Female fruit flies use social cues to make egg clustering decisions

Emily R. Churchill[1], Emily K. Fowler[2], Lucy A. Friend[2], Marco Archetti[3], Douglas W. Yu[2], Andrew F. G. Bourke[2], Tracey Chapman[2*] and Amanda Bretman[1*]

* Corresponding authors

1. School of Biology, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT, UK

2. School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK

3. Department of Biology, Pennsylvania State University, University Park, PA 16802, USA

ORCID information:

Emily R. Churchill – 0000-0003-0910-8600

Emily K. Fowler – 0000-0001-5290-7630

Lucy A. Friend – 0000-0001-5728-9384

Marco Archetti – 0000-0002-8303-1632

Douglas W. Yu – 0000-0001-8551-5609

Andrew F. G. Bourke – 0000-0001-5891-8816

Tracey Chapman – 0000-0002-2401-8120

Amanda Bretman – 0000-0002-4421-3337

Keywords

Cooperative oviposition; Laying rate; Public goods; Density; Fitness; *Drosophila melanogaster*

Abstract

The ability to respond plastically to environmental cues is a key determinant of fitness. In changing environments, females plastically adjust the number and placement of eggs they lay, allowing them to optimise the level of resources available for offspring by minimising over-exploitation whilst also accruing potential cooperative benefits such as through communal larval feeding. This suggests that the pattern of female egg laying should vary across differing social environments, to balance potential for cooperative benefits versus competitive costs of egg clustering. Here we test this prediction in the fruit fly *Drosophila melanogaster*. We developed a mathematical null model, which verified that females lay eggs on substrates in significantly non-random patterns. We then tested the effects of manipulating social density and egg density on the egg laying patterns of mated females. As the density of adult females was increased, females laid more eggs, laid them faster, and laid more eggs in clusters. Females also preferred to place eggs within existing egg clusters -and most egg clusters were of mixed maternity. These results show that females in groups, or females that encounter already laid eggs, adjust their laying to place more eggs in mixed maternity clusters. Overall, the results are consistent with our predictions and reveal that females exhibit active choice in egg investment and egg deposition patterns in response to variation in social environments.

Background

In natural contexts, social environments and availability of resources are highly variable, exposing populations to fluctuating competition. Phenotypic plasticity can play a key role in ensuring individuals maximise their lifetime reproductive success (1, 2). Because optimal strategies may vary with fluctuating levels of competition, individuals that can respond and make appropriate behavioural and physiological adjustments to the prevailing environment should show increased fitness. This should be particularly true in females, who generally exert greater control over reproductive investment in progeny. For example, females can control paternity (3-5), the number and sex ratio of offspring via ejaculate manipulation (6-8), and choice of nesting/oviposition site locations. For females that deposit eggs upon a substrate, egg site location can be a vital determinant of hatching success. Consistent with this, oviposition patterns appear to be plastic in response to environmental variation (9-12).

Competition, cooperation, and cheating amongst females

At increasing population densities, there is expected to be a greater risk of competition but also increased potential for cooperation, and the relative importance of the two is expected to differ as individuals progress through life stages. For example, when food is limiting and competition high, eggs and larvae are typically more vulnerable to cannibalism than are adults, due to their more restricted mobility (13-15). On the other hand, in some species, there is a benefit to being with conspecifics when foraging or hunting. For example, Eurasian Coots (*Fulica atra*) copy each other's behaviours to reduce foraging costs (16), and larvae in metamorphosing species often benefit from increased efficiencies resulting from communal feeding (17, 18). Females can use the presence of conspecifics as a cue to modify reproductive investment in response to changing social conditions. For example, increasing adult female density reduces offspring production and body size in Western Mosquitofish (*Gambusia affinis*) (19). In contrast, in the Willow Leaf beetle (*Plagiodera versicolora*) fitness effects of increasing adult female density can vary with development, resulting in a decrease in egg hatching, but an increase in egg clutch number and duration of laying period (20).

