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Spatially clustered resources increase male aggregation and mating duration in Drosophila melanogaster

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2 and mating duration in *Drosophila melanogaster*

3

4   ABSTRACT

5   In environments where females mate multiply, males should adjust their behaviour and

6   physiology in response to the perceived level of sperm competition in order to

7   maximise their fitness. Evidence of such plasticity has been found in a number of

8   laboratory and field studies, but little is yet known about the cues stimulating these

9   responses in natural populations. One way in which males appear to assess sperm

10   competition risk is through encounter rates with conspecific males. Such encounter

11   rates may be driven by the spatial distribution of resources required by males (i.e. food

12   patches or potential mates), which in turn affects local density. However, explicit links

13   between resource distribution, male encounter rate, and shifts in behaviour related to

14   sperm competition have not been demonstrated. We show that when group size of *D.*

15   *melanogaster* males is held constant, a small decrease in the distance between

16   patches of food resources has striking effects on male behaviour. First, males on

17   clustered resources have a significantly reduced inter-male distance (and hence

18   encounter rate) compared to those on dispersed resources, and second, males from

19   clustered resources show an increase in subsequent non-competitive copulation

20   duration – previously shown to be a reliable indicator of male perception of sperm

21   competition risk – of more than two minutes (13%) compared to those from dispersed

22   resources. The aggregation of resources, operating via increased encounter rate, can

23   stimulate shifts in behaviour affecting male sperm competition performance. Given that

24   the spatial distribution of resources, is typically variable in natural populations (and

25   often unpredictable), selection is likely to favour the evolution of plasticity in sexual

26   behaviour where resource aggregation increases the probability of sperm competition.

27   Keywords

30   Introduction

31   Variation in population density affects the rate at which individuals encounter

32   conspecific competitors and potential mates, with consequences for the strength of

33   sexual selection. One source of variation in local population density is the spatial

34   distribution of critical resources. Clumped resources lead to increased encounter rates

35   with competitors and mates as they gather to access those resources (Emlen & Oring,

36   1977). Where encounter rate is high, investment in traits such as sperm production,

37   courtship, mating duration should be upregulated to maximise reproductive success

38   in a dense social environment (Kokko & Rankin, 2006). Several empirical studies have

39   supported this prediction, including in crickets (Gage & Barnard, 1996), beetles

40   (McCullough, Buzatto, & Simmons, 2018), bugs (García-González & Gomendio,

41   2004), platyhelminths (Giannakara, Schärer, & Ramm, 2016), fish (Candolin &

42   Reynolds, 2002), and rodents (Firman, Garcia-Gonzalez, Simmons, & André, 2018;

43   Ramm & Stockley, 2009).

44   Demonstrating that male encounter rate can stimulate plasticity in sexual traits has

45   generally been achieved by housing males at varying densities in the laboratory, with

46   the most common treatment comparing a singly-housed male with a male housed with

47   one or more conspecifics (Candolin & Reynolds, 2002; Firman et al., 2018; Gage &

48   Barnard, 1996; Lizé et al., 2012; Moatt, Dytham, & Thom, 2013). This extreme

49   manipulation of the total number of potential rivals is not intended to mimic the effects

50   males experience in nature, but rather to demonstrate that such adaptive responses

51   exist. Evidence for how such responses link to more ecologically-realistic stimuli is

52   therefore lacking, although effects of sperm competition have been observed in natural

53   populations – for example in lizards (Kustra, Kahrl, Reedy, Warner, & Cox, 2019) and

54   frogs (Buzatto, Roberts, & Simmons, 2015). Given that patchiness in food resources

4

55 is common in nature, and that resource distribution affects the degree of male-male

56 competition (Emlen & Oring, 1977), small-scale variation in resource distribution that

57 leads to local variation in encounter rate should drive plastic variation in the allocation

58 of resources by males to sexual behaviour described above.

