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

ABSTRACT

In environments where females mate multiply, males should adjust their behaviour and physiology in response to the perceived level of sperm competition in order to maximise their fitness. Evidence of such plasticity has been found in a number of laboratory and field studies, but little is yet known about the cues stimulating these responses in natural populations. One way in which males appear to assess sperm competition risk is through encounter rates with conspecific males. Such encounter rates may be driven by the spatial distribution of resources required by males (i.e. food patches or potential mates), which in turn affects local density. However, explicit links between resource distribution, male encounter rate, and shifts in behaviour related to sperm competition have not been demonstrated. We show that when group size of *D. melanogaster* males is held constant, a small decrease in the distance between patches of food resources has striking effects on male behaviour. First, males on clustered resources have a significantly reduced inter-male distance (and hence encounter rate) compared to those on dispersed resources, and second, males from clustered resources show an increase in subsequent non-competitive copulation duration – previously shown to be a reliable indicator of male perception of sperm competition risk – of more than two minutes (13%) compared to those from dispersed resources. The aggregation of resources, operating via increased encounter rate, can stimulate shifts in behaviour affecting male sperm competition performance. Given that the spatial distribution of resources, is typically variable in natural populations (and often unpredictable), selection is likely to favour the evolution of plasticity in sexual behaviour where resource aggregation increases the probability of sperm competition.

27 Keywords
28 Copulation duration, evolution, mating behaviour, plasticity, resource distribution,
29 sexual conflict, sexual selection, sperm competition

Introduction

Variation in population density affects the rate at which individuals encounter conspecific competitors and potential mates, with consequences for the strength of sexual selection. One source of variation in local population density is the spatial distribution of critical resources. Clumped resources lead to increased encounter rates with competitors and mates as they gather to access those resources (Emlen & Oring, 1977). Where encounter rate is high, investment in traits such as sperm production, courtship, mating duration should be upregulated to maximise reproductive success in a dense social environment (Kokko & Rankin, 2006). Several empirical studies have supported this prediction, including in crickets (Gage & Barnard, 1996), beetles (McCullough, Buzatto, & Simmons, 2018), bugs (García-González & Gomendio, 2004), platyhelminths (Giannakara, Schärer, & Ramm, 2016), fish (Candolin & Reynolds, 2002), and rodents (Firman, Garcia-Gonzalez, Simmons, & André, 2018; Ramm & Stockley, 2009).

Demonstrating that male encounter rate can stimulate plasticity in sexual traits has generally been achieved by housing males at varying densities in the laboratory, with the most common treatment comparing a singly-housed male with a male housed with one or more conspecifics (Candolin & Reynolds, 2002; Firman et al., 2018; Gage & Barnard, 1996; Lizé et al., 2012; Moatt, Dytham, & Thom, 2013). This extreme manipulation of the total number of potential rivals is not intended to mimic the effects males experience in nature, but rather to demonstrate that such adaptive responses exist. Evidence for how such responses link to more ecologically-realistic stimuli is therefore lacking, although effects of sperm competition have been observed in natural populations – for example in lizards (Kustra, Kahrl, Reedy, Warner, & Cox, 2019) and frogs (Buzatto, Roberts, & Simmons, 2015). Given that patchiness in food resources

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

competition risk, an association that has been demonstrated repeatedly in the laboratory (Bretman et al., 2009; Bretman, Fricke, Hetherington, Stone, & Chapman, 2010; Bretman, Westmancoat James, Gage Matthew, & Chapman, 2012; Bretman, Westmancoat, & Chapman, 2013; Mazzi, Kesäniemi, Hoikkala, & Klappert, 2009; Moatt et al., 2013). We predict that: (a) by experimentally manipulating the distribution of food resources, males on clustered resources have a higher mean proximity to rivals (i.e. a higher encounter rate on average), and (b) males on clustered resources will subsequently mate for longer on average, indicating an adaptive response based on perception of increased sperm competition risk.

Methods

All fly rearing and experiments were conducted in a 12 hour light:dark cycle (0800 – 2000 GMT), at 25 °C. *Drosophila melanogaster* used were from a laboratory population (Canton-S), and populations were cultured on 7 ml of a standard agar-based medium of 40 g of yeast per litre, in 40 ml vials. Between 20 and 30 *Drosophila* were housed in each vial. To minimise any effects of inbreeding, drift, and selective sweeps, every seven days the adults from all vials were pooled and randomly redistributed among new vials to start the next generation.