Among females of species that deposit their eggs upon substrates, it is also often found that eggs are laid in conspecific clusters, e.g. in fish (21), birds (22), reptiles, amphibians (23), and invertebrates (24). Egg clustering may increase the local population density of

offspring and thus the potential not only for competition but also cooperation at early life-history stages. Benefits from cooperation could include reduced energy expenditure for ovipositing females by minimising search times, reduced egg predation risk (the dilution effect' hypothesis (25)), increased protection from abiotic challenges, or increased larval communal feeding.

However, cooperative interactions can be exploited by cheater individuals. In some egg-laying species mothers also provide their eggs with diffusible defensive compounds. For example, medfly (*Ceratitis capitata*) females provision the surface of their eggs with antimicrobial peptides, which spread into the surrounding environment, creating a zone of microbial growth inhibition (26). Other examples include in the Green lacewing moth (*Ceraeochrysa cubana*), in which eggs are endowed with an anti-predator alkaloid (27), and in fruit flies (*Drosophila melanogaster*), in which eggs are coated with anticannibalism compounds (28). These defensive substances represent potential public goods (29, 30) as all eggs nearby could benefit, even eggs that do not themselves provide the defensive compound itself. This raises the intriguing possibility that in egg clusters of mixed maternity, there could be 'co-operator' eggs coated with defensive compounds, and non-defended 'cheater' eggs that benefit from their placement within the defensive diffusion radius. In this scenario, cheaters benefit by avoiding the energetic expense of provisioning eggs with defensive compounds. Egg clustering might also provide benefits as well as cheating opportunities through communal feeding. For example, if eggs are laid on harder substrates there could be benefits for all via the collective processing of food, while simultaneously allowing the possibility for cheaters to benefit by utilising resources liberated by the processing of food by their cluster mates. Such co-operators and cheats could stably coexist within a population if the benefits of public goods have a non-linear relationship with fitness (30, 31).

In summary, we expect that natural selection can favour either egg dispersion (competition) or egg-clustering (cooperation and cheating) behaviour. Which of these is selected for will depend on the balance of abiotic and selective forces. The potential fitness benefits of joining an existing egg cluster could depend on key factors such as number, quality, fertility, and age of eggs already present. Based on existing evidence that females can vary the number of eggs they lay according to social density (9, 10, 32) we predict, and test here,

100 the idea that females should use number of eggs and/or number of adult conspecifics as cues
101 to direct their egg-clustering patterns.

102 *Drosophila melanogaster* as a model for understanding variation in oviposition patterns

103 In wild populations, *D. melanogaster* larvae and adults periodically occur at high densities
104 around fallen, fermenting fruit. For females this fruit is both a source of nutrition and
105 potential oviposition sites. However, these key resources are patchy and ephemeral, and they
106 vary in their nutrient availability (density, ripeness, and state of decomposition). Access to
107 such resources is also shaped by the immediate social environments (number of conspecific
108 or heterospecific males and females utilising the same resource) (33). To oviposit in a way
109 that maximises fitness, female *Drosophila* should assess the nutritional quality, degree of
110 interspecific competition, risk of pathogens and parasites present at each oviposition site, as
111 well as the ongoing search costs, and potential benefits and costs (e.g. from overexploitation
112 leading to dispersal) for developing offspring (34).

113 Consistent with this reasoning, female *Drosophila* have a social attraction to
114 conspecifics on oviposition resources (35, 36) which they can detect directly or via
115 pheromones, markings, or the presence of eggs or larvae (37-41). This attraction could arise
116 because it allows females to copy the site-selection choices of others (37, 38, 40) to enable
117 cooperative feeding among larvae (42), or to gain other public goods benefits of egg
118 clustering, as outlined above. Existing evidence suggests that female *Drosophila* can adjust
119 their fecundity responses according to their previous or current social environments, with
120 females laying fewer eggs after pre-mating exposure to conspecifics (9, 43) but laying eggs
121 more quickly within larger current post-mating social groups (32).