59 Laboratory studies have repeatedly demonstrated that *Drosophila melanogaster*

60 *(Drosophilidae Diptera)* males are highly sensitive to the presence of other males, and

61 that they increase their investment in sperm quality and ejaculate size (Garbaczewska,

62 Billeter, & Levine, 2013; Hopkins et al., 2019; Moatt, Dytham, & Thom, 2014),

63 investment in ejaculate composition (Fedorka, Winterhalter, & Ware, 2011; Hopkins et

64 al., 2019; Wigby et al., 2009), and lengthen copulation durations (Bretman, Fricke, &

65 Chapman, 2009) when they perceive an elevated risk of sperm competition. Because

66 *D. melanogaster* feed and breed on fermenting fruit (Begon, 1982), they rely on an

67 inherently patchy resource with individual fruits naturally varying in size and proximity.

68 Sex ratio and local population density of natural populations can vary considerably as

69 a result (Markow, 1988; Soto-Yéber, Soto-Ortiz, Godoy, & Godoy-Herrera, 2018).

70 Such patchiness in natural food resources seems an ideal candidate for the type of

71 ecological variability that might stimulate adjustment in post-copulatory processes in

72 the wild.

73 In this study, we test whether sperm competition-linked responses respond to resource

74 patchiness by exposing male *D. melanogaster* to three different food distributions

75 (clustered, dispersed and a uniform coverage control). In this way we can manipulate

76 local density in an ecologically-realistic way, but without manipulating the number of

77 rivals as previous laboratory studies have done (Bretman et al., 2009; Fedorka et al.,

78 2011; Garbaczewska et al., 2013; Hopkins et al., 2019; Moatt et al., 2014; Wigby et

79 al., 2009). We use the duration of copulation as a proxy for males' perception of sperm

80   competition risk, an association that has been demonstrated repeatedly in the

81   laboratory (Bretman et al., 2009; Bretman, Fricke, Hetherington, Stone, & Chapman,

82   2010; Bretman, Westmancoat James, Gage Matthew, & Chapman, 2012; Bretman,

83   Westmancoat, & Chapman, 2013; Mazzi, Kesäniemi, Hoikkala, & Klappert, 2009;

84   Moatt et al., 2013). We predict that: (a) by experimentally manipulating the distribution

85   of food resources, males on clustered resources have a higher mean proximity to rivals

86   (i.e. a higher encounter rate on average), and (b) males on clustered resources will

87   subsequently mate for longer on average, indicating an adaptive response based on

88   perception of increased sperm competition risk.

89

90   Methods

91   All fly rearing and experiments were conducted in a 12 hour light:dark cycle (0800 –

92   2000 GMT), at 25 °C. *Drosophila melanogaster* used were from a laboratory

93   population (Canton-S), and populations were cultured on 7 ml of a standard agar-

94   based medium of 40 g of yeast per litre, in 40 ml vials. Between 20 and 30 *Drosophila*

95   were housed in each vial. To minimise any effects of inbreeding, drift, and selective

96   sweeps, every seven days the adults from all vials were pooled and randomly

97   redistributed among new vials to start the next generation.

98   Test flies (180 in total – 60 per treatment) were collected from parent vials, each

99   established with six males and six females allowed to breed for 70-98 h. Test flies

100   were removed from parent vials within six hours of eclosion to ensure virginity; prior to

101   this individuals are not sexually mature (Strömnæs & Kvelland, 1962). Flies were

102   immediately aspirated under light ice anaesthesia into treatments. Virgin female flies

103   for mating assays were collected from the same parental vials and aspirated into new

104   vials in groups of four. Females were used in mating assays when they were seven

105   days (+ 6-8 hours) old (Churchill, Dytham, & Thom, 2019).

106   Manipulating resource distributions and patchiness

107   Each replicate for each treatment consisted of four virgin males maintained in a 90

108   mm Petri dish for three days. Food in each of these 45 dishes was arranged in one of

109   three treatments (N = 15): clustered, dispersed or uniform food resource distributions.

110   Clustered and dispersed treatments both contained four plugs (420 mm^3 per patch) of

111   standard food medium (as described above). The size of these patches is within the

112   range of patch sizes where territorial behaviours have previously been observed

113   (Hoffmann & Cacoyianni, 1990).

