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20   Offspring born to related parents often have lower fitness than those born to non-

21 related parents, a phenomenon termed inbreeding depression. While many species have been

22 shown to rely on pre- and/or post-copulatory mate choice to avoid inbreeding, such research

23 has focussed largely on polyandrous rather than monandrous species. The absence of post-

24 copulatory mate choice in monandrous species suggests that pre-copulatory mate choice

25 should play a more important role in inbreeding avoidance. We used a monandrous wolf

26 spider, *Pardosa astrigera,* as a model system to investigate whether (1) male spiders respond

27 differently to sibling and non-sibling females; (2) female spiders respond differently to

28 sibling versus non-sibling males; and (3) inbreeding affects females and their offspring. Male

29 courtship behavior was similar for sibling and non-sibling females; although females were

30 less likely to mate with siblings, over half did mate successfully with their sibs. Sibling-

31 mated females produced fewer offspring from the first eggsac and fewer total offspring, but

32 inbred offspring survived longer in a range of environments than their outbred counterparts.

33 This suggests that the fitness costs of reduced fecundity in sibling-mated females may be

34 offset by higher offspring survivorship. Our results highlight the importance of considering

35 both parent and offspring fitness when addressing the costs of inbreeding, and are the first to

36 document the impact of inbreeding on sexual behaviour and reproductive fitness in a

37 monandrous spider.

40   Inbred individuals are often less fit than outbred individuals, a phenomenon generally

41   resulting from increased homozygosity at loci carrying rare deleterious recessive alleles or

42   exhibiting over-dominance (Charlesworth & Charlesworth, 1987; Lynch, 1991). The fitness

43   costs of inbreeding have been documented in an array of taxa, and exert a strong selective

44   pressure on both mating and reproductive strategies (Bateson, 1982; Escobar et al., 2011;

45   Muller & Muller, 2016; Szulkin, Stopher, Pemberton, & Reid, 2013). The impact of

46   inbreeding on offspring can be altered by the surrounding environment. Varying

47   environmental conditions, for example, can cause stress and often exacerbate the effects of

48   inbreeding (Armbruster & Reed, 2005). These stressors can include suboptimal diets (Fox &

49   Reed, 2011; Freitak, Bos, Stucki, & Sundstrom, 2014) and variation in temperature (Fox &

50   Reed, 2011; Kristensen, Barker, Pedersen, & Loeschcke, 2008), and are widely recognized to

51   exacerbate the fitness costs of inbreeding.

52   An array of mechanisms have evolved for avoiding inbreeding and/or reducing its

53   fitness costs (Firman & Simmons, 2008; Pusey & Wolf, 1996; Ruch, Heinrich, Bilde, &

54   Schneider, 2009). Prior to breeding, sex-biased dispersal from natal habitats decreases

55   inbreeding risk in some species (Keane, 1990; Pusey & Wolf, 1996; Smith, Su, Berger-Tal, &

56   Lubin, 2016), while other species prefer to mate with unrelated partners (Fischer, Karl,

57   Heuskin, Janowitz, & Dotterl, 2015; Thomas & Simmons, 2011; Whitehorn, Tinsley, &

58   Goulson, 2009). The recognition and avoidance of related individuals requires chemical or

59   other cues that are indicative of relatedness (Firman & Simmons, 2008; Pusey & Wolf, 1996;

60   Ruch et al., 2009). In insects, for instance, both mate recognition and pre-mating preference

61   are affected by cuticular hydrocarbons (CHCs; Geiselhardt, Otte, & Hilker, 2009; Thomas &

62   Simmons, 2011) and other compounds (Chuine, Sauzet, Debias, & Desouhant, 2015;

63   Herzner, Schmitt, Heckel, Schreier, & Strohm, 2006). The CHC profiles of several

64   chrysomelid beetle species, for example, affect mate choice and facilitate outbred mating

65   (Geiselhardt et al., 2009). Even if inbreeding does occur, its impact in polyandrous species

66   can be reduced via post-copulatory mechanisms in which differential fertilization success

67   depend on patterns of relatedness rather than intrinsic male quality (Bretman, Wedell, &