122 *Drosophila* eggs can often be found in extremely close proximity, in large groups or
123 clusters (Fig. S1). Hence, after deciding to lay on substrates with existing conspecific eggs,
124 females must also decide whether to lay eggs that join existing clusters. Females generally lay
125 one egg at a time (44), explore oviposition sites between laying, and can retain eggs until an
126 optimal laying site is found (45). Therefore, clustering of eggs suggests that females make
127 repeated egg-laying decisions and that females lay their eggs in non-random distributions.
128 However, whether the temporal or spatial distribution of egg laying is significantly non-

random has not previously been formally tested. In addition, social density is reported to affect the number and location of eggs laid by *D. melanogaster* (9, 10, 32), but nothing is yet known about how it might affect clustering rates or patterns. Here we address these key gaps in our knowledge of the social influences on oviposition patterns.

We first test, by using a mathematical model, a key hypothesis:

- (H1) that female *D. melanogaster* do indeed place their eggs non-randomly within a food patch

We then conducted experiments to determine key drivers of the significantly non-random egg deposition observed. We did this by testing whether the decisions to join existing clusters responded to experimentally manipulated adult female group size and egg presence or density.

Due to the potential for resource competition, cooperation, or public goods benefits, we predicted that, as adult female social density increased, females should:

- (H2) decrease laying latency (if copying other's laying decisions (38))
- (H3) invest more in egg production (either through laying more eggs ((46)), or fewer eggs of higher quality)
- (H4) alter egg-clustering pattern (if clustering balances cooperative larval feeding, whilst avoiding competition)
- (H5) incur fitness benefits via increased egg clustering (increased egg-adult viability of highly clustered eggs)
- (H6) lay eggs in clusters of mixed -maternity to potentially gain public goods-related benefits

In addition to adult presence (H2-6), we also predicted that the presence of existing egg clusters should elicit comparable responses in egg laying (as females should use similar density cues). Due to the expected advantages of clustering (17, 18, 25-28, 42), and assuming

154 benefits increase with cluster size, we hypothesised that (H7) females should be more willing
155 to lay their eggs within existing clusters, and more eggs should be laid in larger clusters as the
156 number and size of existing clusters present increases.

157 **Methods**

158 Our experimental strategy was first to construct a model, parameterised with data, to test
159 the hypothesis that females exhibit non-random egg placement (H1). Having confirmed that
160 egg laying patterns were significantly non-random, we then conducted experiments to test
161 the effect of social group size on the rate and likelihood of egg laying, egg clustering patterns
162 (H2-H4) and fitness (egg-adult viability as a function of egg clustering (H5)). An additional
163 experiment was then conducted using females that produced dyed eggs, to measure the
164 frequency of egg clusters with mixed maternity (H6). Finally, we tested the effect of exposing
165 standard sized groups of females to environments containing variable numbers of egg
166 clusters, and recording the rate and pattern of egg laying (H7).

167 *Fly husbandry and rearing of experimental flies*

168 Fly rearing and all experiments were conducted at 25°C on a 12h light:dark cycle (09:00-21:00
169 GMT). Wild type flies originating from a Dahomey laboratory population maintained in cages
170 were used throughout. We collected eggs from cages using Petri dishes (*Sarstedt*
171 *#82.1473.001*) filled with grape juice agar-based medium (50g agar (*Fisher Scientific*
172 *#10048991*), 600ml red grape juice (*Young's Brew red wine enhancer*), 42ml Nipagin solution
173 (methylparaben, 10% w/v solution, dissolved in 95% Ethanol) per 1.1l H_2O). Once eggs had
174 hatched, we transferred first instar larvae to a 40ml plastic vial (*Sarstedt #58.490*) containing
175 7ml of a standard sugar-yeast-agar (SYA) medium (100g brewer's yeast (*Buy Wholefoods*
176 *Online*), 50g sugar (*Tate & Lyle*), 15g agar, 30ml Nipagin solution, and 3ml propionic acid
177 (*Fisher Scientific #10193190*) per litre of medium), at a standard density of 100 larvae per vial
178 (i.e., 'standard density vials'). Experimental flies were then collected under light ice
179 anaesthesia, within six hours of eclosion to ensure virginity.