114 Dispersed food discs were placed at four equidistant points around the circumference

115 of the Petri dish; these were 50 mm apart along the edge of the square, 70 mm apart

116 on the diagonal (illustrated in Fig. 2). Clustered discs were placed in the centre of the

117 Petri dish, in a square arrangement with each food disc in direct contact with adjacent

118 discs. The uniform treatment was an even layer of 45 ml standard medium covering

119 the bottom of the dish (to the same height as the four food patches in the previous two

120 treatments): volume and surface area were both greater in the uniform than the two

121 patchy treatments, but given the number of flies food was assumed to be available *ad*

122 *libitum* in all. All treatments were maintained in 12L:12D at 25 °C, and the four male

123 flies per treatment remained in these conditions for 70 hours (+/- 1 h) until aged to

124 three days.

125

126 Quantifying male spacing behaviour

127 Treatment enclosures were placed in one of two identical incubators maintained at 25

128 °C and on the same 12:12 L:D cycle as the stock flies. Each incubator was fitted with

129 a Raspberry Pi (www.raspberrypi.org) connected to an 8MP Raspberry Pi Camera

130 module (v2; www.thepihut.com). Two to three Petri dishes, placed in a balanced

131 arrangement across all treatment combinations, were placed directly under each

132 camera. We used frame capture software ('raspistill') to collect one image every 15

133 minutes from 0800-2000 GMT (during the light part of the cycle). We captured the x-y

134 coordinates of each male at each time point using ImageJ's multiple point selector tool

135 (Schneider, Rasband, & Eliceiri, 2012), and then converted these into a set of six

136 Euclidean pairwise distances between the four males (24670 measurements across

137 the three treatments and all time points). For 325 out of the 4290 individual time-point

138 photographs (7.6%) we were unable to accurately locate at least one male on the

139   image. To minimize the effect of missing data on the number of time points included

140   per replicate, the unit of analysis was the mean (rather than the raw data) of the

141   distances between each pair for each time point.

142

143   Reproductive behavioural assays

144   After 70 h in treatment, each male from each Petri dish was allowed one opportunity

145   to mate with a virgin female and mating behaviours were observed (N = 15; 60

146   individuals). The male and female were aspirated into a standard food vial

147   supplemented with ~0.03 g active yeast granules. The space in the vial was limited to

148   $7cm^3$ by pushing the vial bung down into the vial to reduce encounter latency.

149   Courtship latency was defined as the time from which the pair were first introduced

150   until the male initiated his first wing extension. Latency to copulate (courtship duration)

151   started at the time of the first wing extension, and ended with a male's successful

152   mounting attempt. Copulation duration was recorded from successful mounting until

153   the pair were fully separated.

154   Not every male courted (uniform: 81.8%; clustered: 86.4%; dispersed: 95.6%), and not

155   all courting males mated (uniform: 75.0%; clustered: 86.8%; dispersed: 83.3%). We

156   observed each pair for a maximum of 90 minutes after the pair had been introduced,

157   and recorded failure to court and/or failure to mate after this time.

158

159   Statistical analysis

160   Sample sizes were 15 replicates (N = 60 *Drosophila*) for each of the three treatments,

161   of which 11 from each treatment (33 in total) were photographed to collect spacing

162   data. The effect of treatment on total inter-male distance was analysed using linear

9

163   mixed effects models, with plate included as a random effect in all models to account

164   for the non-independence of the four males in a single treatment replicate. Time point

165   (numbered sequentially from first to last measurement and treated as continuous) was

166   modelled as a fixed effect.

167   Treatment effects on mating related traits were analysed using linear mixed effects

168   models, with replicate plate entered as a random effect to account for the fact that

169   mating data were available for (up to) four males per plate. Time point) and treatment

170   were initially entered as interacting predictor variables; if the interaction was non-

171   significant we re-ran the model with both variables entered as main effects. We used

172   the R package lmerTest (Kuznetsova, Brockhoff, & Christensen, 2017) to generate p

173   values using the Satterthwaite approximation for degrees of freedom. To assess the

174   effect of treatment on binomial variables (courtship success, copulation success) we

175   used generalised linear mixed models with a binomial error distribution, and replicate

176   plate nested within treatment to account for possible plate effects.

