 However, laboratory strains of house mice are typically maintained under an enforced  
121 monogamous mating regime. The Uwa:MMD colony of wild-derived mice was  
122 established in 1997 at the Animal Resources Centre (ARC) (Murdoch, Western

123 Australia) with 90 breeding pairs sourced from six wild, Australian *Mus domesticus*  
124 populations. From 1997 to 2002 the colony was maintained as an outbred population  
125 under the Poiley outbreeding system (Poiley 1960), after which the outbreeding  
126 regime changed to a dedicated program based on the coefficient of inbreeding  
127 (Firman and Simmons 2010). Consequently, the Uwa:MMD colony was maintained  
128 under a strict monogamous mating regime for approximately 30 generations prior to  
129 the establishment of the selection lines. Male/female pairs ( $n = 60$ ) reproduced to  
130 establish a base population from which our selection lines were derived, for which we  
131 referred to the ARC colony pedigree to ensure that close relatives did not breed.

132         At the University of Western Australia the animals were maintained in a  
133 constant temperature room under identical conditions (24°C; reversed 14:10 hour  
134 light-dark cycle), and food and water was provided *ad libitum*. We established four  
135 monogamous and four polygamous lines with 18 males and 18 females in each.  
136 Subsequently, 18 males and 18 females contributed to each generation. In the  
137 monogamous lines, almost all selection on adult fitness was eliminated by ensuring  
138 that every male and female pair contributed one son and one daughter to the next  
139 generation (Shabalina et al. 1997). Although greatly relaxed, selection may not have  
140 been completely eliminated because sometimes a pair did not mate or produce  
141 offspring. In the polygamous lines, adult females had equal fitness (two offspring),  
142 and adult males had equal mating success but not equal fertilisation success due to  
143 sperm competition and/or cryptic female choice. Four polygamous lines were  
144 established with 18 females and 18 males, but potentially <18 sires. In the  
145 polygamous lines, the same three males mated with the same three females. Thus,  
146 males in the polygamous lines competed for fertilizations, and the number of males  
147 who contributed to successive generations was determined by the relative paternity

148 success of each male. This mating design ensured that the effective population sizes  
149 of the monogamous lines were potentially greater than the polygamous lines at each  
150 generation. Therefore, any observed fitness benefits associated with sperm  
151 competition would be conservative, as the polygamous lines would be expected to  
152 have higher inbreeding coefficients than the monogamous lines (documented at  
153 generation 12; Firman & Simmons 2011). As with the monogamous lines, one male  
154 and one female were selected at random from each polygamous line litter and used to  
155 produce the next generation. Consequently, while postcopulatory sexual selection on  
156 males was reinstated in the polygamous lines, natural selection and precopulatory  
157 sexual selection remained absent in both treatments.

158 Anatomical measurements and sperm quality assays initially performed after  
159 eight generations of experimental evolution revealed that males had diverged in sperm  
160 number without changes in testes size (Firman and Simmons 2010; Table S1 in the  
161 online supplementary material). Males from the polygamous lines produced more  
162 sperm, had a greater proportion of motile sperm, and sperm with greater swimming  
163 velocities than males from the monogamous lines, as would be expected following  
164 selection via sperm competition (Firman and Simmons 2010). Further, testes size  
165 among males from the different selection regimes had not changed following 16  
166 (Firman et al. 2011) or 18 (Table S1; Table S2) generations of selection. Males from  
167 the 24<sup>th</sup> generation were used here to assess evolutionary change in the spatial  
168 organisation of the testes. Due to time and resource restrictions, we were limited to  
169 using a randomly selected sample of three monogamous ( $n = 10$  males/line) and three  
170 polygamous ( $n = 10$  males/line) selection lines.

171 Variation in the social environment can induce plasticity in sperm production in  
172 house mice (Ramm and Stockley 2009; Firman et al. 2013). Consequently, the



173 animals used in this experiment were reared under identical conditions. Thus, after  
174 being weaned from their mother at three weeks of age, each male was housed in an  
175 individual cage until they reached sexual maturity (12 weeks of age). To ensure that  
176 males acquired the appropriate olfactory cues during their sexual development they  
177 were placed within close proximity to cages housing females. All males were virgins  
178 at the time of sacrifice.