180 *Egg-clustering model to test for non-random egg deposition (H1)*

181 To ascertain whether the proportion of observed egg clustering differed from a null
182 expectation (H1), we produced a model to calculate random distributions of eggs in a

183 standard vial. We defined a 'cluster' as any group of two or more eggs in which the main
184 bodies of the eggs were in direct contact (Fig. S1). In the model, given that the observed egg
185 to dish area is 1/5770, we assume that there are 5770 positions available for egg laying; there
186 is a total number (parameter Ɛ) of eggs per vial. Assuming that all eggs are laid sequentially
187 and at random, we calculated the expected size of clusters (defined as the number of eggs
188 per cluster) for any number of total eggs laid (Ɛ).

189 The preference for clustering eggs next to an existing egg (hereafter 'clustering
190 preference') is defined by the probability of a clustering decision. Let us call K the probability
191 that a fly will lay an egg next to an already existing egg. After the first egg is placed at random,
192 where Ɛ > 1, the next egg is either laid in a random position (probability of probability of 1 -
193 K) or next to an existing egg (probability of K); in the latter case, the position of the egg is
194 chosen at random amongst only positions with existing eggs.

195 The expected number of eggs per cluster can be predicted based on K and Ɛ. When K
196 = 0 (no preference for egg clustering), very few clusters with 2 eggs occur and virtually no
197 clusters with more than 2 eggs (2.5% with Ɛ = 233 (the maximum number of eggs observed in
198 our experiment); 1% with Ɛ = 100; no clusters for Ɛ below 70). Therefore, if Ɛ << 5770 (as is
199 the case in our experiment), randomly laid eggs form almost exclusively singly laid eggs. When
200 K = 1 (absolute preference for egg clustering), all eggs are clustered in one large cluster in a
201 single position.

202 At intermediate values of K, some variation in cluster size emerges. We simulated the
203 expected number and size of egg clusters for different values of K from 0 to 1 in intervals of
204 0.1 (using the average of 10 different simulations per K value). We then compared these
205 expected distributions to the empirically observed distributions of eggs laid by females from
206 the differing social density treatments (groups of 1, 2, 4, or 8) described below, to estimate
207 the most likely preference K leading to that distribution. For each fly, we performed a
208 Kolmogorov-Smirnov test (implemented in Mathematica 13. 1 (49)) between the expected
209 and observed distribution, for all K values. We considered the K value yielding the highest
210 (least significant) P values to be the most likely K value for that fly.

211 *Effects of adult social density on egg laying rate, egg number, egg-clustering decisions and fitness*
212 *(egg-adult viability) (H2-H5)*

213 We investigated how variation in adult social group size affected egg laying decisions:
214 specifically, the speed of egg laying (H2), the number of eggs laid (H3), egg clustering patterns
215 (H4), and fitness (egg-adult viability of clustered versus non-clustered eggs) (H5). The data
216 from this experiment were also used to parameterise the model, in order to test for non-
217 random egg laying patterns (H1). Experimental flies were collected and housed in same-sex
218 groups of 10 in standard vials. At four days post-eclosion, females were randomly assigned to
219 one of the following social group treatments: 1, 2, 4, or 8 females and kept in these conditions
220 for 48h (N = 30 vials each treatment; Fig. S1). Males were held for six days until they were
221 used for matings.