68   Tregenza, 2004; Firman & Simmons, 2008; Fitzpatrick & Evans, 2014).

69   Research exploring inbreeding avoidance has primarily addressed polyandrous

70   species, organisms capable of employing both pre- and post-copulatory mate choice strategies

71   (Cornell & Tregenza, 2007; Firman & Simmons, 2008; Tregenza & Wedell, 2002; Welke &

72   Schneider, 2009). This focus reflects the genetic benefits likely necessary for polyandry to

73   evolve in species where females derive little or no material benefit from males (reviewed in

74   Simmons, Beveridge, Wedell, & Tregenza, 2006). In contrast, inbreeding in monandrous

75   species has received far less attention. Because monandrous females only mate once within a

76   single reproductive episode, inbreeding avoidance must occur via pre-copulatory mechanisms

77   (Hosken, Stockley, Tregenza, & Wedell, 2009). In situations where inbreeding is costly,

78   monandrous species may thus possess especially effective pre-copulatory barriers. The

79   strength of these barriers may, however, vary by sex: because male fitness is relatively

80   unaffected by inbreeding, they should be more tolerant of sibling matings than females

81   (Duthie, Lee, & Reid, 2016).

82   The wolf spider *Pardosa astrigera* Koch is widely distributed in East Asia. Male

83   courtship consists of two distinct behaviours, body shaking and foreleg raising (Wu, Jiao, &

84   Chen, 2008). Olfaction plays a key role in male courtship. Males initiate courtship in

85   response to pheromones associated with female dragline silk, and males can distinguish silk

86   cues from individuals differing in sex and mating status (Xiao et al., 2015). While female *P.*

87   *astrigera* are monandrous, the polygynous males can copulate with as many as five virgin

88   females at 24h intervals (Jiao et al., 2011; Wu et al., 2008). While inbreeding depression has

89   not previously been addressed in this species, prior research into its courtship and mating

90   behavior make it an ideal model system for addressing such questions.

91   We report work investigating inbreeding avoidance through courtship behavior and

92   the impact of inbreeding on reproductive output and offspring survival in the monandrous

93   wolf spider *P. astrigera*. We compared male courtship behaviors in response to dragline silk

94   of sibling and non-sibling females to test for male pre-copulatory kin discrimination. We also

95   conducted non-choice mating experiments to compare the likelihood of sibling and non-

96   sibling mating. In addition, we measured post-mating female reproductive output (both

97   number and size of offspring) to determine the cost of inbreeding on female fitness. Finally,

98   we compared the survival of inbred versus outbred offspring across a range of temperatures.

99   We predicted that strong pre-copulatory barriers exist to sibling mating, that these barriers are

100   stronger in females than in males, that inbreeding reduces both maternal and offspring fitness,

101   and that higher temperatures increase the impact of inbreeding on the offspring.

102   **Methods**

103   Subadult *P. astrigera* of the overwintering generation were collected in April 2012

104   from Ma'anshan Forest Park, Wuhan, Hubei Province, China. Spiders were housed

105   individually in opaque Plexiglass enclosures (5.0 × 5.0 × 7.5 cm, l×w×h) at 25 ± 0.5 ºC with

106   60 ± 10% relative humidity ('RH') and on a 14:10 light:dark ('l:d') cycle. Spiders were

107   supplied with water ad libitum and fed every 3 days with a mixture diet of *Drosophila*

108   *melanogaster* and mosquitoes (*Culicidae*). Individuals were checked daily for subadult

109   molting in order to determine the exact date of adulthood. We used randomly-selected adult

110   spiders to create the ten male:female pairs used to generate ten families. Mated females were

111   maintained as above. We randomly selected and reared 30 spiderlings from each eggsac; each

112   spiderling was reared individually in a glass tube (1.5 cm diameter). Spiderlings were

113   supplied with water *ad libitum* and fed every two days with a mixture of *D. melanogaster* and

114   mosquitoes. Once the spiders matured, similarly-sized females in their third day of adulthood

115   were selected for silk collection and/or behavioural trials. All spiders were virgin and used

116   only once; all adult spiders, except for those females whose lifespan was measured (details

117   below) were released following their involvement in the experiment.

Experiment 1: Male response to sibling/nonsibling female silk

119   Silk was collected by placing each female in a 9-cm diameter glass petri dish lined

120   with filter paper (15 cm diameter; Double Ring brand, Hangzhou, Zhejiang, China) for 12 h.