177

178   Animal welfare note

179   Although *Drosophila* are not currently subject to any ethical restrictions in the United

180   Kingdom, we took precautions to minimise injury and stress by controlling larval

181   density during development, handling flies minimally and using only light ice

182   anaesthesia, and by euthanizing flies at the end of the experiment while they were

183   under anaesthesia.

184

185

186 Results

187 Effect of food distribution on inter-male spacing

188 The spatial distribution of food patches significantly influenced the mean pairwise

189 distance between the four males in the treatment, and this interacted with the time

190 course of exposure to treatment (treatment*time: $F_{2,4239}$ = 286, P = 2.20e^{-11}; Fig. 1;

191 Table 1). On the final day of treatment the time effect had stabilized (treatment*time

192 $F_{2,525}$ = 1.134, P = 0.3224), leaving a significant main effect of treatment on pairwise

193 distance between males ($F_{2,30}$ = 32.268, P = 3.33e^{-8}; interaction removed; Table 1).

194 Post-hoc testing confirmed that on this final day, pairwise distances among males in

195 the dispersed treatment (44.02 ± 0.66 mm SE) and the uniform treatment (39.35 ±

196 0.93 mm SE) were both significantly greater than among males in the clustered food

197 treatment (22.79 ± 0.86 mm SE; dispersed vs clustered $F_{1,20}$ = 57.8, P = 2.53e^{-7};

198 uniform vs clustered: $F_{1,20}$ = 27.9, P = 3.63e^{-5}; time remained in these models as a

199 main effect). There was no significant difference in mean pairwise distance between

200 males in the uniform and dispersed treatments ($F_{1,20}$ = 3.9, P = 0.061).

201

202 Effect of food distribution on mating behaviour

203 Among those males that mated, copulation duration was significantly affected by food

204 distribution previously experienced by males ($F_{2,42.5}$ = 3.96, P = 0.026; Fig. 2).

205 Analysing the effect of treatment on the mean mating duration across all males in a

206 replicate – a more conservative measure – confirmed a significant difference in mating

207 durations between treatments ($F_{2,42}$ = 4.22, P = 0.021). Males from the clustered

208 treatment mated for significantly longer (1170 ± 28 s SE) than those from the dispersed

209 treatment (1029 ± 28 s SE), a difference of 2 minutes 20 seconds ($F_{1,28}$ = 6.59, P =

210 0.016). Copulation duration of males from the uniform treatment did not significantly

11

211   differ from either of the other treatments (uniform copulation duration 1107 ± 23 s SE;

212   vs. dispersed: $F_{1,28.5}$ = 2.22, P = 0.146; vs. clustered $F_{1,28.5}$ = 1.96, P = 0.172).

213   However, despite these observed differences between clustered and dispersed

214   treatments, the mean distance between males while in the treatment did not

215   significantly affect copulation duration in any of the three treatments (all P > 0.101).

216   In total, 159 of 180 males (88.3%) courted the female. There was no significant effect

217   of treatment on the proportion of males that courted (generalized linear model with

218   binomial errors and plate nested within treatment; χ^2 = 118, P = 0.376). Similarly, 144

219   (80%) of males mated, and this was not influenced by treatment (χ^2 = 175, P = 0.286).

220   Neither the latency to start courting ($F_{2,39.3}$ = 0.201 P = 0.818) nor the latency to start

221   copulation ($F_{2,30.4}$ = 1.257, P = 0.299), differed significantly among the three

222   treatments.

223

224   Discussion

225   The high degree of plasticity in mating-related traits in male *Drosophila* is well

226   established (Churchill et al., 2019; Davies, Schou, Kristensen, & Loeschcke, 2019;

227   Droney, 1998; Fricke, Bretman, & Chapman, 2008; Jensen, McClure, Priest, & Hunt,

228   2015; Lefranc, 2000; Lüpold, Manier, Ala-Honkola, Belote, & Pitnick, 2010; Morimoto

229   & Wigby, 2016; Ormerod et al., 2017; Schultzhaus, Nixon, Duran, & Carney, 2017).