Test flies (180 in total – 60 per treatment) were collected from parent vials, each established with six males and six females allowed to breed for 70-98 h. Test flies were removed from parent vials within six hours of eclosion to ensure virginity; prior to this individuals are not sexually mature (Strömnæs & Kvelland, 1962). Flies were immediately aspirated under light ice anaesthesia into treatments. Virgin female flies for mating assays were collected from the same parental vials and aspirated into new vials in groups of four. Females were used in mating assays when they were seven days (+ 6-8 hours) old (Churchill, Dytham, & Thom, 2019).

Manipulating resource distributions and patchiness

Each replicate for each treatment consisted of four virgin males maintained in a 90 mm Petri dish for three days. Food in each of these 45 dishes was arranged in one of three treatments (N = 15): clustered, dispersed or uniform food resource distributions. Clustered and dispersed treatments both contained four plugs (420 mm^3 per patch) of standard food medium (as described above). The size of these patches is within the range of patch sizes where territorial behaviours have previously been observed (Hoffmann & Cacoyianni, 1990).

Dispersed food discs were placed at four equidistant points around the circumference of the Petri dish; these were 50 mm apart along the edge of the square, 70 mm apart on the diagonal (illustrated in Fig. 2). Clustered discs were placed in the centre of the Petri dish, in a square arrangement with each food disc in direct contact with adjacent discs. The uniform treatment was an even layer of 45 ml standard medium covering the bottom of the dish (to the same height as the four food patches in the previous two treatments): volume and surface area were both greater in the uniform than the two patchy treatments, but given the number of flies food was assumed to be available *ad libitum* in all. All treatments were maintained in 12L:12D at 25 °C, and the four male flies per treatment remained in these conditions for 70 hours (+/- 1 h) until aged to three days.

Quantifying male spacing behaviour

Treatment enclosures were placed in one of two identical incubators maintained at 25 °C and on the same 12:12 L:D cycle as the stock flies. Each incubator was fitted with a Raspberry Pi (www.raspberrypi.org) connected to an 8MP Raspberry Pi Camera module (v2; www.thepihut.com). Two to three Petri dishes, placed in a balanced arrangement across all treatment combinations, were placed directly under each camera. We used frame capture software ('raspistill') to collect one image every 15 minutes from 0800-2000 GMT (during the light part of the cycle). We captured the x-y coordinates of each male at each time point using ImageJ's multiple point selector tool (Schneider, Rasband, & Eliceiri, 2012), and then converted these into a set of six Euclidean pairwise distances between the four males (24670 measurements across the three treatments and all time points). For 325 out of the 4290 individual time-point 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

mixed effects models, with plate included as a random effect in all models to account for the non-independence of the four males in a single treatment replicate. Time point (numbered sequentially from first to last measurement and treated as continuous) was modelled as a fixed effect.

Treatment effects on mating related traits were analysed using linear mixed effects models, with replicate plate entered as a random effect to account for the fact that mating data were available for (up to) four males per plate. Time point) and treatment were initially entered as interacting predictor variables; if the interaction was non-significant we re-ran the model with both variables entered as main effects. We used the R package lmerTest (Kuznetsova, Brockhoff, & Christensen, 2017) to generate p values using the Satterthwaite approximation for degrees of freedom. To assess the effect of treatment on binomial variables (courtship success, copulation success) we used generalised linear mixed models with a binomial error distribution, and replicate plate nested within treatment to account for possible plate effects.

Animal welfare note

Although *Drosophila* are not currently subject to any ethical restrictions in the United Kingdom, we took precautions to minimise injury and stress by controlling larval density during development, handling flies minimally and using only light ice anaesthesia, and by euthanizing flies at the end of the experiment while they were under anaesthesia.