121   All females were starved for 12 h beforehand to reduce faecal contamination. All silk was

122   used within 18-24 h after its collection, a period of time over which silk-borne spider cues do

123   not degrade under natural conditions (Baruffaldi, Costa, Rodriguez, & Gonzalez, 2010;

124   Costa, Curbelo, & Perez-Miles, 2015).

125   We randomly selected similarly-sized virgin males ($N = 65$, 5-7 individuals per

126   family) aged 5-10 days post-maturation and assigned each to one of the two female silk

127   stimulus treatments. Male body size did not differ significantly between treatments ($t_{61} =$

128   0.75, $P = 0.45$). Thirty-three males were exposed to silk from a female in the same family

129   (sibling), and 32 males were exposed to silk from a female from a different family (non-

130   sibling); silk from a given female was only used for one male.

131   Behavioural trials were carried out in a cylindrical glass container open at both ends

132   (10.5-cm diameter, 12-cm length). After setting the cylindrical glass container on the silk-

133   covered filter paper, a single male was gently introduced onto the stimulus filter paper with a

134   glass tube from above and its courtship behaviour videotaped (HDR-CX580E Sony video

135   camera) for five min. We chose this cut-off period because preliminary experiments revealed

136   that male spiders exposed to silk either began courtship rapidly (within two minutes) or never

137   engaged in courtship behavior (Roberts & Uetz, 2004). Each arena was cleaned after each

138   trial with 70% ethanol and left to air dry. Videos were analysed using Observer v. 4.1 (Noldus

139   Information Technology, Wageningen, The Netherlands), a software package for behavioural

140   data analysis. On the basis of work reported in Wu et al. (2007, 2008) and Jiao et al. (2009),

141   the following courtship behaviours were analysed: (a) time to the start of body shaking and

142   (b) foreleg raising; (c) The number of body shaking and (d) foreleg raising events per minute.

143   Data from all trials was analysed to determine whether the likelihood of courting

144   behavior was affected by female relatedness. For analysis of specific courtship behaviors,

145   data from trials where such behaviors did not occur within five minutes were excluded from

146   analysis.

147   **Experiment 2: Male and female responses to siblings and non-siblings**

148   We paired individual virgin females ($N = 120$; 12 spiders from each family) in their

149   third day of adulthood with individual virgin males 5-10 days into adulthood; 60 male-female

150 pairs were siblings, and the other sixty pairs were non-siblings. All spiders belonged to one of

151 the ten families. We recorded behavioural data on male courtship as per experiment #1, and

152 also whether mating occurred within 30 minutes. Data for replicates in which no mating

153 occurred was used to analyse mating likelihood in treatments but not included in other

154 mating-dependent analyses (described below). Forty-two females mated with non-sibling

155 males and 31 females with sibling males; each mating produced an eggsac. The unit of

156 replication for analysis of mating behavior was individual mating pairs ($N = 73$).

157 **Experiment 3: Impact of inbreeding on female fecundity and offspring survival**

158 We held mated females individually under the conditions described above, and

159 checked daily for an eggsac. Although all 73 females produced eggsacs, 20 cannibalized their

160 eggsacs prior to hatching; eggsacs from the remaining 53 spiders (26 sibling and 27 non-

161 sibling) hatched successfully. The size (measured as carapace width) of female spiders did

162 not differ between treatments ($t_{45} = 0.51$, $P = 0.61$). We removed the eggsacs of five

163 randomly-chosen sibling-mated spiders and eight non-sibling-mated spiders for an unrelated

164 experiment, leaving a total of 40 eggsac-producing females (21 sibling and 19 non-sibling,

165 representing all ten families). For each female, we recorded time (days) from mating to first

166 eggsac production and from first eggsac production to hatching. After the first eggsac was

167 produced, each female was kept alive and fed *ad libitum* until death to measure their lifespan

168 and see if they produced additional eggsacs. Offspring from these eggsacs plus the number of

169 offspring from the first eggsac determined total offspring production per female.

170 After recording the number of offspring emerging from the first eggsac, we preserved

171 five randomly-selected offspring from it in 70% alcohol for carapace width measurements.