230   Variation in these traits is highly sensitive to conspecific male density in a manner

231   which suggests that males adjust investment in anticipation of the intensity of sperm

232   competition they are likely to encounter during mating (Bretman et al., 2009). However,

233   how this level of plasticity relates to the variation in density and resource distributions

234   observed in natural populations remains unknown, and laboratory studies tend to

235   manipulate density in ways that seem unlikely to occur frequently in nature (e.g. singly-

236   housed males compared to a high density of males in a single vial).

237   We show that manipulating food patchiness while keeping group size constant has a

238   similar effect on a sperm competition-related trait – both in direction and magnitude –

239   as manipulating local density directly, and that these effects can be observed even

240   over very small spatial scales. Other studies on this species have found an

241   approximately two-minute increase in mating duration in high density males compared

242   to low density males (Bretman et al., 2009; Bretman et al., 2010; Bretman et al., 2013).

243   Given that wild *D. melanogaster* encounter a patchy resource that is likely to alter male

244   encounter rates at a similar scale to that demonstrated here (Markow, 1988; Soto-

245   Yéber et al., 2018), we suggest that fine-scale variation in these environmental cues

246   might influence male allocation of resources to traits associated with sperm

247   competition, and thus mating success, in wild-living *Drosophila*.

248   Although the effect on mating duration is a repeatable indicator of male perception of

249   sperm competition risk, the benefits of this behaviour to males remains uncertain. In

250   many species, increased mating duration has been linked to increased sperm transfer

251   and offspring production (Edvardsson & Canal, 2006; Engqvist & Sauer, 2003;

252   Sakaluk & Eggert, 1996). In *Drosophila* the consequences of longer copulation

253   durations are less clear, with some studies reporting an association with increased

254   fitness (Bretman et al., 2009; Garbaczewska et al., 2013; Price, Lizé, Marcello, &

255   Bretman, 2012), while others have not found a link (Bretman et al., 2012; Dobler &

256   Reinhardt, 2016). Whether males on the clustered food resource would have a higher

257   fitness than those on dispersed resources remains to be tested, but will almost

258   certainly depend on mating order effects and the competing male's history of exposure

259   to rivals (Bretman et al., 2012). However, our objective here was not to examine fitness

260   consequences, but rather to demonstrate that males alter their perceptions of likely

261   sperm competition risk based on small-scale changes in the spatial distribution of

262   resources.

263   Interestingly, the effect of food distribution on male distribution behaviour and sexual

264   investment was observed in the absence of females. Females often follow social cues,

265   and their grouping behaviour is promoted by aggregation pheromones (Bartelt,

266   Schaner, & Jackson, 1985; Duménil et al., 2016). By comparison, given their low

267   feeding rate once adult (Wong, Piper, Wertheim, & Partridge, 2009), males are thought

268   to aggregate near food resources primarily to seek mating opportunities. That these

269   groups of males responded in their individual positioning to the distribution of food

270   even in the absence of females is intriguing, and leaves open the question of the

271   relative importance of female social cues compared to the direct response of males to

272   food resources. In general however, studies manipulating male density have tended

273   to exclude females from the treatment phase (e.g. Bretman et al. (2009); Bretman et

274   al. (2010); Lizé et al. (2012); Moatt et al. (2013); Price et al. (2012); and Rouse and

275   Bretman (2016)), meaning the effects of inter-sexual interactions on plastic responses

276   to density is relatively unexplored.

277   This study adds to a small number of studies that demonstrate the effects of

278   environmental heterogeneity on *Drosophila* behaviour. Yun, Chen, Singh, Agrawal,

279   and Rundle (2017) demonstrated that female fitness was higher in more spatially

280   complex laboratory environments as a result of a reduction in sexual interactions and

281   consequent mitigation of male harm. Similar effects have been demonstrated when

282   laboratory populations were presented with a refuge: female remating rates declined

283   substantially (Byrne, Rice, & Rice, 2008). Such rapid shifts in behaviour, driven by

284   ecological patchiness, have rarely been included in laboratory assays, but may have

285   major effects on the demography and growth rate of populations exposed to spatial

286   patchiness, through their effects on male reproductive skew and therefore effective

287   population size. Such effects may have important evolutionary and ecological

288   consequences in relatively patchy parts of a species' distribution, for example by

289   increasing sexual conflict over shared resources (Pilakouta, Richardson, & Smiseth,

290   2016), or reducing maximum sustainable rates of evolution (Bridle, Kawata, & Butlin,

291   2019; Bridle, Polechová, & Vines, 2009).

