1   **Local mate competition modifies the costs of mating in a mostly**

2   **monandrous parasitoid wasp**

3   **Running title: LMC modifies the costs of mating**

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Lay summary

Multiple mating by females is widespread but the factors that promote its evolution are not

well understood. We show that in the parasitoid wasp *Nasonia vitripennis* mating multiply is

costly becausemultiply mated females lay fewer fertilised eggs and more unfertilised eggs.

These unfertilised eggs develop into sons, whilst fertilised eggs develop into daughters, and

female *Nasonia* typically produce very female-biased sex ratios. . However, the cost of

producing more sons than is optimal is reduced when females lay eggs on hosts together. We

suggest that this constraint maintains single mating in dispersed natural conditions when lots

of females rarely lay eggs together, but permits multiple mating to evolve under mass rearing

conditions.

Abstract

The costs and benefits of mating are frequently measured in order to understand why females

mate multiply. However, to separate the factors that initiate the evolution of polyandry (from

monandry) from the factors that maintain it, we must ascertain how the environmental

context changes the economics of mating. Here we show how context-dependent costs of

mating can lead to the evolution of polyandry in a species that is monandrous in the wild, the

parasitoid wasp *Nasonia vitripennis*. We have previously shown that when females have

insufficient time between mating and gaining access to hosts for oviposition, they appear

unable to process sperm effectively and end up overproducing sons (i.e. laying unfertilised

eggs, since *Nasonia* is in haplodiploid). This overproduction of sons is costly due to selection

on sex allocation in this species. Although *N. vitripennis* is monandrous in the wild,

polyandry evolves under laboratory culture despite this sex allocation cost. In this study we

show why: when groups of females oviposit together, as they do in laboratory culture,

44   selection on sex allocation via Local Mate Competition (LMC) is reduced, increasing the

45   reproductive value of sons. This relaxes the fitness cost of male production. Overproduction

46   of sons still occurs, but it is penalised less in terms of fitness than when females oviposit

47   alone, under high LMC conditions, as they typically do in the field. Our results highlight how

48   the costs and benefits of mating can vary under different ecologically relevant conditions, in

49   this case the spatio-temporal distribution of resources and competitors, promoting the

50   evolution of polyandry from monandry, and vice versa.

51

52   **Introduction**

53   Polyandry, or the multiple mating by females to different males, is widespread in nature, with

54   profound consequences for sexual selection and mating system evolution (Pizzari & Wedell

55   2013; Taylor et al. 2014). In the insects, direct or material benefits that females gain from

56   mating multiply (e.g. nutritious nuptial gifts and seminal fluid components) are common and

57   contribute to the high incidence of polyandry (Arnqvist & Nilsson 2000). However,

58   polyandry in the insects is not without its costs, and the trade-off between the costs and

59   benefits of mating often result in female fitness being optimised at intermediate mating rates

60   (Arnqvist & Nilsson 2000). These costs and benefits are not fixed though, and can depend on

61   the environment in which mating takes place. Here we explore how the environmental

62   context can alter the economics of mating and drive mating system evolution.

63

64   We often think of the costs and benefits of mating and, by extension, polyandry, as more or

65   less fixed as is the case for some direct benefits and for the costs that result from damage

66   during copulation (Daly 1978; Thornhill & Alcock 1983; Chapman 2001; Elzinga et al.

67   2011). Often, however, the costs and benefits of mating depend upon the context in which

68   mating takes place. For instance mating can reduce the time available for foraging or

69   oviposition, and can increase the risk of predation. In these cases, the costs of mating increase

70   when there are such opportunities for foraging or oviposition, or when predation risk is

71   elevated (Daly, 1978; Thornhill & Alcock, 1983; Rowe, 1994).

72

73   In terms of context-dependent benefits, mating multiply can allow females to procure limited

74   resources that are not otherwise available in the environment. For instance, where water or

75   key nutrients are not readily available, polyandry can provide females with a relatively

76   greater benefit through nutritive ejaculate components, than in nutrient/water rich

77   environments (e.g. *Callosobruchus maculatus* Fox 1993; *Tribolium castaneum* Droge-Young

78   et al. 2016; *Pisaura mirabilis* Toft & Albo 2015). In these cases, resource-limited females

79   benefit more from polyandry, but the opposite can also occur. For instance, female

80   *Drosophila melanogaster* with more abundant resources increase their fecundity by mating

81   multiply, while resource-limited females do not. This is thought to be because high condition

82   females have higher potential fecundity, and so they need to mate multiply to acquire

83   sufficient sperm to maximize their fertility (Morimoto et al. 2016; Amitin & Pitnick 2007).

84

85   Explicitly considering how context can alter the costs and benefits of mating is key to

86   understanding why and how polyandry varies across time and space, and the extent to which

87   plasticity in female mating rate is favoured. Changes to the environment will alter the relative

88   costs and benefits of mating, which might then lead to evolutionary transitions between

89   monandry and polyandry. The gregarious parasitoid wasp *Nasonia vitripennis* is a valuable

90   model species to study the evolutionary transition from monandry to polyandry because it is

91   mostly monandrous in the wild, but polyandry evolves when these wasps are maintained

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92   under laboratory culture conditions (van den Assem & Jachmann 1999; Burton-Chellew et al.

93   2007, 2008; Grillenberger et al. 2008). In this study, we aim to address how the

94   environmental context alters the costs and benefits of polyandry in *N. vitripennis.* In doing so,

95   we hope to shed light on how changing ecological conditions (from the dispersed field

96   environment to the highly aggregated laboratory environment) alter the costs and benefits of

97   mating, and whether this change might contribute to the evolutionary transition from

98   monandry to greater polyandry seen in this species.

99

100   Previous work in *N. vitripennis* has shown that polyandrous females are constrained in their

101   production of female offspring (i.e. fertilised eggs that become daughters, as in haplodiploid

102   species such as *N. vitripennis;* Boulton & Shuker 2015a,b; for a discussion of the possible

103   mechanism, see Discussion). This reduces female fitness by compromising her sex allocation

104   under Local Mate Competition (LMC; Hamilton 1967). LMC occurs when mating is

105   localised and occurs between kin, a situation that is common in the wild in gregarious

106   parasitoids such as *N. vitripennis* (Grillenberger et al. 2008; Burton-Chellew et al. 2008).

107   When a single female (termed a "foundress") oviposits on a patch of hosts, LMC will be high

108   because brothers will compete to mate with their sisters. In this scenario, the foundress will

109   maximise her grand-offspring production by laying only enough sons to inseminate her

110   daughters (Martel et al. 2016). Under high LMC, producing a highly female biased sex ratio

111   will result in greater fitness, and so in this situation monandry is expected to be favoured over

112   polyandry, as monandrous females will be more able to produce such highly female biased

113   sex ratios.