172   We divided the remaining offspring of the first eggsac into three groups. Spiderlings

173   were kept in 1.5-cm diameter glass tubes with no water and held at one of three temperatures

174   (15, 25 and 30 ºC) without food nor water (60 ± 10% RH, 14:10 light:dark cycle). These

175   temperatures were chosen to reflect the mean, high, and absolute highest temperatures spiders

176   might experience at this point in the year. While 25º C temperatures are ideal for spider

177   development when water is provided *ad libitum*, in the absence of water such high

178   temperatures speed desiccation and death. Survival was checked twice daily. The survival of

179   all offspring of a female at a given temperature was averaged; the unit of replication was

180   mean offspring survival per female per temperature ($N = 120$).

181   **Ethical note**

182   Animal care in all experiments complied with the current laws and standards of China

183   (Bayne & Wang, 2014).

184   **Data analysis**

185   Data were analyzed by fitting a generalized linear mixed model (glmm) with the

186   appropriate link function (e.g. Gaussian, Poisson, binomial) using penalized quasi-likelihood

187   (PQL) ('glmmPQL' function in MASS package, Venables & Ripley, 2002) in R (R

188   Development Core Team, 2017). Family nested within treatment (i.e. sibling and non-sibling)

189   was used in all models as a random effect to account for the non-independence of multiple

190   individuals from a given family. A Wald χ^2 test was used to extract χ^2 and *P*-values on the

191   glmm model using the 'Anova' function in the 'car' package (Fox & Weisberg, 2011).

192   Additionally, data on mean offspring size from experiment three was analyzed by including

193   mating treatment in all models as a fixed effect with female carapace width (a proxy for body

194 size) as a covariate. Data on mean offspring survival from experiment three was also

195 analyzed as above but with the addition of a fixed main effect (temperature) and a

196 temperature*mating interaction.

197 **Results**

198 Twenty-one of 33 males responded to sibling silk, and 22 of 32 males responded to

199 non-sibling silk; the proportion of non-responding males did not differ between treatments

200 ($\chi^2_1 = 0.50$, $P = 0.48$). Males did not differentiate between sibling and non-sibling females

201 when exposed to either silk cues (Fig. 1, top panel) or directly to the females themselves (Fig.

202 1, bottom panel). The start of courtship behaviors such as foreleg raising or body shaking was

203 unaffected by female relatedness, whether conveyed via silkborne cue (Figs. 1A and 1B,

204 respectively; χ^2_1, both $P > 0.5$) or direct female exposure (Figs. 1E and 1F; both $P > 0.5$).

205 There were also no treatment differences in the frequency of courtship behaviors in both the

206 silk-cue (Figs. 1C and 1D; both $P > 0.4$) and direct exposure (Figs. 1G and 1H; both $P > 0.3$)

207 experiments.

208 Despite similar male courtship behavior, mating occurred more often between

209 unrelated individuals (70% of pairings) than between siblings (52%; $\chi^2_1 = 4.26$, $P = 0.039$).

210 The time from mating to first eggsac production (Fig. 2A) and from production to hatching

211 (Fig. 2B) was similar for both sibling and non-sibling pairings ($\chi^2_1 = 0.43$ and 0.31,

212 respectively, both $P > 0.05$). The fecundity of sibling-mated females, however, was much

213 lower than that of non-sibling mated ones: they produced 41% fewer offspring in their first

214 eggsac (Fig. 2C; $\chi^2_1 = 24.8$, $P < 0.001$) and 44% fewer offspring in total (Fig. 2D; $\chi^2_1 = 34.2$,

215 $P < 0.001$). Five of 27 non-sibling mated females produced a second eggsac, while only two

216 of 26 sibling-mated females did so; this difference was not, however, significant ($\chi^2_1 = 1.40$, P

217 $= 0.24$). There were no treatment-level differences in the longevity of mated adult females

218 ($\chi^2_1 = 0.07$, $P = 0.80$).