222 At 6 days post-eclosion, males were introduced to all the female social group
223 treatment vials for 2h at a density of 3:2 males:females (or 2:1 for isolated females) to enable
224 choice (Fig. S1). To ensure that this was a set up that would result in all females successfully
225 mating within the 2h period allowed, we separately recorded female mating frequency in an
226 identical experimental set up. In this we observed that 97.3% of females mated, with no
227 significant difference in mating frequency between treatments ($X^2 = 1.79$, d.f. = 3, $p = 0.617$).

228 After the 2h mating period in the main experiment, we transferred all mated females
229 to fresh standard vials to observe egg laying decisions. Every 2 – 3h, we counted the number
230 of eggs laid, number of egg clusters, and size of egg clusters. Observations were made at
231 14:00, 16:00, 19:00, and 22:00 on day 1 of laying, and 10:00 and 12:00 on day 2 (i.e., 2, 4, 7,
232 10, 22, and 24h after the end of the mating period, respectively). Differences in counts
233 (number of eggs, number of clusters, and size of clusters) between timepoints were calculated
234 to give overall egg laying latencies, numbers and clustering rates (H2-H4). We calculated the
235 proportion of eggs in clusters at each timepoint by summing the total number of eggs in each
236 cluster and dividing by the overall total number of eggs laid in the vial. All females were
237 removed after 12:00 on day 2 and vials were then kept for 14 days, to count the total number
238 of eclosed adults from each of the social exposure treatments (H5).

239 *Testing for mixed maternity of egg clusters (H6)*

240 To determine whether egg clusters were of single or mixed maternity when more than one
241 female was present (H6), we conducted an additional experiment, to test the egg clustering
242 patterns of groups of 4 females. In each treatment group there was one wild type (Dahomey)
243 focal and 3 non-focal females from the *scarlet* strain (recessive *scarlet* eye colour mutation
244 backcrossed into the Dahomey wild type > 4 times). These non-focal *scarlet* females were fed
245 Sudan Black B dye that stained laid eggs, allowing the eggs of focal versus non-focal females
246 to be identified. First instar larvae of Dahomey wild type and *scarlet* strains were raised in
247 cultures of 100 larvae per vial as described above. Focal Dahomey females were collected
248 from cultures reared and maintained on standard medium, whereas *scarlet* females were
249 derived from cultures containing Sudan Black B dye (100g brewer's yeast, 50g sugar, 15g agar,
250 30ml Nipagin solution, 3ml propionic acid, and 1.4g Sudan Black B powder (*Sigma-Aldrich,*
251 cat#199664) dissolved in 14ml corn oil (*Mazola* 100% pure corn oil) per litre of medium). Focal
252 Dahomey virgin females were collected from the standard medium vials, and non-focal *scarlet*
253 virgin females were collected from Sudan Black B vials and maintained on that same medium
254 until use. Upon collection, all females were housed in groups of 10 and then Dahomey focal
255 females were housed in groups of 4 from 5 days post-eclosion. At 4 days post-eclosion, *scarlet*
256 females were wing clipped for identification, and also housed 4 per vial. Dahomey and *scarlet*
257 males for matings were collected from standard medium vials and housed in groups of 10
258 until use.

259 Seven days post-eclosion, males were transferred into the female vials for mating: 8
260 Dahomey males were added to each group of 4 Dahomey females, and 6 *scarlet* males for
261 each group of 4 *scarlet* females. The flies were given 4 hours to mate.

262 One female from each of the Dahomey vials was then transferred to fresh standard
263 media alongside three *scarlet* dye-fed females at 12:00 GMT (N = 29). We then counted the
264 number of eggs laid at 10.5h post-mating. Eggs were categorised as clustered or not clustered
265 (Fig. S1) and according to whether they were laid by a focal or non-focal female. This allowed
266 us to calculate the proportion of eggs that were clustered, and whether the cluster contained
267 eggs from both focal and non-focal females.