179

### 180 **TESTES HISTOLOGY AND IMAGE ANALYSIS**

181 Males were sacrificed via lethal injection and stored at -20°C. Prior to dissection, the  
182 bodies were defrosted and weighed. A single testis from each male was chosen at  
183 random and fixed in 10% buffered formal saline. Each testis was dehydrated through  
184 a series of graded ethanol baths, chloroform baths, and paraffin, and then embedded in  
185 paraffin wax. The specimens were sectioned through the midline, and centre sections  
186 of the testis were mounted on slides. The slides were stained with Gill's haematoxylin  
187 and Eosin and viewed under a BX50 (Olympus) light microscope (×10 objective).  
188 Five different images per testis were captured using an Olympus DP72 camera  
189 attached to the microscope. Testis tissue covered the entire area in each image (Fig.  
190 2).

191 Each image was visualized using the image analysis software ImageJ. We  
192 quantified the proportion of sperm-producing tissue within each image. Thus, we  
193 measured and recorded the area of each seminiferous tubule (including the tubule  
194 lumen) within each image (see Fig. S1 in the online supplementary material). We then  
195 calculated the proportion of sperm-producing tissue per image:  $\text{sum}[\text{seminiferous}$   
196  $\text{tubule area}]/\text{total image area}$ . The 300 images were distributed randomly among four  
197 different investigators, and each investigator was blind to the treatment from which

198 the images were sourced.

199

## 200 **STATISTICAL ANALYSES**

201 To confirm that investigator bias did not influence our measurements and results, a set  
202 of eight images were measured by the four investigators, which allowed us to perform  
203 a repeatability analysis on those measurements. A Levene's Test was applied to test  
204 the equality of variances in the proportion of sperm-producing tissue among the  
205 replicate lines (*SciStat Calc* 2013). We used JMP statistical software to perform a  
206 nested analysis of variance (ANOVA) with replicate lines nested within selection  
207 treatments (random effect) to account for non-independence of replicate lines. Effect  
208 size analysis was calculated using the library "compute.es" in R. All means are  
209 presented  $\pm 1$  SE.

210

## 211 **Results**

### 212 **SPERM NUMBER AND TESTES SIZE MEASUREMENTS**

213 The repeatability analysis revealed that there was no measurement bias among the  
214 investigators. There was significantly more variation between images than within  
215 images ( $F_{7,24} = 17.171$ ,  $P < 0.001$ ;  $R = 0.500$ , calculated following Becker 1984).  
216 We calculated the mean proportion of sperm-producing tissue for each male. A  
217 Levene's Test revealed that the variances in the mean proportion of sperm-producing  
218 tissue were equal among the replicate lines ( $n = 6$ ,  $W = 0.778$ ,  $P = 0.570$ ). Thus, we  
219 looked for an effect of selection history among males from the different experimental  
220 populations.

221 A nested ANOVA revealed significant variation in the proportion of total tubule  
222 area among males from the polygamous and monogamous selection lines (Table 1).

223 The mean difference in the proportion of sperm-producing tissue between treatments  
224 was 4.6%. Males from the polygamous lines had testes with greater proportions of  
225 sperm-producing tissue ( $n = 3$ ,  $0.747 \pm 0.008$ ) compared with males from the  
226 monogamous lines ( $n = 3$ ,  $0.701 \pm 0.009$ ) (Fig. 1; Fig. 2). We calculated a  
227 standardized mean difference effect size (Cohen's  $d$ ) and the associated 95%  
228 confidence intervals (CIs) to gauge the magnitude of the observed effect of selection  
229 history ( $d = 4.66$  [0.29, 9.03]) on the proportion of total tubule area. The effect size is  
230 large, and the 95% CIs give us confidence in rejecting the hypothesis that selection  
231 history had no effect on tubule area.