219 The offspring of sibling and non-sibling pairings were of similar size (1.28 ± 0.006

220 [SE] and 1.27 ± 0.007 mm carapace width, respectively; $\chi^2_1 = 1.79$, $P = 0.18$). Offspring in

221 the sibling treatment survived an average of 23% longer (9.3 ± 0.20 [SE] and 7.1 ± 0.13 days;

222 $\chi^2_1 = 33.0$, $P < 0.001$) across all three temperature treatments than those in the non-sibling

223 treatment (Fig. 3). Spiderling survival declined as temperature increased ($\chi^2_2 = 111$, $P <$

224 0.001), and there was a significant mating*temperature interaction $\chi^2_2 = 10.3$, $P = 0.006$).

225 This interaction reflected the fact that the survival advantage of inbred offspring generally

226 decreased as temperature increased; inbred offspring survived 28% longer in the 15ºC

227 treatment, 19% longer in 25ºC, and 22% longer in 30ºC (Fig. 3).

228 **Discussion**

229 Contrary to our predictions, we found only weak pre-copulatory inbreeding avoidance

230 in *P. astrigera*. Male spiders, by not responding differently to silk or courting female cues,

231 showed no evidence of kin discrimination (Fig. 1). Female spiders mated at a higher rate with

232 unrelated individuals, but over half still mated successfully with male siblings. While weak

233 sibling avoidance suggests a minimal cost to inbreeding, the fecundity of sibling-mated

234 females was reduced (Fig. 2). Experimental assessment of their offspring, however, revealed

235 that although they were the same size as their outbred congeners, the offspring of sibling-

236 mated females survived ~20% longer under a range of environmental conditions (Fig. 3).

237 These findings highlight the importance of assessing both parental and offspring fitness when

238   exploring the costs of inbreeding.

239   The fact that females bred less often with sibling males demonstrates their ability to

240   detect relatedness via chemical or other cues; mate recognition via such cues often plays a

241   key role in inbreeding avoidance (Geiselhardt et al., 2009; Herzner et al., 2006; Lihoreau &

242   Rivault, 2010; Thomas & Simmons, 2011). In many spider species, males employ silk-

243   mediated cues for species, sex, and mating status recognition (Gaskett, 2007; Xiao et al.,

244   2015). Given this, we were surprised to find no evidence for male pre-copulatory mate choice

245   in response to either females or their silk. This result likely reflects the fact that male *P.*

246   *astrigera* are polygynous and compete fiercely with each other for mating opportunities (Jiao

247   et al., 2011). Because the males can remate, they have little to lose from inbreeding and

248   should seek to maximize mating opportunities even under strong inbreeding depression

249   (Duthie et al., 2016).

250   The inbreeding-related decline in female fecundity is consistent with results from a

251   wide range of taxa (Charlesworth & Charlesworth, 1987; Hedrick & Garcia-Dorado, 2016;

252   Pusey & Wolf, 1996). The >40% reduction in offspring number is especially harmful in a

253   monandrous species like *P. astrigera*, since females cannot compensate via subsequent

254   matings with higher-quality partners. Given these high costs, it may seem surprising that over

255   half of the females in the sibling group chose to mate. One explanation for this result may

256   involve our decision to employ a no-choice design in our mating assays. A recent meta-

257   analysis (Dougherty & Shuker, 2015) found stronger mating preferences in choice

258   experiments where females were exposed to different mates. If this is the case in *P. astrigera*,

259   our results may underestimate the strength of female mate preference. Alternately, sex-biased

260   dispersal prior to reproductive maturity has been shown to reduce the likelihood of

261   inbreeding in some species (Keane, 1990; Pusey & Wolf, 1996; Smith et al., 2016). If such

262   sex-biased dispersal occurs in this species, it may reduce the need for females to strongly

263   discriminate against related individuals. Finally, the weak sibling avoidance we observed may

264   highlight the importance of viewing the costs of inbreeding depression within the larger

265   context of female inclusive fitness - and specifically, the higher survival of inbred offspring.

266   There is considerable evidence that the offspring of sibling matings are equally or

267   more sensitive to environmental variation than their outbred congeners, presumably because

268   the stress associated with that variation increases the expression of deleterious recessive

269   alleles (Armbruster & Reed, 2005; Fox, Stillwell, Wallin, Curtis, & Reed, 2011; Kristensen et

270   al., 2008). We were thus surprised to find that inbred offspring survived longer than outbred

271   ones across a range of temperatures (Fig. 3). One explanation for this pattern, the idea that

272   density-dependent resource competition may disproportionately affect spiderlings from larger

273   clutches (Wise, 2006), is unlikely since hatched spiderlings were immediately confined to

274   individual glass tubes.