268 *Effects of existing egg cluster sizes on female egg-clustering decisions (H7)*

269 To assess whether the size of egg clusters already present in an environment alters
270 subsequent female laying decisions (H7), we conducted a final experiment in which we
271 presented focal females with a laying environment that already contained varying numbers
272 of eggs and egg clusters. To create the egg clusters, singly laid eggs were collected from SYA
273 medium-filled Petri dishes and transferred into vials to create four different egg cluster sizes:
274 1, 4, 7, and 10 eggs per cluster. Females have a strong tendency to lay eggs at vial edges (Table
275 S2; (9, 47, 48)), hence we mimicked this tendency by placing all egg clusters at the edge of the
276 egg treatment vials.

277 To obtain the Dahomey females and males for the experiment, we collected virgin flies
278 from standard density vials. Prior to the tests, females were maintained alone, males in
279 groups of four per vial. At seven days post-eclosion, males and females were paired and
280 observed to ensure they had all successfully mated. After mating, groups of 4 females were
281 then transferred to each of the 4 types of egg cluster treatment vials (1 egg: N = 20 vials; 4
282 eggs: N = 17 vials; 7 eggs: N = 20 vials; 10 eggs: N = 21 vials). After the introduction of the focal
283 females, vials were checked at 30 min intervals from 13:00 until 21:30, at which point if
284 females had not laid eggs they were removed. If eggs were laid by the introduced focal
285 females, we counted the number of eggs laid and categorised them in relation to the existing
286 egg cluster present (clustered or not clustered). All vials were then frozen at -20°C for imaging.

287 Vials were imaged to record inter egg distances, using a video camera (Sony Handycam
288 HDR-CX405). Using ImageJ's multiple point selector tool (50) we captured x-y coordinates of
289 each egg and converted these into Euclidean pairwise distances. Selected coordinates were
290 taken from the edge points of eggs that were in closest proximity, resulting in clustered eggs
291 having a distance value of zero.

292 *Statistical analysis*

293 All statistical analyses were conducted using R v 4. 2. 2 (51), and mean values and standard
294 deviations are reported within the text. Graphs were produced using packages 'ggplot2',
295 'cowplot' and 'grid' (52, 53), with plotted standard errors calculated using 'Rmisc' (54).

We analysed the effect of egg and adult social density on the likelihood that females laid eggs and produced adult offspring, by using generalised linear models (GLMs) with binomial errors. We used the package 'AER' (55) to test for overdispersion, and where that was found, we used GLMs with a quasi-Poisson distribution to account for this over-dispersion and analyse the effect of adult density on the number of offspring produced. Linear models were used to investigate the effect of adult density on egg-clustering patterns (egg laying and clustering rates), and total eggs laid was included as a fixed effect to account for increased likelihood of clustering when more eggs are present in the environment.

When analysing variables throughout multiple timepoints, we used linear mixed effects models; the timepoint was included as a fixed effect in the model to account for differences in rates and the test vial was included as a random effect – using functions in packages 'lme4' (56) and 'lmerTest' (57). We tested for Pearson's product-moment correlations between the proportion of eggs laid in clusters and subsequent egg-adult viability. In all cases, post-hoc tests (models including only pairwise comparisons) were used to identify significant differences in the six pairwise comparisons available.

In the first experiment investigating the effects of social density, there was variation in sampling effort between treatments, due to the differences in female group sizes (and lack of identification of focal females). To account for this, all significant results from the initial analyses were reanalysed with a randomised subset of data so that the number of individual flies (rather than number of vials) per treatment were similar. In this reduced subset, all vials from solitary females were included (N = 30 females), half of those in pairs (N = 30 females), eight of those from groups of 4 (N = 32 females), and four of those from groups of 8 (N = 32 females). These analyses were congruent with those of the initial analyses, suggesting the initial analyses were robust.

Further details on the model syntax for all analyses and all data are available from the Dryad Digital Repository (10.5061/dryad.mgqnk9971).