114

115   In mass culture conditions, however, LMC is reduced as many females will lay eggs together,

116   including on the same host (a situation termed superparasitism: Godfray 1994) and so the

117   predicted sex ratio approaches equality. As such, sons are not constrained to mate only with

118   their sisters, and their reproductive value increases (Hamilton 1967; Werren 1980; Shuker &

119   West 2004). Under these conditions, we expect polyandry to be freer to evolve as the sex

120   allocation 'cost' observed in previous experiments becomes negligible.

121

122   So far, the costs and benefits of mating in *N. vitripennis* have only been measured under

123   controlled, high LMC conditions. Although this has been useful and informative, it does not

124   allow us to explicitly contrast the fitness costs and benefits of polyandry under typical mass

125   culture versus field conditions (Boulton & Shuker 2015a,b; Boulton & Shuker 2016). Here,

126   we present the results of two experiments that investigate the sex allocation cost of polyandry

127   under high (single foundress) and low (10 foundress) LMC conditions (experiment 1), and

128   when ovipositing on previously parasitized hosts (superparasitism; experiment 2). Finally, we

129   address whether the relaxation of this sex allocation cost might be sufficient to permit the

130   evolution of polyandry in *N. vitripennis*, by calculating the fitness costs of a range of

131   'mutant' sex allocation strategies under typical laboratory culture conditions.

132

133   **Methods**

134   *Study species*

135   *Nasonia vitripennis* (Hymenoptera: Pteromalidae) is a generalist gregarious parasitoid of

136   numerous species of muscomorphan diptera (Whiting 1967). As with all Hymenoptera,

137   *Nasonia* is haplodiploid. Under haplodiploidy, females are able to lay either a fertilised or an

138   unfertilised egg, producing either a daughter or a son respectively. Fourteen days after eggs

139   are laid (for development at 25°C), adult wasps emerge from the host puparium (Whiting

140   1967). Mating occurs on emergence, often between siblings, leading to Local Mate

141   Competition (LMC; Hamilton 1967, see above). Males display a stereotyped pre- and post-

142   copulatory courtship display; the former causes the female to become receptive to mating

143   (opening her genital pore to allow copulation) and the latter reduces her receptivity to

144   additional matings (although the effectiveness of post-copulatory courtship is reduced in lab-

145   adapted polyandrous lines; van den Assem & Visser 1976; Burton-Chellew et al. 2007; van

146   den Assem & Jachmann 1999). In these experiments, we prevented males from engaging in

147   post-copulatory courtship for every mating across all treatments in order to increase the

148   proportion of females that were receptive to additional matings (preventing or allowing post-

149   copulatory courtship does not influence offspring production or the sex ratio; Boulton &

150   Shuker 2015a). Females in monandrous treatments mated once with a virgin male, and

151   polyandrous females mated twice with a separate virgin male on each occasion. We deemed

152   two matings to be sufficient in this case for the polyandry treatment as *N. vitripennis* females

153   rarely accept a third mating in quick succession, even when post-copulatory courtship is

154   prevented (Boulton & Shuker 2015a).

155

156   *Laboratory culture*

157   For the following experiments wild-type individuals from the outbred strain HVRx were used

158   as focal females. When post-copulatory courtship is permitted this strain satisfies the criteria

159   for monandry (Torres-Avila et al. 2004) as <25% of females accept a second mating. HVRx

160   was derived from five field lines collected from Hoge Veluwe National Park in The

161   Netherlands in 2001 (van de Zande 2014). We maintain HVRx as outbred by mixing

162   parasitized *Calliphora vicina* (Diptera: Calliphoridae) pupae taken from six replicate cultures

163   each generation. Stock cultures are kept at 25°C on a 12: 12 light dark cycle, and every 16

164   days hosts (N = 40 per replicate) are provided to newly emerged wasps (around 40 females

165   and <8 males). For the following experiments we also used females from the red-eyed mutant

166   line STDR as competitors. STDR is maintained in a similar way to HVRx, but parasitized

167   pupae are not mixed (it is not an outbred strain). Focal females only mated with HVRx males

168   and competitor females only with STDR males.

169

170   *Standardisation of wasp rearing*

171   In order to standardise the larval environment, and as such the size and age of wasps used in

172   experiments, we reared all focal individuals and competitors from a grandparental generation

173   isolated from stock populations. We provided 'grandmothers' (of the focal females'

174   offspring) with equivalent sized hosts for the same time period (48 hours). To obtain HVRx

175   males, host pupae were opened two days before the next generation of adult wasps was due to

176   emerge and 216 virgin female pupae removed, isolated and provided with three *C. vicina*

177   hosts on adult eclosion. The virgin male offspring of this grandmother generation emerged

178   two weeks later and were maintained in groups of brothers until they were used in the

179   experiments. Focal (HVRx) and competitor (STDR) females were obtained in a similar way

180   by isolating and providing 360 (per strain) mated grandmothers (that had emerged with males

181   two days previously) with three *C. vicina* as hosts. In the next generation, competitor (STDR;

182   red-eyed) females were allowed to eclose and mate for 24 hours, before being isolated.

183   HVRx focal females were isolated from hosts as pupae before adult eclosion to ensure

184   virginity.

185

186   In experiment 1, both polyandrous and monandrous females were provided with hosts

187   immediately after their last (or only) mating. In experiment 2, we further explore the

188   possibility that the timing of mating with respect to oviposition influences the sex allocation

189   cost. This allowed us to test whether the time interval between mating and oviposition

190   influences sex allocation. Taken together, these two experiments test whether the sex

191   allocation cost is a true cost of polyandry (occurring only when multiple ejaculates interact)

192   or whether it occurs after any successful insemination.

193

194   *Experiment 1: The costs of mating when LMC varies*

195   In experiment 1, we tested how the number of matings (one or two) influenced the sex

196   allocation cost of polyandry when females allocate sex either alone (high LMC) or in a group

197   of 10 co-foundresses (low LMC). On the first day of the experiment, nine STDR females per

198   replicate (x 60 replicates per treatment) were put in stock tubes without males. This was to

199   ensure that all competitor females had sufficient time to process sperm after their most recent

200   matings (van den Assem & Feuth-de-Bruijn 1977). On day two, 120 HVRx females were

201   isolated into small petri dishes with a single virgin male and observed until mating took

202   place. After copulation, the male was brushed off her using a paintbrush to prevent him

203   engaging in post-copulatory courtship. These females mated again, with a different male, on

204   day three and were assigned to the polyandrous treatment. Monandrous females (N = 120)

205   were given access to their first and only male on day three, such that monandrous and

206   polyandrous females experienced the same interval between mating and being provided with

207   hosts to oviposit upon.

208

209   Immediately after the final mating (one for monandrous females, two for polyandrous

210   females) focal females were provided with six *C. vicina* pupae as hosts in one of two possible

211   LMC regimes, either high LMC (alone with hosts) or low LMC (with nine STDR females

212   and hosts). Thus, the factorial design comprised four treatments (1) monandrous, high LMC

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213   (N = 46); (2) monandrous, Low LMC (N = 43); (3) polyandrous, high LMC (N = 47); (4)

214   polyandrous, low LMC (N = 45). Initial sample sizes (N = 60 per treatment) here, and again

215   in experiment 2, differ from final sample sizes reported due to death/escape of focal females

216   during experiments and exclusion of data from replicates where mostly diapause larvae

217   (where sex is not easily distinguishable) were laid.