232

### 233 *Discussion*

234 Testes size evolution via postcopulatory sexual selection is well evidenced  
235 (Gomendio et al. 1998; Simmons 2001; Birkhead et al. 2009). Both inter- and intra-  
236 species comparisons have shown that sperm competition typically favours an increase  
237 in the amount of spermatogenic tissue, and thus larger testes (e.g. Gage 1994;  
238 Hosken 1997; Byrne et al. 2002; Firman and Simmons 2008; Dzminiski et al. 2009).  
239 However, in addition to gross testes size, the relative proportion of sperm-producing  
240 tissue within the testes could also be an important factor determining spermatogenic  
241 investment (Ramm and Schärer 2014). Certainly, the capacity of the mammalian  
242 testes to produce sperm varies considerably across taxa; among six rodent species the  
243 proportion of sperm-producing tissue within the testes has been shown to range from  
244 33% to 90% (Russell et al. 1990). Within *Mus*, the level of sperm competition (as  
245 estimated by relative testes size) correlates with both the number of sperm produced  
246 (Montoto et al. 2011) and the proportion of seminiferous tubules within the testes  
247 (Montoto et al. 2012). Similar evolutionary associations between the intensity of

248 sperm competition and testes tissue organization have been observed in comparative  
249 studies of two groups of birds, the New World blackbirds (Icteridae) and the  
250 Australian passerines (Maluridae) (Lüpold et al. 2009; Rowe and Pruett-Jones 2011).  
251 Here, we show that male house mice from experimental populations evolving with  
252 sperm competition have testes with greater proportions of sperm-producing tissue  
253 compared to the testes of males from populations maintained under enforced  
254 monogamy.

255         An increase in the proportion of spermatogenic tissue in the testes of males from  
256 the polygamous lines could be ascribed to selection acting on standing genetic  
257 variation in testes tissue organisation. Alternatively, as we reinstated sperm  
258 competition in a source population that had experienced a long history of monogamy,  
259 in which deleterious mutations might have accumulated, our result could be explained  
260 by sperm competition successfully purging mutations that influenced testes  
261 phenotype. While theory predicts that sexual selection on males can substantially  
262 reduce the accumulation of mildly deleterious mutations (McLearn and Manning  
263 1985; Whitlock and Agrawal 2009), experimental evidence among different  
264 invertebrate species is contradictory (e.g. Radwan 2004; Hollis et al. 2009; Arbuthnott  
265 and Rundle 2012; Almbro and Simmons 2014). To date, there have been no direct  
266 tests of whether mutation accumulation influences testes tissue composition, however  
267 it is interesting to note that a recent study of the dung beetle *Onthophagus taurus*  
268 found that sexual selection was ineffective in removing deleterious mutations that  
269 reduced testes mass (Almbro and Simmons 2014).

270         Deleterious recessive mutations can be exposed under conditions of inbreeding  
271 (Charlesworth and Charlesworth 1999). Consequently, if individuals from our  
272 monogamous selection lines were ‘carrying’ high mutation loads they might be

273 expected to experience greater fitness consequences from reproducing with relatives  
274 compared with individuals from the polygamous lines. Within-line experimental  
275 matings performed after 16 generations of selection revealed that full-sibling parental  
276 pairs and unrelated parental pairs had equivalent fitness (Firman *et al.* 2011). Further,  
277 the resulting ‘inbred’ polygamous and ‘inbred’ monogamous male offspring had, on  
278 average, comparable testes sizes and sperm numbers (Firman *et al.* 2011). Thus,  
279 although we are unable to eliminate conclusively that sperm competition purged  
280 deleterious alleles that influenced testes phenotype and suppressed testes function,  
281 available data suggests that this was not the case. Regardless, we have provided  
282 compelling evidence that sperm competition selects for enhanced levels of sperm-  
283 producing tissue, either by selecting against males with high mutation loads, or by  
284 acting on standing genetic variation. Certainly, these two mechanisms are not  
285 mutually exclusive and are likely to co-occur in both experimental and natural  
286 populations.

287       A series of previous investigations performed using these lineages of house  
288 mice had revealed that males from populations evolving via sperm competition  
289 produced more sperm compared with males from populations evolving under  
290 monogamy, but that changes in sperm production were not associated with changes in  
291 testes size (Firman and Simmons 2010; Firman *et al.* 2011). Moreover, the increase in  
292 sperm production resulted in enhanced success in sperm competition (Firman and  
293 Simmons 2011). We do not have testes size data from males from the 24<sup>th</sup> generation,  
294 however testes size had not diverged after eight (Firman and Simmons 2010), 16  
295 (Firman *et al.* 2011) or 18 generations of selection. Thus, it is likely the increase in  
296 sperm production among males from the polygamous lines is attributable to an  
297 increase in the proportion of sperm-producing tissue, which was documented here.