Results

Egg laying patterns of females were significantly non-random (H1)

Across the four social treatments, clustering preferences (values of K that, based on the Kolmogorov-Smirnov test, yielded no significant difference between the simulated and observed distributions) were between 0.3 and 0.5 (Table S1).

For all social treatments with two or more females, patterns of egg clustering in the empirical data were significantly more clustered than those predicted by the null model (H1; Wilcoxon Signed Rank test comparisons with simulations using K = 0; paired: $z(6) = -2.29$, $p = 0.0220$, $r = 0.866$, N = 7 vials; groups of four: $z(25) = -4.47$, $p = 7.95 \times 10^{-6}$, $r = 0.876$, N = 26 vials; groups of eight: $z(27) = 4.63$, $p = 3.63 \times 10^{-6}$, $r = 0.875$, N = 28 vials). Therefore, we conclude that the egg-clustering patterns of all grouped females followed a non-random distribution (Table S1 and Fig. 1). However, solitary females did not cluster their eggs significantly more than the null model predicted ($z(2) = -1.36$, $p = 0.174$, $r = 0.786$, N = 3 vials), suggesting that their laying patterns are random – although it is worth noting that this effect could be due to low power as clustering preference values are similar to the grouped treatments. Clustering preferences also varied among social treatments: solitary and paired females had a significantly lower clustering preference in comparison to females housed in groups of 4 or 8 (Table S1 and Fig. 1). The findings support H1, that eggs are laid in non-random patterns. In addition, the results suggest that egg clustering patterns are plastic and responsive to the female's social environment.

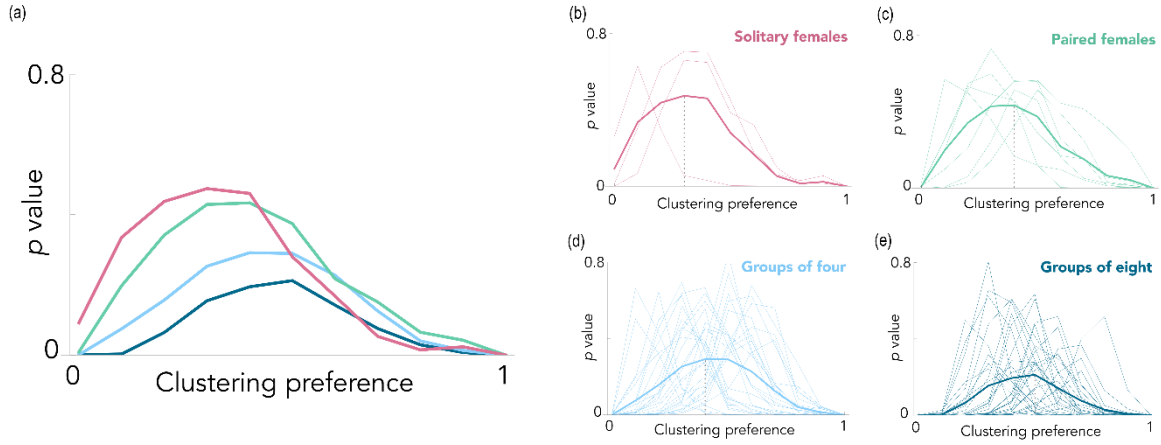


Figure 1. **Egg clustering is non-random and increases with increasing adult social density.** (a) Comparison of the clustering proportion model outputs and empirical egg laying patterns observed in *D. melanogaster,* for four social group sizes (1 (pink), 2 (green), 4 (light blue), and 8 (dark blue) adult females per group). The average Kolmogorov-Smirnov P values at K (clustering preference) values ranging from 0 to 1 in 0.1 increments – where the null distribution is K = 0 (no eggs clustered), and K = 1 (all eggs laid in one large cluster). The best-fit values of K are those that produced the least significant (highest P value) difference between simulated and observed data. (b-e) The Kolmogorov-Smirnov P values for individual replicates in the four social group sizes (1, 2, 4, and 8 adult females), with the values for each vial shown in the pale, thin lines and the average value indicated with a bold line. The vertical grey dotted lines show clustering preference values.