Results

Effect of food distribution on inter-male spacing

The spatial distribution of food patches significantly influenced the mean pairwise distance between the four males in the treatment, and this interacted with the time course of exposure to treatment (treatment*time: $F_{2,4239}$ = 286, P = 2.20e^{-11}; Fig. 1; Table 1). On the final day of treatment the time effect had stabilized (treatment*time $F_{2,525}$ = 1.134, P = 0.3224), leaving a significant main effect of treatment on pairwise distance between males ($F_{2,30}$ = 32.268, P = 3.33e^{-8}; interaction removed; Table 1). Post-hoc testing confirmed that on this final day, pairwise distances among males in the dispersed treatment (44.02 ± 0.66 mm SE) and the uniform treatment (39.35 ± 0.93 mm SE) were both significantly greater than among males in the clustered food treatment (22.79 ± 0.86 mm SE; dispersed vs clustered $F_{1,20}$ = 57.8, P = 2.53e^{-7}; uniform vs clustered: $F_{1,20}$ = 27.9, P = 3.63e^{-5}; time remained in these models as a main effect). There was no significant difference in mean pairwise distance between males in the uniform and dispersed treatments ($F_{1,20}$ = 3.9, P = 0.061).

Effect of food distribution on mating behaviour

Among those males that mated, copulation duration was significantly affected by food distribution previously experienced by males ($F_{2,42.5}$ = 3.96, P = 0.026; Fig. 2). Analysing the effect of treatment on the mean mating duration across all males in a replicate – a more conservative measure – confirmed a significant difference in mating durations between treatments ($F_{2,42}$ = 4.22, P = 0.021). Males from the clustered treatment mated for significantly longer (1170 ± 28 s SE) than those from the dispersed treatment (1029 ± 28 s SE), a difference of 2 minutes 20 seconds ($F_{1,28}$ = 6.59, P = 0.016). Copulation duration of males from the uniform treatment did not significantly

differ from either of the other treatments (uniform copulation duration 1107 ± 23 s SE; vs. dispersed: $F_{1,28.5}$ = 2.22, P = 0.146; vs. clustered $F_{1,28.5}$ = 1.96, P = 0.172). However, despite these observed differences between clustered and dispersed treatments, the mean distance between males while in the treatment did not significantly affect copulation duration in any of the three treatments (all P > 0.101).

In total, 159 of 180 males (88.3%) courted the female. There was no significant effect of treatment on the proportion of males that courted (generalized linear model with binomial errors and plate nested within treatment; χ^2 = 118, P = 0.376). Similarly, 144 (80%) of males mated, and this was not influenced by treatment (χ^2 = 175, P = 0.286). Neither the latency to start courting ($F_{2,39.3}$ = 0.201 P = 0.818) nor the latency to start copulation ($F_{2,30.4}$ = 1.257, P = 0.299), differed significantly among the three treatments.

Discussion

The high degree of plasticity in mating-related traits in male *Drosophila* is well established (Churchill et al., 2019; Davies, Schou, Kristensen, & Loeschcke, 2019; Droney, 1998; Fricke, Bretman, & Chapman, 2008; Jensen, McClure, Priest, & Hunt, 2015; Lefranc, 2000; Lüpold, Manier, Ala-Honkola, Belote, & Pitnick, 2010; Morimoto & Wigby, 2016; Ormerod et al., 2017; Schultzhaus, Nixon, Duran, & Carney, 2017). Variation in these traits is highly sensitive to conspecific male density in a manner which suggests that males adjust investment in anticipation of the intensity of sperm competition they are likely to encounter during mating (Bretman et al., 2009). However, how this level of plasticity relates to the variation in density and resource distributions observed in natural populations remains unknown, and laboratory studies tend to manipulate density in ways that seem unlikely to occur frequently in nature (e.g. singly-housed males compared to a high density of males in a single vial).

We show that manipulating food patchiness while keeping group size constant has a similar effect on a sperm competition-related trait – both in direction and magnitude – as manipulating local density directly, and that these effects can be observed even over very small spatial scales. Other studies on this species have found an approximately two-minute increase in mating duration in high density males compared to low density males (Bretman et al., 2009; Bretman et al., 2010; Bretman et al., 2013). Given that wild *D. melanogaster* encounter a patchy resource that is likely to alter male encounter rates at a similar scale to that demonstrated here (Markow, 1988; Soto-Yéber et al., 2018), we suggest that fine-scale variation in these environmental cues might influence male allocation of resources to traits associated with sperm competition, and thus mating success, in wild-living *Drosophila*.