218   *Experiment 2: Costs of mating under superparasitism*

219   In experiment 2, we tested whether disrupted sex allocation after re-mating occurred as a

220   direct result of the number of matings, or whether the timing of mating with respect to

221   oviposition increased the likelihood that polyandrous females pay this cost (i.e. in Boulton &

222   Shuker 2015b). Focal females only had access to previously parasitized hosts (i.e. they were

223   the superparasite), allowing us to determine whether experimental females deviated from the

224   optimum predicted sex ratio according to their relative clutch size (based on the model of

225   Werren 1980). Briefly, the second foundress is predicted to increase the proportion of

226   daughters as she lays more eggs relative to the first foundress. When she lays only a single

227   egg, LMC is absent (there are no brothers to compete with) and she should lay a son. As she

228   lays more eggs on the host, LMC increases (because brothers represent competitors for

229   mates) and so the superparasite is predicted to lay more daughters (Werren 1980). The fully

230   factorial design comprised four treatments: (1) twice mated (polyandrous) females that were

231   given hosts 24 hours after their final mating (P24, N = 48); (2) once mated (monandrous)

232   females that were given hosts 24 hours after their final mating (M24, N = 50); (3) twice

233   mated females that were given hosts immediately after their final mating (P0, N = 37); (4)

234   once mated females that were given hosts immediately after their final mating (M0, N = 48).

235   On the first day of the experiment, 288 mated STDR (competitor) females were isolated.

236   Sixty virgin HVRx females were mated with 60 virgin HVRx males and post-copulatory

237 courtship was prevented (these females would be used for treatment P24). Twenty-four hours

238 later, each isolated STDR female was given a single *C. vicina* host on which to oviposit for

239 24 hours. On the same day, the P24 females were mated a second time with a virgin male.

240 Another 120 virgin HVRx females were also mated (again without post-copulatory courtship)

241 to virgin males, and these females comprised the M24 and P0 treatments. On the third day,

242 the P0 females mated again to virgin males. Finally, another 60 HVRx virgin females mated

243 with HVRx virgin males to make up the M0 treatment. The STDR females were then

244 removed from their hosts and each focal HVRx female received one of these pre-parasitised

245 hosts to superparasitise.

246 For both Experiments 1 and 2, one-way escape tubes were fitted to the tubes containing the

247 ovipositing females 60 minutes after providing hosts. This facilitates female dispersal and

248 limits forced super-parasitism (Werren 1983; Shuker et al.2004; 2007). After 24 hours, we

249 removed all foundresses and the hosts were incubated in small vials at 25°C until the

250 offspring emerged and died. Offspring were then sexed and genotyped by eye colour in order

251 to assess the fitness of focal females depending on the number or timing of matings and the

252 level of LMC.

253

254

255 *Estimating fitness costs*

256 In experiments 1 and 2, we calculated the relative fitness, which we define here as the fitness

257 of each focal female as a percentage compared to a female that allocated sex in line with the

258 predicted optimum under the same conditions ($W_{focal}/W_{predicted}$ x 100).

259

260   *Experiment 1*

261   In experiment 1 the optimal sex ratio (S*; proportion of sons) was calculated according the

262   number of foundresses using the following formula (Hamilton 1967):

263   Equation 1:

264
$$S^* = \frac{(N-1)(2N-1)}{[N(4N-1)]}$$

265

266   where N is the number of foundresses contributing offspring to a patch. In the low LMC (10

267   foundress) condition, the optimal predicted sex ratio was 0.438 (proportion of sons). In single

268   foundress conditions, the optimal proportion of sons is assumed to be 0.045 (the formula

269   gives a value of 0 because it does not take into account the necessity of producing at least one

270   son to ensure daughters are inseminated). We calculated 0.045 as the mean sex ratio required

271   to ensure insemination of all daughters based on estimates from two previous studies that

272   showed a single *N. vitripennis* male can inseminate between 15 and 30 females (van den

273   Assem 1976; Werren 1983; reviewed in Martel et al. 2016).

274   To calculate fitness for females in experiment 1 we used the following equation (Hamilton

275   1979):

276   Equation 2:

277
$$W = \frac{1}{2}\left[\frac{S_2}{S_2 + (N-1)S_1}\right][(N-1)(1-S_1)(1-S_2)] + \left[\frac{N}{2N-1}\right][1-S_2]$$

278   where N is the number of foundresses, S_1 is the ESS (evolutionary stable strategy) sex ratio

279   (produced by all other females) and S_2 is the focal female sex ratio (proportion of sons). We

280   calculated fitness for focal HVRx females W_{focal} and fitness for females that produced the

281   predicted 'optimum' sex ratio (equation 1) under the same conditions as the focal female

282    $W_{predicted}$. When fitness was calculated for females that allocated sex as predicted ($W_{predicted}$) S_1

283   and S_2 were equal. We then calculated the percentage relative fitness for the focal female as

284    $W_{focal}/W_{predicted} \times 100$.

285

286   *Selection on sex allocation*

287   To explore how the high foundress numbers that are typical in the laboratory can influence

288   selection on adaptive sex allocation, we calculated the fitness for a range (0.1-1) of mutant

289   sex allocation strategies relative to the optimal predicted strategy (when clutch size does not

290   vary) for foundress numbers from 1-40 (equation 1). We used equation 2 (from Hamilton

291   1979) to calculate fitness for females that either allocated sex optimally ($W_{predicted}$) or

292   produced an arbitrary sex ratio between 0.1 and 1 (W_{mutant}). We calculated the percentage

293   fitness for a mutant female compared to an 'optimal' female under the same conditions

294   ($W_{mutant}/W_{predicted} \times 100$).

295

296   *Experiment 2*

297   The exact sex ratio a female should produce depends upon where in the sequence she lays

298   (first or last; Werren 1980). For focal females that were the superparasite in experiment 2, we

299   calculated the sex ratio if a female allocated sex in line with the predicted optimum according

300   to their relative clutch size (relative to that of the first foundress; STDR). To do this we used

301   the following equation (adapted from Werren 1980; Shuker & West 2004):

302

303   Equation 3:

304
$$S_2^* = \frac{\sqrt{[2(1+F)(1+2F)(1+T)S_1]} - 2(1+2F)S_1}{2T(1+2F)}$$

13

305   where T = number of eggs laid by the first (STDR) female/ number of eggs laid by the

306   second (HVRx) female, F = Inbreeding coefficient (= 0.197 from the most recent and

307   complete study of *N. vitripennis* population genetics in the wild; Grillenberger et al. 2008). S_1

308   = sex ratio of the initial (STDR) brood.