292   There are some intriguing dynamics operating in the inter-male distances in the early

293   stages of the treatment period: in particular, males on the dispersed food patches

294   initially experience lower inter-male distances than those on the clustered food (Fig.

295   1). This effect does not match what we expected to see among males attempting to

296   defend individual patches, and is the opposite to the pattern observed on the final days

297   of treatment. Inspection of photographs from this treatment suggests that males on

298   the dispersed food patches initially cluster together away from food before sorting

299   themselves into individual territories focussed around each patch. Territorial behaviour

300   in *D. melanogaster* has previously been observed under laboratory conditions, and

301   appears to be driven by boundaries of food sources (Lim, Eyjólfsdóttir, Shin, Perona,

302   & Anderson, 2014) so it is possible that multiple distinct territories could be established

303   under these conditions. However, it remains unclear what is driving the initial clustering

304   behaviour.

305   Our results demonstrate a clear link between small-scale patchiness of resources and

306   sexual behaviours that suggest that males are sensitive to sperm competition risk,

307   mediated by changes in male-male encounter rate. While density effects on male

308   mating duration have been demonstrated several times, we have placed this response

309   in a biologically meaningful context by demonstrating a link to ecological factors that

310   are very likely to be at play in wild-living populations.

311

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317

318   Author contributions

319   ERC, MDFT, and JRB designed the study; ERC performed all experiments; ERC and

320   MDFT analysed the data; all authors contributed to writing the manuscript.

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489

490

491   Figure 1. Mean inter-fly distance (mean of 6 pairwise distances between 4 focal flies

492   per plate, averaged across 11 replicate plates) over time. Black = uniform treatment

493   (evenly distributed food); red = clustered food patches; blue = dispersed food patches.

494   Bars show standard errors of the mean for each time point across all 11 treatment

495   replicates. Grey blocks indicate period of dark (2000 - 0800 GMT), and are not to

496   scale.

497

498   Table 1. Details of statistical parameters from linear mixed models analyses outlined

499   in the results. Model outputs are presented in the order they appear in the text.

500   Response variables and data subsetting are outlined in the subheadings, predictor

501   variables in the 'Parameter' column.

502

503   Figure 2. The effect of food resource spatial distribution on the duration of subsequent

504   copulation. Means (black dot) and 95% confidence intervals of copulation duration

505   (seconds). Sample sizes: clustered 49 (11 males did not mate), uniform 44 (16),

506   dispersed 51 (9). The treatment effect on mating duration remains significant when the

507   two mating duration values below 600s in the dispersed treatment are excluded from

508   the analysis ($F_{2,40.9}$ = 3.55, P = 0.038).