275   One likely explanation for our results involves the trade-off between offspring number

276   and per-offspring investment predicted for sibling matings (Duthie et al., 2016). Since inbred

277   offspring share more alleles with their parents than outbred offspring, each successful inbred

278   offspring increases parental inclusive fitness more than its outbred congener and is thus more

279   'worthy' of parental resource investment. As a consequence, the inclusive fitness of

280   inbreeding parents that invest resources in fewer offspring may equal or exceed that of

281   outbreeding parents that produce more less-provisioned offspring (Duthie et al., 2016). Were

282   this the case, we might expect offspring size to differ. Although spiderling carapace width

283   was negatively correlated with offspring per eggsac, there were no between-treatment

284   differences. Inbreeding parents may allocate more nutrients to eggs (Wilder, 2011) or employ

285   other forms of investment (e.g., parental care; Pilakouta & Smiseth, 2016). Future research

286   might address whether such alternate forms of parental provisioning occur in this system.

287   Our results are also consistent with the hypothesis that inbreeding in *P. astrigera*,

288   while harmful to parental fecundity, benefits one or more traits that prolong offspring

289   survival. The effects of inbreeding are often trait-specific, with some traits strongly affected

290   and others remaining similar to those found in outbred congeners (Kristensen et al., 2008;

291   Pilakouta & Smiseth, 2016; Valtonen, Roff, & Rantala, 2011). Given this, it is unsurprising

292   that inbreeding can increase the benefit of some life history traits. In the cricket *Teleogryllus*

293   *commodus*, for example, inbred individuals exhibit higher macroparasitic immunity than

294   outbred individuals (Gershman et al., 2010). Similarly, male *Litoria peronii* frogs that mate

295   with sibling females sire more offspring in sperm competition (Sherman, Wapstra, Uller, &

296   Olsson, 2008). These benefits can also be sex-specific: inbreeding in the beetle

297   *Callosobruchus maculatus* increases male - but shortens female - lifespan (Bilde, Maklakov,

298   Meisner, la Guardia, & Friberg, 2009). In our case, an increase in desiccation tolerance or

299   modifications to similar traits might provide inbred spiderlings a survival advantage

300   consistent with our results.

301   While inbreeding is generally harmful, its costs can vary substantially both between

302   and within species (Aviles & Bukowski, 2006; Szulkin et al., 2013); theory predicts an

303   optimal balance between inbreeding and outbreeding (Kokko & Ots, 2006; Puurtinen, 2011;

304   Richard, Losdat, Lecomte, de Fraipont, & Clobert, 2009). Our results reveal unexpectedly

305   weak inbreeding avoidance in a monandrous spider and demonstrate that sibling mating

306   reduces maternal fecundity but increases offspring survival in a range of environmental

307   conditions. These findings highlight the importance of viewing maternal fecundity in the

308   larger context of inclusive fitness; a relatively low degree of inbreeding avoidance may

309   reflect a trade-off between parental and offspring fitness. This is especially important for

310   monandrous organisms that, by definition, cannot employ post-copulatory mechanisms to

311   reduce the impact of inbreeding. In such species, weak sibling avoidance may be indicative of

312   inbreeding-related tradeoffs: future research should explore both the conditions that

313   necessitate pre-copulatory mate choice strategies and determine its strength.