Although the effect on mating duration is a repeatable indicator of male perception of sperm competition risk, the benefits of this behaviour to males remains uncertain. In many species, increased mating duration has been linked to increased sperm transfer and offspring production (Edvardsson & Canal, 2006; Engqvist & Sauer, 2003; Sakaluk & Eggert, 1996). In *Drosophila* the consequences of longer copulation durations are less clear, with some studies reporting an association with increased fitness (Bretman et al., 2009; Garbaczewska et al., 2013; Price, Lizé, Marcello, & Bretman, 2012), while others have not found a link (Bretman et al., 2012; Dobler & Reinhardt, 2016). Whether males on the clustered food resource would have a higher fitness than those on dispersed resources remains to be tested, but will almost certainly depend on mating order effects and the competing male's history of exposure to rivals (Bretman et al., 2012). However, our objective here was not to examine fitness consequences, but rather to demonstrate that males alter their perceptions of likely sperm competition risk based on small-scale changes in the spatial distribution of resources.

Interestingly, the effect of food distribution on male distribution behaviour and sexual investment was observed in the absence of females. Females often follow social cues, and their grouping behaviour is promoted by aggregation pheromones (Bartelt, Schaner, & Jackson, 1985; Duménil et al., 2016). By comparison, given their low feeding rate once adult (Wong, Piper, Wertheim, & Partridge, 2009), males are thought to aggregate near food resources primarily to seek mating opportunities. That these groups of males responded in their individual positioning to the distribution of food even in the absence of females is intriguing, and leaves open the question of the relative importance of female social cues compared to the direct response of males to food resources. In general however, studies manipulating male density have tended

to exclude females from the treatment phase (e.g. Bretman et al. (2009); Bretman et al. (2010); Lizé et al. (2012); Moatt et al. (2013); Price et al. (2012); and Rouse and Bretman (2016)), meaning the effects of inter-sexual interactions on plastic responses to density is relatively unexplored.

This study adds to a small number of studies that demonstrate the effects of environmental heterogeneity on *Drosophila* behaviour. Yun, Chen, Singh, Agrawal, and Rundle (2017) demonstrated that female fitness was higher in more spatially complex laboratory environments as a result of a reduction in sexual interactions and consequent mitigation of male harm. Similar effects have been demonstrated when laboratory populations were presented with a refuge: female remating rates declined substantially (Byrne, Rice, & Rice, 2008). Such rapid shifts in behaviour, driven by ecological patchiness, have rarely been included in laboratory assays, but may have major effects on the demography and growth rate of populations exposed to spatial patchiness, through their effects on male reproductive skew and therefore effective population size. Such effects may have important evolutionary and ecological consequences in relatively patchy parts of a species' distribution, for example by increasing sexual conflict over shared resources (Pilakouta, Richardson, & Smiseth, 2016), or reducing maximum sustainable rates of evolution (Bridle, Kawata, & Butlin, 2019; Bridle, Polechová, & Vines, 2009).

There are some intriguing dynamics operating in the inter-male distances in the early stages of the treatment period: in particular, males on the dispersed food patches initially experience lower inter-male distances than those on the clustered food (Fig. 1). This effect does not match what we expected to see among males attempting to 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

Author contributions

ERC, MDFT, and JRB designed the study; ERC performed all experiments; ERC and MDFT analysed the data; all authors contributed to writing the manuscript.