309   We then used the following equation (adapted from Werren 1980) to calculate the fitness of

310   each focal female (W_{focal}) based on the sex ratio she laid (S_2) and her fitness had she allocated

311   sex as predicted (S_2*) in equation 3 ($W_{predicted}$).

312   Equation 4:

313    $$W = \left[\frac{TS_2}{S_1 + TS_2}\right][1 - S_1 + T(1 - S_2)]\left[\frac{1+F}{2}\right] + T(1 - S_2)\left[\frac{1 + 3F}{2}\right]$$

314   where S_2 = actual sex ratio produced by the focal (HVRx) female (T, S_1 and F are as above in

315   equation 3). S_2* was used in place of S_2 to calculate a females predicted fitness had she

316   allocated sex optimally ($W_{predicted}$). We then calculated relative percentage fitness for each

317   focal female compared to a female that produced the same clutch size but allocated sex as

318   predicted ($W_{focal}/W_{predicted} \times 100$).

319

320   *Statistical analysis*

321   To test whether the number of matings and level of LMC (in experiment 1) and the number

322   of matings and timing of mating with respect to oviposition (in experiment 2), as well as any

323   interaction effects, had any effect on offspring production of the focal (HVRx) female, we

324   used two-way GLMs with a Poisson error structure and a logit link function in R (lme4; R

325   Studio, Inc., Boston, MA: Bates et al. 2015). To test whether the level of LMC or the number

326   (and timing) of matings (and interaction effects) influenced the sex ratio produced, GLMs

327   with a binomial error structure were used. Similarly, two-way GLMs with a Poisson error

14

328   structure were used to test whether treatment influenced relative fitness in experiments 1 & 2.

329   For experiment 2, STDR clutch size was included as a fixed factor in all models. Where data

330   were found to be overdispersed (based on a dispersion parameter greater than 1) quasi-

331   models were used to account for overdispersion in the data.

332

333   For experiment 2, we extended the basic analysis of the sex ratio (including only treatment

334   effects) to demonstrate to what extent the sex ratio departed from the predicted optimum for

335   each focal female (Werren 1980). To do this, we fitted a binomial GLM where the outcome

336   variable was the sex ratio and the predictors were the relative clutch size (T = HVRx clutch

337   size/STDR clutch size), the quadratic function of relative clutch size (relative clutch size^2),

338   and the interaction terms for relative clutch size:treatment and relative clutch size^2:treatment.

339   The quadratic function of relative clutch size was used as we expect a curvilinear relationship

340   between the relative clutch size and the sex ratio (Werren 1980). A significant interaction

341   effect between relative clutch size^2 and treatment would suggest that across treatments

342   females allocate sex differently according to clutch size. In order to test exactly how the

343   number and timing of matings influenced the pattern of sex allocation, we used a one-way

344   GLM with a Gaussian error structure. The dependent variable was the residual sex ratio,

345   which was extracted from a sex ratio model (including data from focal females from all

346   treatment groups) with only relative clutch size and relative clutch size^2 as fixed factors. We

347   also calculated the predicted 'optimal' sex ratio over the full range of clutch sizes (see

348   equation 3 below, Werren, 1980) in this model. We then used pairwise comparisons (LSD

349   tests) to compare the sex ratios produced by females in each treatment group to their sex ratio

350   that they are predicted to produce given their clutch size and the clutch size of the first female

351   to oviposit. See supplementary table S1 for a summary of statistical tests used in experiments

352   1 and 2.

353   **Results**

354   *Experiment 1: The costs of multiple mating when LMC varies*

355   In experiment 1, polyandrous females produced significantly fewer offspring than

356   monandrous females ($F_{1,180} = 5.53$, $p = 0.02$), regardless of whether females were ovipositing

357   alone (high LMC) or with nine other co-foundresses (low LMC; interaction between LMC

358   level and mating treatment: $F_{1,177} = 0.01$, $p = 0.93$). More generally, individual females

359   unsurprisingly laid fewer eggs each when ovipositing in groups ($F_{1,180} = 34.53$, $p < 0.0001$;

360   Figure 1 A).

361

362   In terms of sex allocation, there was no significant effect of the number of matings on the sex

363   ratio (Quasibinomial GLM: $F_{1,180} = 3.12$, $p = 0.08$) in either the high or low LMC conditions

364   (interaction effect $F_{1,177} = 0.4$, $p = 0.53$; Figure 1 B).

365   As expected, females did adjust the sex ratio they produced according to the LMC regime

366   ($F_{1,180} = 37.12$, $p < 0.0001$), producing more female-biased sex ratios when ovipositing alone,

367   although in all treatment combinations the sex ratios produced were less female-biased than

368   those predicted by LMC theory. This disrupted sex allocation results in a loss of fitness

369   compared to a female that allocates sex optimally in the same conditions (Figure 1 C).

370   However, there was no significant effect of the number of matings on relative fitness ($F_{1, 179} =$

371   1.13, $p = 0.29$), nor any significant interaction effect between the number of matings and

372   LMC regime ($F_{1,179} = 1.62$, $p = 0.20$). The less female-biased sex ratios produced by focal

373   females were less penalised under low LMC however, where focal females had higher

374   relative fitness ($F_{1,179} = 48.54$, $p < 0.0001$; Figure 1 C).

375

376   *Selection on sex allocation*

377 We found that at very high foundress numbers (around 40 is common in laboratory culture)

378 fitness begins to plateau across all mutant strategies, suggesting that selection on adaptive sex

379 allocation will be weaker under these conditions (Figure 2).

380

381 *Experiment 2: Costs of mating under superparasitism*

382 Neither the number nor the timing of mating had any effect on successful parasitism (number

383 of matings: binomial GLM $\chi^2_{1,168} = 1.07$, p = 0.30, timing of mating: $\chi^2_{1,168} = 0.45$, p = 0.50,

384 interaction effect $\chi^2_{1,168} = 0.75$, p = 0.38; no effect of STDR clutch size $\chi^2_{1,169} = 3.04$, p =

385 0.08). Nor did the number or the timing of mating have a significant effect on clutch size of

386 the focal female (number of matings: QuasiPoisson GLM $F_{1,168} = 2.75$ p = 0.10, timing of

387 mating: $F_{1,168} = 0.02$, p = 0.89, interaction effect $F_{1,168} = 0.78$, p = 0.38). However, in this

388 model STDR clutch size had a highly significant effect on focal female clutch size ($F_{1,169} =$

389 34.35, p < 0.0001), which may mask any variation due to focal female mating treatment.