509

510
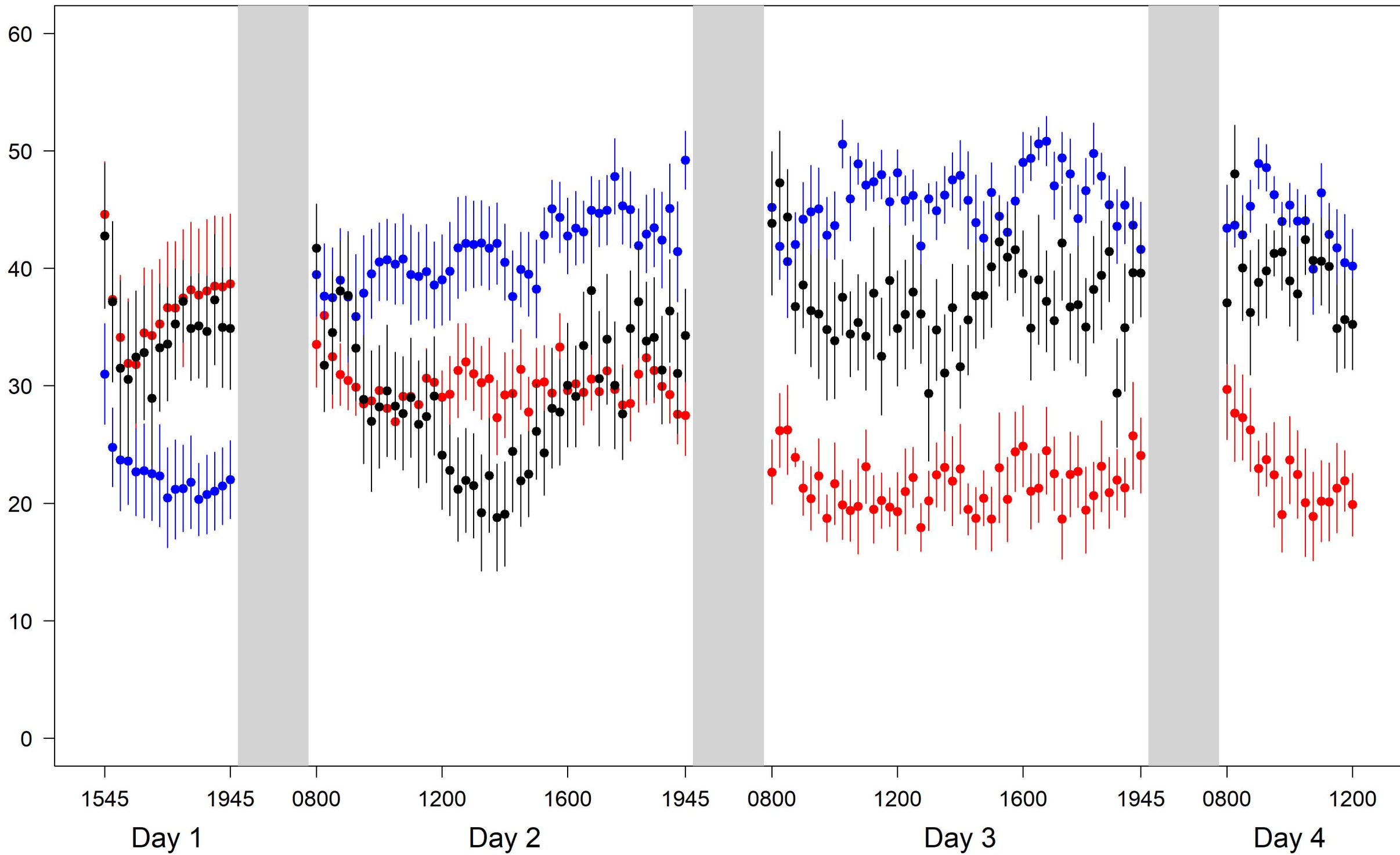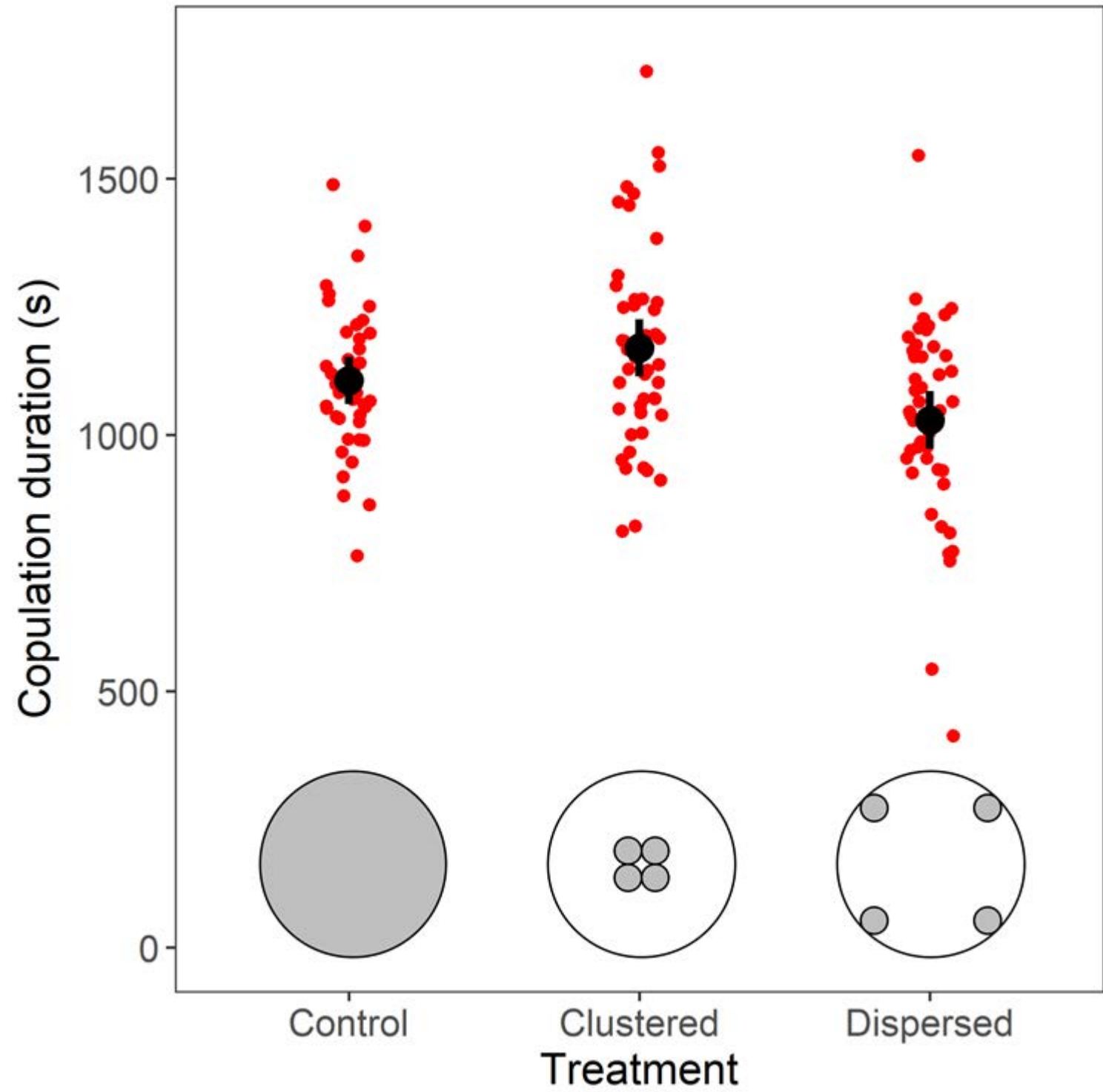
Parameter	Estimate	SE	T	p
Pairwise distance between males: full duration of treatment				
Clustered (intercept)	35.14	1.85	18.978	<0.0001
Uniform	-6.305	2.618	-2.408	0.021
Dispersed	-3.930	2.617	-1.501	0.142
Time sequence	-0.127	0.008	-14.946	<0.0001
Uniform*time	0.207	0.012	17.225	<0.0001
Dispersed*time	0.276	0.012	23.025	<0.0001
Pairwise distance between males: final day of treatment[a]				
Clustered (intercept)	22.794	1.983	11.493	<0.0001
Uniform	16.560	2.777	5.963	<0.0001
Dispersed	21.224	2.777	7.643	<0.0001
Copulation duration				
Clustered (intercept)	1170.9	35.28	33.19	<0.0001
Uniform	-64.7	51.12	-1.266	0.2124
Dispersed	-140.31	49.89	-2.813	0.0075
Copulation duration; outliers removed[b]				
Clustered (intercept)	1170.55	31.98	36.60	<0.0001
Uniform	-64.45	46.46	-1.387	0.173
Dispersed	-121.13	45.48	-2.66	0.0112
Courtship latency				
Clustered (intercept)	925.5	176.37	5.247	<0.0001
Uniform	-157.78	249.9	-0.631	0.531
Dispersed	92.17	245.2	-0.376	0.709
Copulation latency				
Clustered (intercept)	954.33	183.00	5.215	<0.0001
Uniform	-254.07	262.09	-0.969	0.340
Dispersed	154.10	255.73	0.603	0.552

a non-significant time*treatment term removed

b two outliers in the dispersed treatment with copulation duration values < 600 seconds removed