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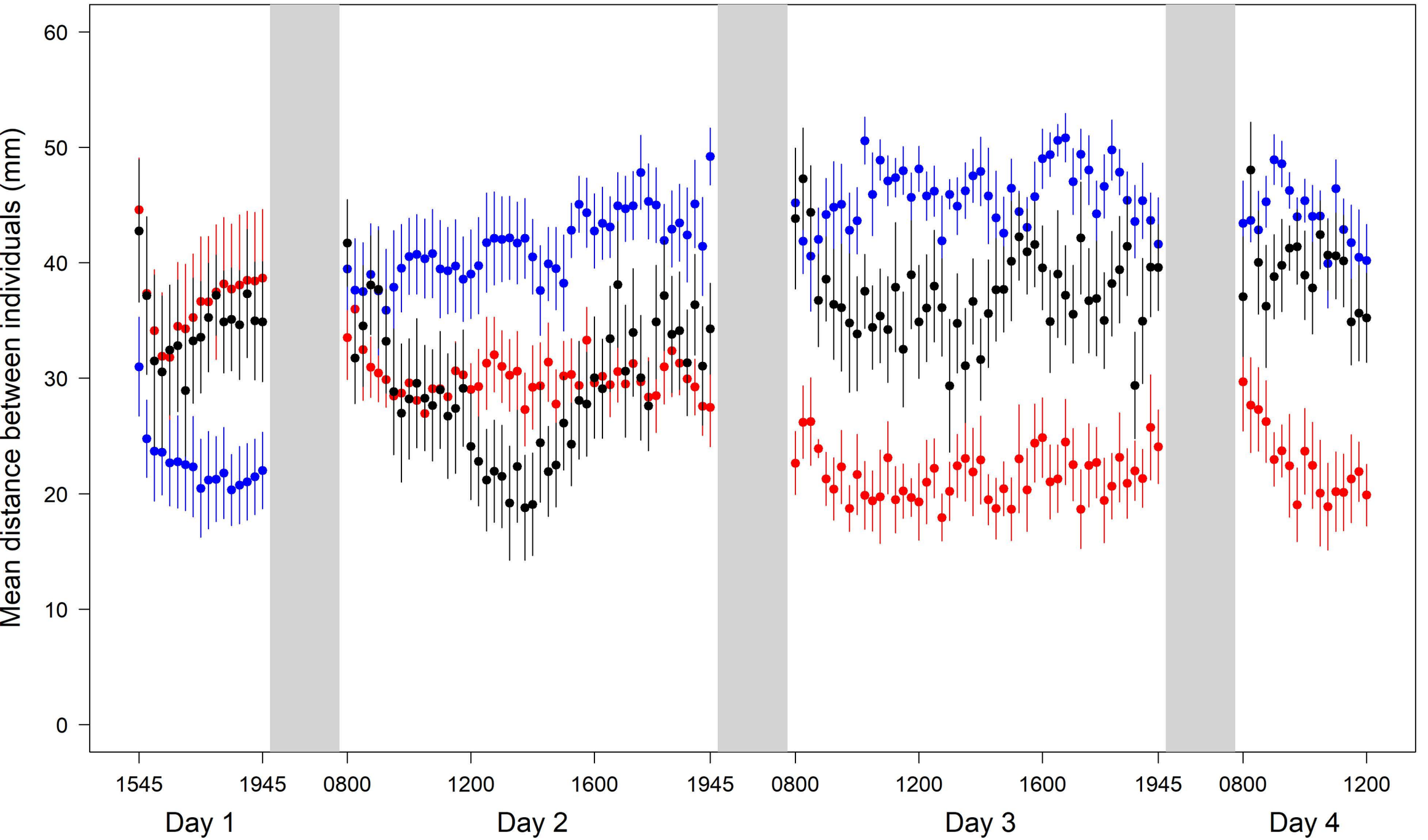
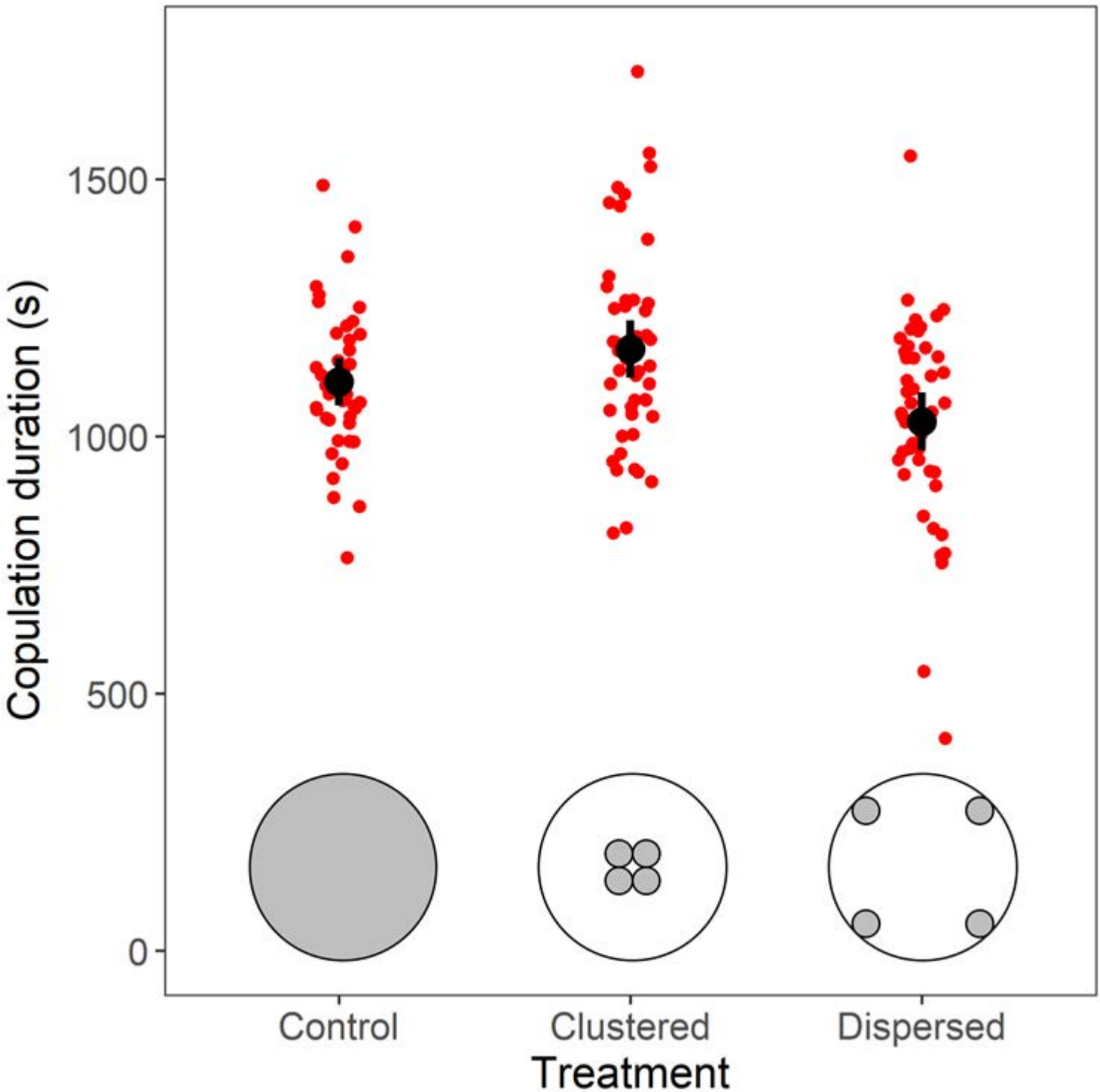
Figure 1. Mean inter-fly distance (mean of 6 pairwise distances between 4 focal flies per plate, averaged across 11 replicate plates) over time. Black = uniform treatment (evenly distributed food); red = clustered food patches; blue = dispersed food patches. Bars show standard errors of the mean for each time point across all 11 treatment replicates. Grey blocks indicate period of dark (2000 - 0800 GMT), and are not to scale.

Table 1. Details of statistical parameters from linear mixed models analyses outlined in the results. Model outputs are presented in the order they appear in the text. Response variables and data subsetting are outlined in the subheadings, predictor variables in the 'Parameter' column.

Figure 2. The effect of food resource spatial distribution on the duration of subsequent copulation. Means (black dot) and 95% confidence intervals of copulation duration (seconds). Sample sizes: clustered 49 (11 males did not mate), uniform 44 (16), dispersed 51 (9). The treatment effect on mating duration remains significant when the two mating duration values below 600s in the dispersed treatment are excluded from the analysis ($F_{2,40.9} = 3.55$, $P = 0.038$).


Mean distance between individuals (mm)
60
50
40
30
20
10
0
1545
1945
0800
1200
1600
1945
0800
1200
1600
1945
0800
1200
Day 1
Day 2
Day 3
Day 4


Copulation duration (s)
1500
1000
500
0
Control
Clustered
Dispersed
Treatment

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