390

391 Crucially, females that were provided hosts immediately after mating produced more male-

392 biased sex ratios (Quasibinomial GLM: $F_{1,106} = 77.05$, p < 0.0001). Although there was no

393 main effect of the number of matings on the sex ratio ($F_{1,106} = 1.88$, p = 0.17), there was a

394 significant interaction between mating number and when hosts were provided, because

395 monandrous females laid significantly more sons than polyandrous females, but only when

396 they were provided with hosts immediately after ovipositing (M0; interaction effect $F_{1,106} =$

397 6.99, p = 0.009; effect of STDR clutch size $F_{1,107} = 4.24$, p = 0.04). Additionally, treatment

398 had a significant effect on the pattern of sex allocation with respect to clutch size (interaction

399 effect: relative clutch size^2*treatment: $F_{4,216} = 4.64$, p < 0.001; figure 3 A). Analysis of the

400 residuals for the model that included the predictors relative clutch size and relative clutch

17

401 size^2 showed that females given hosts immediately after mating produced sex ratios that were

402 significantly more male biased than the predicted optimum, regardless of mating number

403 (M0: t = 8.57, p < 0.0001; P0 t = 4.03, p < 0.0005; M24: t = -2.96, p < 0.05, t = P24: t = -

404 1.80, p = 0.07, p values corrected using the LSD test, Figure 3 B). A twenty-four hour delay

405 between mating and oviposition increased daughter production and allowed M24 and P24

406 females to allocate sex closer to the theoretical predictions (note however that M24 females

407 actually overproduced daughters compared to the prediction).

408

409 The overproduction of sons that occurred when females oviposited immediately after mating

410 led to a significant reduction in fitness (compared to a female laying the same clutch size, but

411 allocating sex optimally; QuasiPoisson GLM: $F_{1,93}$ = 21.24 p < 0.0001, Figure 4). Again,

412 however, there was no effect of mating number on relative fitness ($F_{1,94}$ = 0.01, p = 0.92;

413 interaction effect between timing and number of matings $F_{1,95}$ = 0.0001, p = 0.99).

414

415

416 **Discussion**

417 We typically study the costs and benefits of polyandry in species that are already

418 polyandrous. Whilst this is often the most logistically feasible approach, it means that we

419 typically cannot distinguish what maintains polyandry from what favours its origin (Boulton

420 & Shuker 2015a). Here we have shown that in the mostly monandrous wasp *Nasonia*

421 *vitripennis*, disrupted sex allocation constitutes a significant cost of mating, but the severity

422 of this cost depends on: (1) the timing of mating with respect to oviposition; (2) the level of

423 local mate competition (LMC). In particular, we found that polyandry need not be more

424 costly than monandry. If females oviposit soon after mating then they are unable to lay highly

425 female-biased sex ratios that are optimal under high levels of LMC. Sex allocation seems to

426 be disrupted because females require sufficient time to process sperm after mating, be it from

427 one or two ejaculates (Boulton et al. 2017). The timing of mating with respect to oviposition

428 therefore contributes to female fitness by influencing sex allocation, but by accepting

429 additional matings, polyandrous females are more likely to experience disrupted sex

430 allocation for longer and so polyandry can become more costly than monandry, as we have

431 seen in our previous studies (i.e. Boulton & Shuker 2015b; 2016; Boulton et al. 2017).

432 Additionally, we found that under low LMC conditions, disrupted sex allocation becomes

433 less costly in terms of relative fitness because the reproductive value of sons increases and

434 selection on adaptive sex allocation is weaker. A female that has accepted a mating soon

435 before ovipositing, which is more likely under polyandry than monandry (Boulton & Shuker

436 2015b; 2016; Boulton et al. 2017), will have lower fitness under high LMC than under low

437 LMC

438 The key finding from our first experiment was that the number of matings had no effect on

439 sex allocation. In this experiment, all females (monandrous or polyandrous) were provided

440 hosts immediately after mating. In both high (alone) and low (nine co-foundresses) LMC

441 conditions, all females laid around 30% more sons than predicted. In experiment 2, we

442 extended this observation: when oviposition occurred immediately after mating, regardless of

443 mating number, females produced too many sons and experienced reduced fitness. If, on the

444 other hand, females experienced a twenty-four hour interval between mating and oviposition,

445 they were able to allocate sex closer to the predicted optimum and consequently achieved

446 higher fitness relative to an 'optimally' behaving female.

447

448   Our results clearly show that the fitness costs of mating in *N. vitripennis* also vary according

449   to the level of LMC. This is one of the advantages of this system, in that the fitness

450   consequences of sex allocation (West 2009) can be easily quantified. In experiment 1, all

451   females experienced disrupted sex allocation, but in terms of fitness, those that were kept

452   with nine other foundresses (low LMC) did better than females that were maintained alone

453   with hosts (high LMC). This is because although all females produced too many sons, the

454   reproductive value of males increases as LMC decreases. Moreover, by calculating the

455   relative fitness of a range of fixed mutant sex allocation strategies under foundress numbers

456   ranging from 1-40, we can see that under typical mass culture conditions, when foundress

457   numbers are very high (~40), selection on individual sex ratios is weakened (provided that

458   the population sex ratio is at, or close to, equilibrium; Figure 4). This confirms the insight

459   that the Fisherian sex ratio equilibrium is a locally stable one (West 2009). Although the

460   results of experiment 2 show that sex allocation is inevitably disrupted if the delay between

461   copulation and oviposition is insufficient, the results of experiment 1 and Figure 4 show that

462   the fitness consequences of this disruption are less severe under low LMC and by extension

463   under standard mass culture conditions.

464

465   The fitness consequences of altering the LMC regime have also been shown in the

466   haplodiploid spidermite, *Tetranychus urticae*. In this species, females maintained for

467   successive generations under high LMC demonstrate less plasticity in the sex ratio that they

468   produce, while females maintained under low LMC are able to flexibly produce sex ratios in

469   line with theory (Macke et al. 2011). In the current study, and in *T. urticae*, the level of LMC

470   has repercussions that extend beyond sex allocation directly – on the mating rate in *N.*

471   *vitripennis* and on sexual conflict in *T. urticae* (Macke et al. 2014).

472

473   We can view the significance of our results from two perspectives. First, we can consider

474 *Nasonia* as a model for the origin of polyandry, to explore the circumstances in which female

475 multiple mating can evolve. Second we can view our results in the context of how monandry

476 is maintained in this and other species. In terms of the former, polyandry has evolved

477 repeatedly in strains of *N. vitripennis* maintained in laboratory culture (Burton-Chellew et al.

478 2007). Presumably this evolutionary change occurs as a result of changes to the optimal

479 mating rate, which is underpinned by the relative costs and benefits of mating. *N. vitripennis*

480 females have previously been shown to gain a direct benefit from polyandry, but only when

481 mating multiply with virgin males (Boulton & Shuker 2015b). It is worth noting that this

482 result was not replicated here, as polyandrous females actually had lower fecundity in

483 experiment 1. This likely reflects the shorter timescale over which this current experiment

484 was conducted, as in Boulton & Shuker (2015b) the benefit of polyandry accrued over

485 several bouts of oviposition. It seems to be the case, then, that an intrinsic benefit of mating,

486 which occurs perhaps due to differences in ejaculate expenditure, is context-dependent upon

487 the mated status of the male. In particular, when access to virgin males in increased – as

488 under laboratory conditions – multiple mating appears to be favoured. In this study, we have

489 shown that two more context-specific factors, namely the temporal relationship between

490 oviposition and mating, and the level of local mate competition, can also alter the costs and

491 benefits of mating in *N. vitripennis*.