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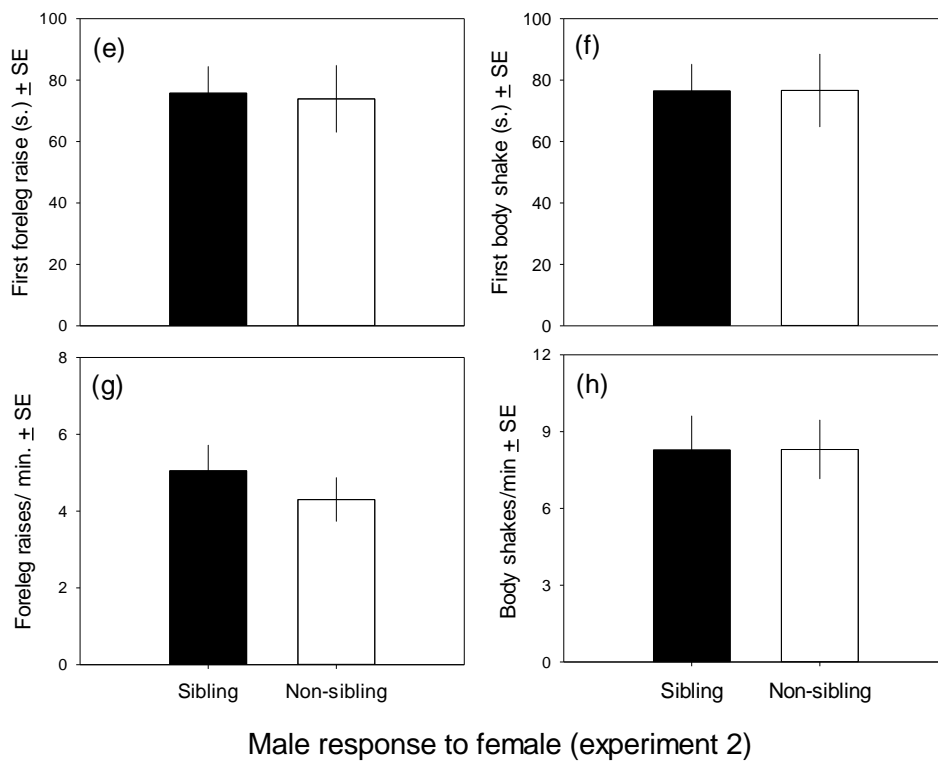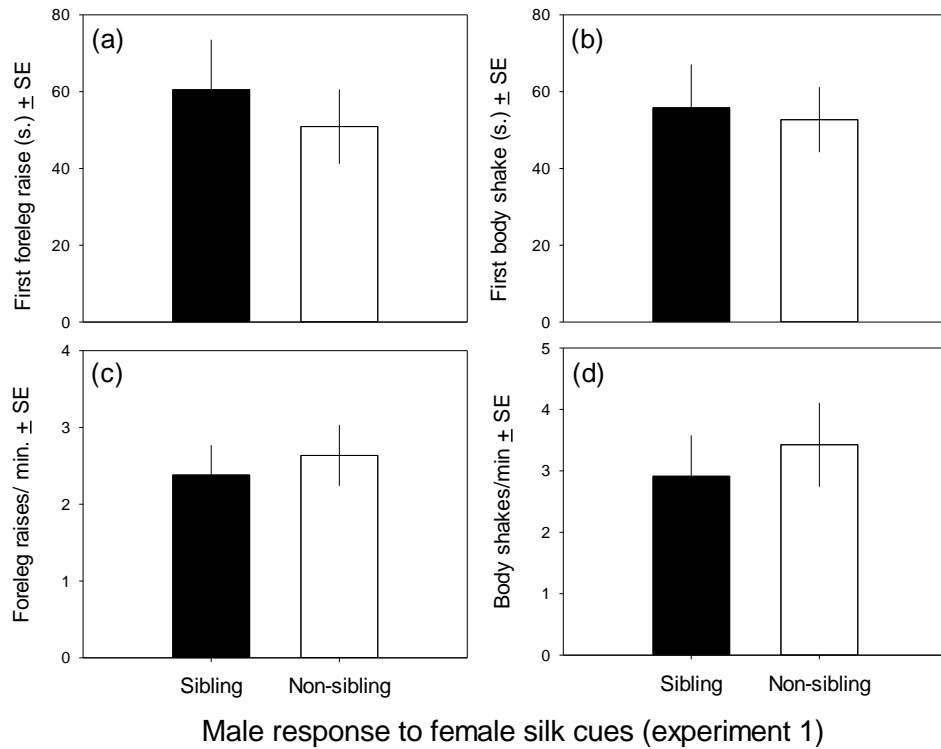
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458   **Figure Legends**