298 Sperm production is also influenced by the efficiency of the sperm-producing  
299 tissue, for example the rate at which individual sperm can be manufactured. In  
300 mammals, the duration of spermatogenesis is defined as the ‘seminiferous epithelium  
301 cycle length’ (SECL), describing one complete series of cell associations that occurs  
302 in the seminiferous epithelium (Clermont 1972). Across species, variation in SECL  
303 has been shown to correlate negatively with relative testes size, supporting the idea  
304 that sperm competition selects for a faster rate of spermatogenesis (Pierce and Breed  
305 2001; Paraponov et al. 2008; Ramm and Stockley 2010). In addition to evolutionary  
306 responses, variation in the speed of spermatogenesis might also account for the  
307 adaptive plasticity in sperm production that has been observed in response to local  
308 sperm competition conditions (delBarco-Trillo and Ferkin 2004; Ramm and Stockley  
309 2009; Firman et al. 2013). Our findings on house mice have important implications  
310 for the general assumption that larger testes confer greater rates of sperm production,  
311 and emphasise the need for explorations beyond a simple measure of testes size and  
312 into testes tissue organisation and function (Ramm and Schärer 2014).

313 As almost all male sex hormone production occurs in the testes interstitium  
314 (Stocco and McPhaul 2006), we contend that an increase in the proportion of  
315 seminiferous tubules and a reduction in the amount of interstitial tissue could have  
316 important implications for precopulatory sexual selection. Testosterone, which is  
317 produced by the Leydig cells, plays a critical role in the promotion of secondary  
318 sexual characters (Dufau 1996). For example, testosterone-dependent urine scent-  
319 marking behaviour in male house mice is indicative of dominance status and territory  
320 defense (Desjardins et al. 1973; Hurst 1990), and is assessed by females to gauge the  
321 quality of potential mates (Wolff 1985, Penn and Potts 1998). Variation in  
322 testosterone levels can lead to changes in the major urinary proteins that mediate the

323 release of pheromones (Harvey et al. 1989). Therefore, a reduction in testes interstitial  
324 tissue, and consequently a reduction in the density of Leydig cells, could result in a  
325 reduction in testosterone production and influence the quality or frequency of scent-  
326 marks produced by males. A recent study has revealed that sexually receptive females  
327 spent more time associating with males from the monogamous populations compared  
328 to males from the polygamous populations, suggesting that these males have a  
329 precopulatory advantage which could be attributable to an intrinsic quality associated  
330 with the scents that they produce (Firman 2014). The divergence in testes tissue  
331 composition among males evolving with and without sperm competition reported here  
332 certainly warrants further research into evolutionary trade-offs between pre- and  
333 postcopulatory sexually selected traits.

334         In summary, we have documented divergence in testes tissue composition  
335 among house mice from populations that had evolved under a polygamous or  
336 monogamous mating regime for 24 generations. Our statistical analysis revealed that  
337 males that had evolved with sperm competition had testes with a significantly greater  
338 proportion of seminiferous tubules compared with males that had evolved under  
339 enforced monogamy. Our finding accounts for the previously reported divergence in  
340 sperm number and sperm competitiveness that had been observed in the absence of  
341 evolutionary changes in testes size (Firman and Simmons 2010; Firman and Simmons  
342 2011). This study has important implications for the general, mostly untested  
343 assumption that testes size is a strong predictor of sperm production, and emphasizes  
344 the need for future research to explore the evolutionary implications of sperm  
345 competition for testicular efficiency.

346

347

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355

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593 **Table 1.** ANOVA comparing the proportion of sperm-producing tissue in the  
 594 testes of males that have evolved under either a polygamous or monogamous  
 595 selection regime.