492

493   The context-specific nature of disrupted sex allocation determines the costs of mating in *N.*

494 *vitripennis*. Although sex allocation is disrupted by the timing and not the number of matings,

495 females that accept additional matings are more likely to overproduce sons compared to

496   resistant females (Boulton & Shuker 2015a, 2016; Boulton et al. 2017), presumably due to

497   the greater delay between mating and oviposition that resistance provides. The risk of failing

498   to allocate sex appropriately may thus constrain the evolution of polyandry in some wild

499   populations. In mass culture conditions though, where LMC is low, disrupted sex allocation

500   is less costly for fitness, so females that accept an additional mating soon before host

501   provisioning will not suffer such significant costs (i.e. Boulton & Shuker 2015a). As such,

502   maintenance of *N. vitripennis* under mass culture conditions may essentially 'free' females

503   from an important cost of mating that constrains the evolution of multiple mating in the wild.

504

505   These processes may not be exclusive to the dichotomy between laboratory versus field

506   however, since certain field populations may be structured such that the sex allocation costs

507   of mating are reduced. For instance, if host patches are aggregated, but sparse, it may be that

508   LMC is low and host search times are long, thus females are not constrained by how recently

509   they have mated. This possibility could be tested by measuring the propensity to re-mate of

510   field-collected virgins from differently structured populations of *N. vitripennis*. Testing field-

511   collected versus laboratory cultured females would also help to elucidate whether (and how)

512   selection acts to counter the sex allocation cost of mating. For example, field-collected

513   females – even though more likely to be monandrous – may be better able to alleviate the sex

514   allocation cost, for instance by processing sperm for daughter production more rapidly after a

515   single mating, than females from laboratory populations.

516

517   Second, our results can be considered in the context of how monandry is maintained in

518   species. Parasitoid wasps are one of the few groups where more or less monandrous females

519   are the norm (Ridley 1988; Boulton et al 2014). If monandry need not necessarily be the

22

520 default null mating system for females (Kokko & Mappes 2013), perhaps we need to reflect a

521 little more on groups where monandry is common. Our work here, and previously, suggests

522 that multiple mating may be costly in terms of reduced efficiency of sperm use and the

523 resulting compromised sex allocation – a trait that is key to fitness in parasitoids (Boulton et

524 al. 2017; Godfray 1994; West 2009). This cost may be widespread across parasitoids where

525 efficient sperm use – in some cases involving the release of sperm on a sperm-by-sperm basis

526 to fertilise each egg (Flanders 1956; Wilkes 1961) – may be very important. As such, sex

527 allocation and sperm use constraints may play an important and underappreciated role in

528 maintaining monandry. The drawback is that sperm limitation, through encountering sperm-

529 depleted males, may likewise be a common cost of monandry (Boulton et al 2014; Martel et

530 al 2016; King 2018). In summary, sperm use dynamics may be crucial to understanding the

531 interplay between monandry and polyandry in parasitoid wasps.

532

533 The maintenance of monandry has broader significance in cousins of the parasitoid wasps,

534 namely the social Hymenoptera, given that monandry (and monogamy more generally) is

535 emerging as a key prerequisite for the evolution of eusociality (Hughes et al. 2008; Boomsma

536 2009; 2013; Fromhage & Kokko 2011; Davies et al. 2016; see also the recent paper by Smith

537 et al 2018 on monandry in social ambrosia beetles). Monogamy maximises within-brood

538 relatedness, helping to set the scene for social evolution, and evidence suggests that eusocial

539 groups evolved from monogamous ancestors, at least in some cases (Boomsma 2013). The

540 extent to which the sperm-use constraints we have considered here influenced monandry in

541 these species is currently unknown, but it remains a possibility for haplodiploid species in

542 which there is selection for facultative sex allocation (West 2009).

543

544 To conclude, our results highlight that to understand the optimal mating rate, we must

545 examine the costs and benefits of mating across ecologically appropriate contexts. As we

546 suggest here, a cost that is contingent on the timing of mating may indirectly influence the

547 optimal mating rate. In the current study, the spatio-temporal distribution of resources and

548 competitors, here hosts and co-foundresses, can also influence the costs and benefits of

549 mating, but many other environmental factors may have important roles to play, for instance

550 predation risk and feeding opportunities (Rowe, 1994), as well as female condition and

551 access to resources (Toft & Albo 2015; Droge-Young et al. 2016; Morimoto et al. 2016). A

552 more comprehensive assessment of how environmental factors can influence the economics

553 of mating may explain discrepancies between optimal and observed mating rates and, more

554 broadly, increase our understanding of how mating systems evolve in response to

555 environmental change.

561

562 **Data accessibility**

563 Analyses reported in this article can be reproduced using the data provided by Boulton et al

564 (2018).

565

566 Conflict of interest: The authors have no conflict of interest to declare.

567

568

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Figure legends

Figure 1 (A) Polyandrous females produced fewer offspring than monandrous females (error

bars = 95% CI) **(B)** There was no effect of the number of matings (1, monandry, or 2,

polyandry) on the sex ratio (proportion sons). The black dotted line shows the predicted sex

ratio for high and low LMC (local mate competition) conditions (error bars = binomial CI)

(C) Focal females had higher relative fitness under low LMC, but there was no effect of

mating number on fitness in experiment 1 (error bars = 95% CI).

Figure 2 The relative fitness (as a percentage compared to females allocating sex optimally)

of a range of mutant sex allocation strategies varies with foundress number according to

Hamilton's (1967, 1979) theory of local mate competition (LMC). The fitness profile of

mutant sex allocation strategies for foundress numbers ranging from 1-40 is given, which

represents different levels of LMC (from high to low). At high foundress numbers (around 40

is typical in mass culture) the sex ratio produced by a mutant female has less of an impact on

715   relative fitness (increasing from dark to light) suggesting that selection on adaptive sex

716   allocation will be weaker under such conditions

717

718

719 **Figure 3** Females that mate immediately before ovipositing produce more male biased sex ratios, that are further from the optimum predicted by

720 relative clutch size **(A)** The timing of mating had a significant effect on the pattern of sex allocation with respect to clutch size (the line of best

721 fit is quadratic with respect to clutch size). **(B)** Pairwise analysis of the residuals revealed M0 and P0 females produced significantly more male

722 biased sex ratios than predicted (the black line represents the optimum predicted sex ratio * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$; error bars =

723 95% CI).