459   Figure 1. Male courtship behaviors in response to sibling versus non-sibling females.
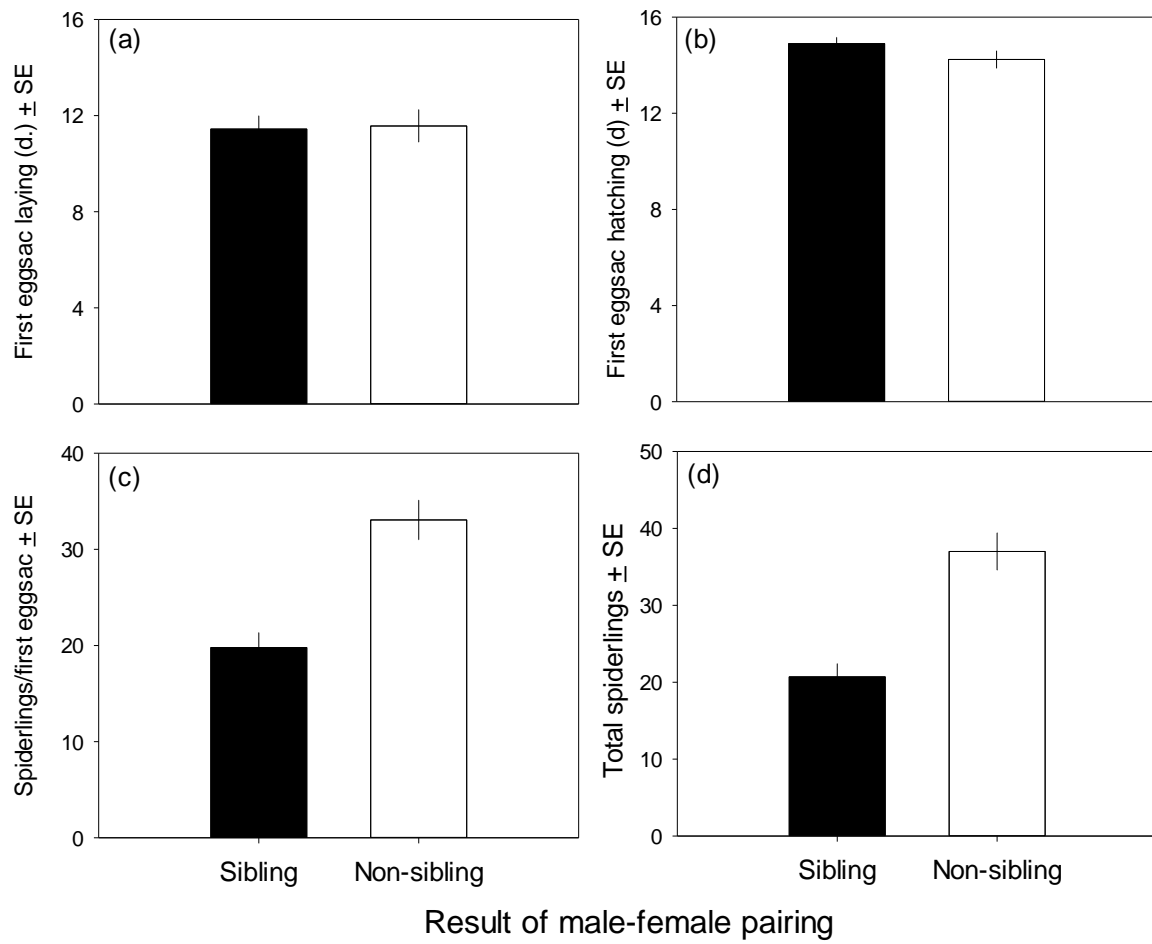
460   Panels A-D: courtship in the presence of silkborne cues produced by sibling (filled bars) and

461   non-sibling (open bars) females. Panels E-H: courtship in the physical presence of sibling and

462   non-sibling females.

463   Figure 2. Female reproduction (A-B) and fecundity (C-D) following mating with

464   sibling (filled bars) and non-sibling (open bars) males.

465   Figure 3. Survival of offspring (days) from sibling-mated females (filled circles) and

466   non-sibling mated females (open triangles) held without food or water at 15, 25, and 30 $^{\circ}$C.

467   Figure 1.

Male response to female silk cues (experiment 1)

Male response to female (experiment 2)

468

469

470   Figure 2.

Result of male-female pairing

471

472

473　　Figure 3.

474