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Effect	SS	df	MS	<i>F</i>	<i>P</i>
Selection history	0.034	1	0.033	32.602	0.005
Line[selection history]	0.004	4	0.001	0.243	0.913
Error	0.223	54			

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601 **Figure 1.** The mean proportion of seminiferous tubules in the testes of house  
 602 mice from monogamous (3) and polygamous (3) selection lines (as measured  
 603 from images of histological preparations).

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607 **Figure 2.** Example images displaying the difference in the density of  
 608 seminiferous tubules in the testes of house mice from monogamous (1-3) and  
 609 polygamous (4-6) selection lines.

Figure 1.

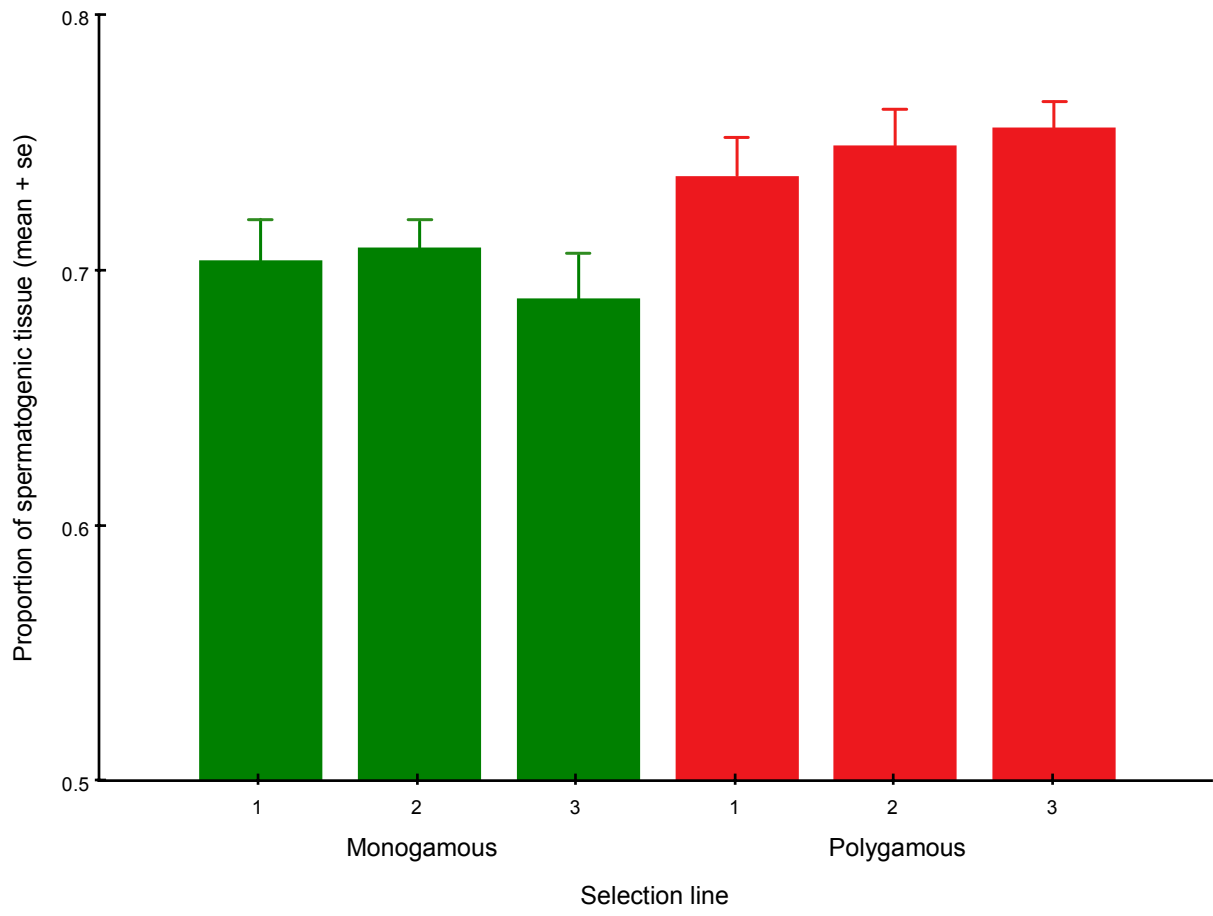


Figure 2.