724

725 **Figure 4** Females that were allowed to oviposit immediately after mating (0) had reduced fitness (relative to a female producing the same

726 number of offspring but allocating sex optimally) compared to females that had a 24-hour delay between mating and ovipositing (24). There was

727 no effect of mating number on relative fitness (error bars = 95% CI).
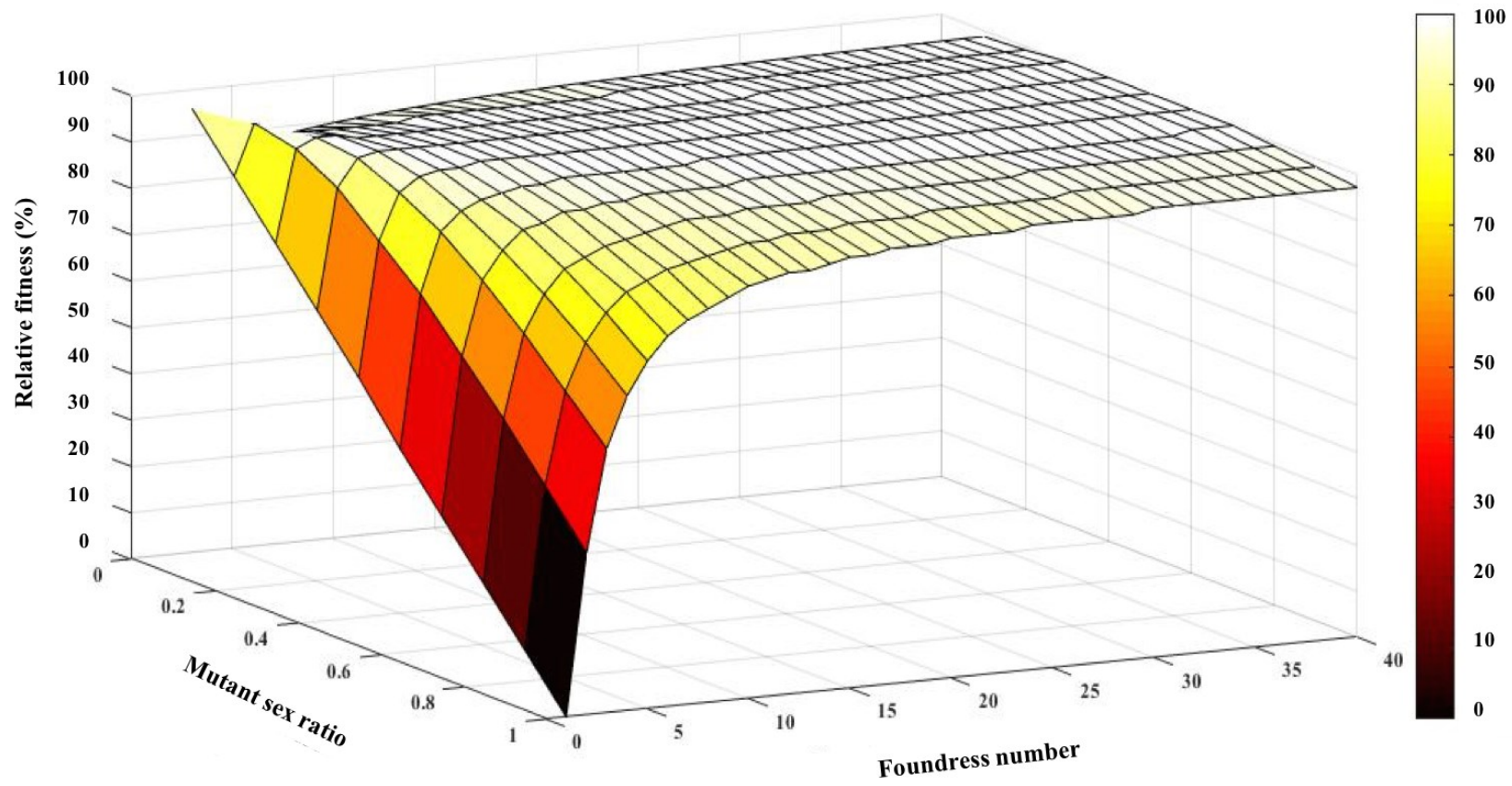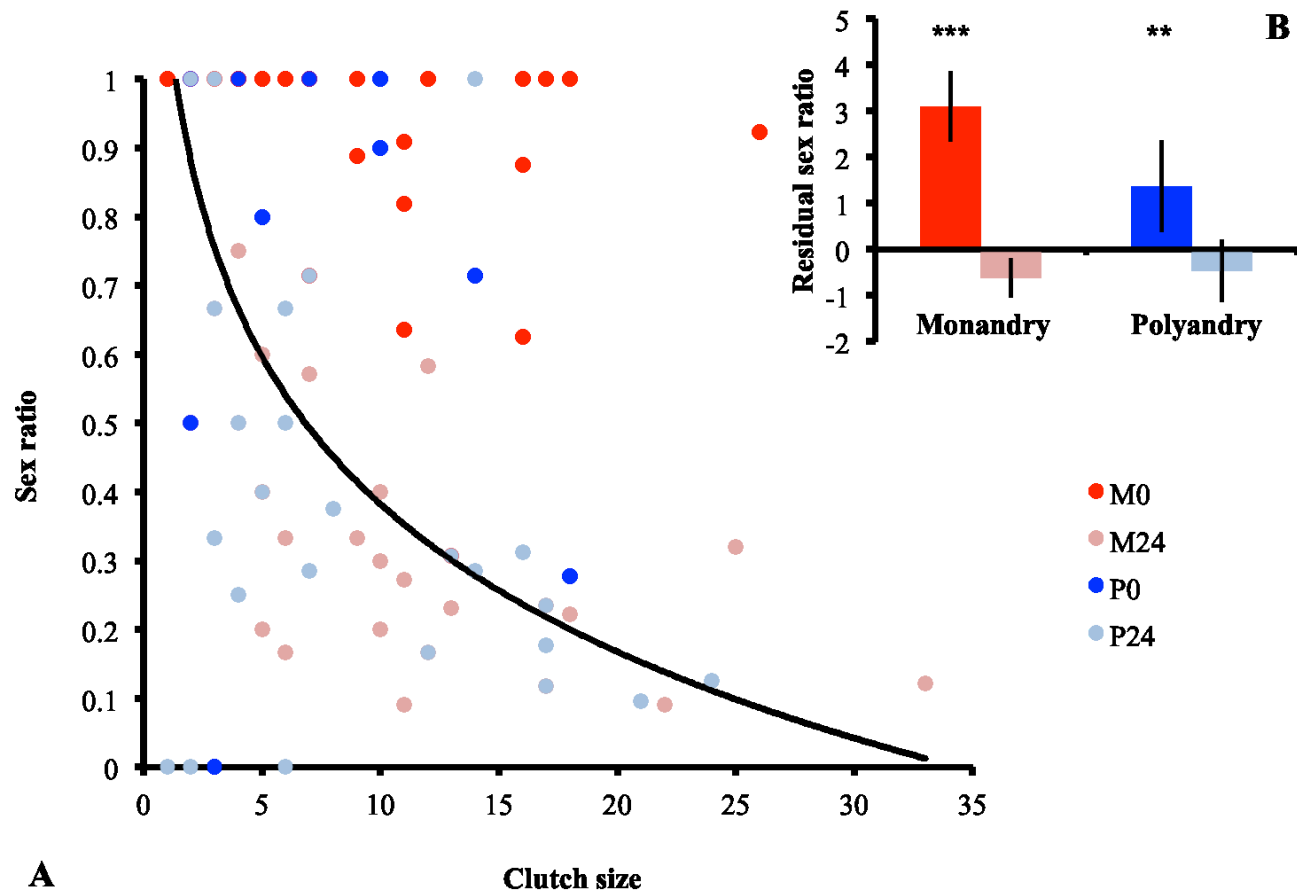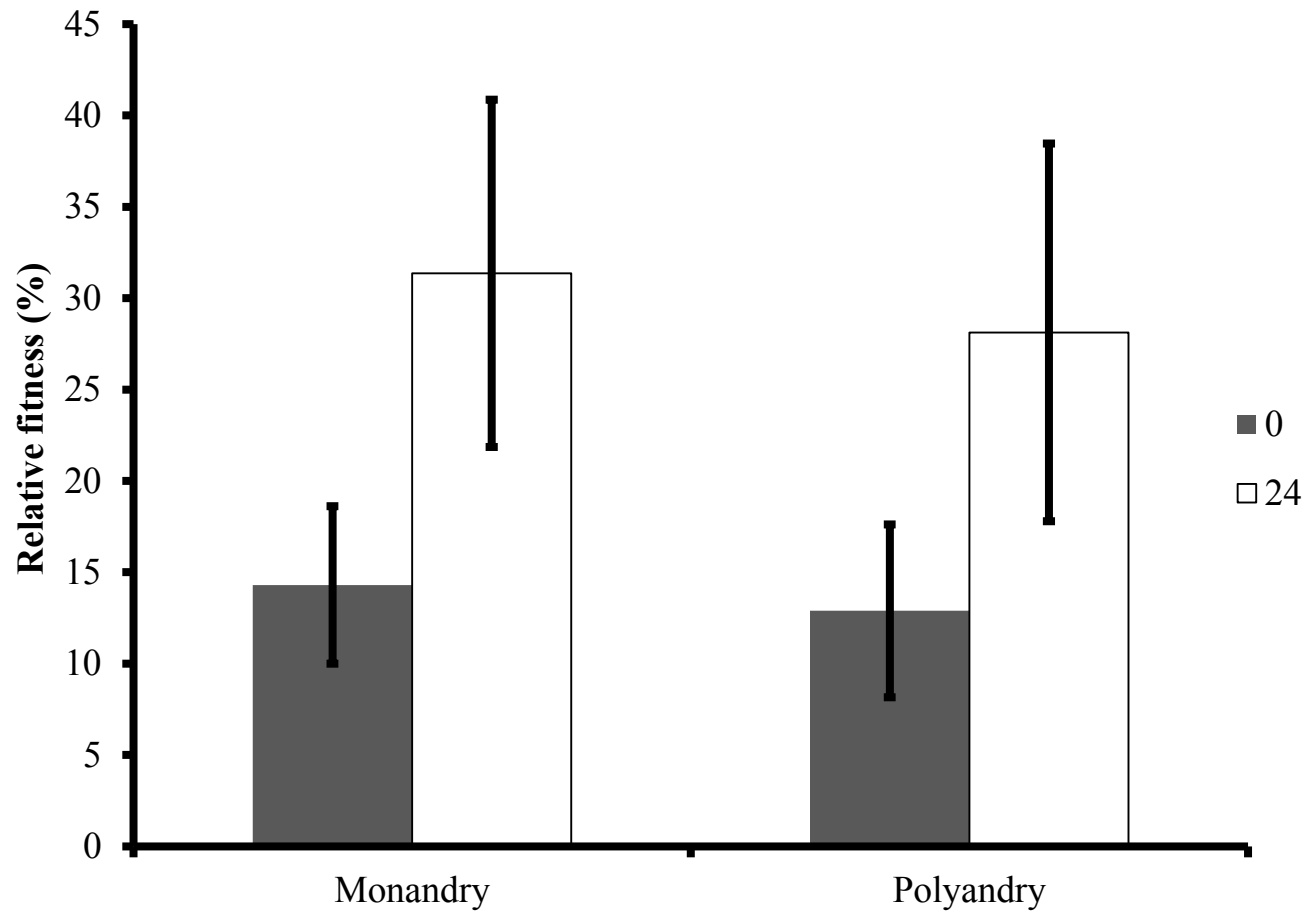
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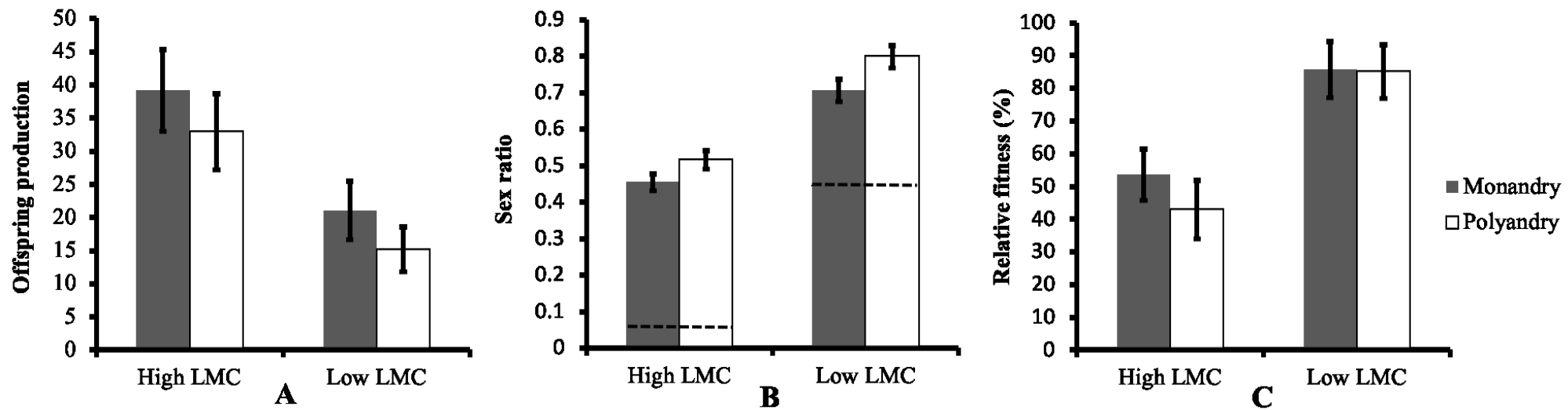
Figure 1

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735 **Figure 2**

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Figure 3

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740 **Figure 4**