Females in larger social groups laid eggs at a faster rate (H2), laid more eggs (H3), and clustered their eggs more (H4), but showed no differences in fitness (egg-adult survival, H5)

In line with H2 and H3, females in larger social groups were significantly more likely to lay eggs (X^2 = 20.2, d.f. = 116, p = 1.51 x 10^{-4}). Eggs were present in 100% of vials housing eight females, a significantly higher proportion than for vials containing a solitary female (66.7%, X^2 = 15.9, d.f. = 58, p = 6.76 x 10^{-5}) or those with two females (83.3%, X^2 = 7.39, d.f. = 58, p = 0.00657). There were also significantly more vials containing eggs from the four female treatment (96.7%) in comparison to the solitary female treatment (X^2 = 10.2, d.f. = 58, p = 0.00140).

Females in larger social groups also laid more eggs per female than did the females from the other treatments (solitary females: 7 ± 10 eggs per female (N = 30 vials); pairs: 9 ± 9 eggs per female (N = 30 vials); groups of four: 18 ± 8 eggs per female (N = 30 vials); groups of eight: 19 ± 5 eggs per female (N = 30 vials); $F_{3, 116}$ = 265, p = 2.93 x 10^{-8}; Fig. 2a). Consistent

with this, post-hoc tests showed that females in solitary and paired social densities laid fewer eggs than females in groups of four and eight (all $p << 0.001$). To control for the increased sampling effort in treatments with larger groups of females, the analysis was repeated with a randomised subset of data to make number of flies (rather than the number of vials) in each treatment similar. The results of these analyses were comparable with the main analysis (larger social groups showed significantly higher likelihood of egg laying: $X^2 = 50.0$, d.f. = 54, $p = 0.0271$ and significantly higher numbers of eggs laid: $F_{3, 54} = 137$, $p = 0.00427$).

An analysis of the data at the final timepoint (24h post-mating) supported H4 and showed that females housed in larger groups laid a higher proportion of their eggs in clusters (solitary females: 22.6% ± 26.9% (N = 20 vials); pairs: 38.1% ± 28.9% (N = 25 vials); groups of four: 62.7% ± 19.2% (N = 29 vials); groups of eight: 73.7% ± 13.3% (N = 30 vials); total number of eggs laid included in model: $F_{3, 99} = 28.4$, $p = 2.53 \times 10^{-13}$; all pairwise comparisons $p < 0.05$; Fig. 2b). We included the total number of eggs laid in the model as we found that there was a positive relationship between the number of eggs and the proportion of eggs that were clustered ($F_{1, 99} = 6.66$, $p = 0.0113$), although this was not the key driver of social density effects on clustering proportions. In all grouped female treatments, the proportion of clustering also increased over time (individual vial included in model as a random effect: pairs: $F_{1, 58} = 19.1$, $p = 5.26 \times 10^{-5}$; groups of four: $F_{1, 95} = 75.8$, $p = 9.99 \times 10^{-14}$; groups of eight: $F_{1, 111} = 74.7$, $p = 4.45 \times 10^{-14}$). There was no significant effect of time on the proportion of clusters in eggs laid by solitary females ($F_{1, 40} = 1.15$, $p = 0.289$).

Analyses of the number of clusters and size of the largest cluster followed a similar pattern and both increased with increasing social density (number: $F_{3, 376} = 463$, $p = 2.20 \times 10^{16}$; size: $F_{3, 299} = 58.3$, $p = 2.20 \times 10^{-16}$; Figs. S2-3), and over time (number: $F_{1, 376} = 631$, $p = 2.20 \times 10^{-16}$; size: $F_{1, 299} = 67.1$, $p = 7.62 \times 10^{-15}$). These results were again robust to differences in sampling effort. When the analysis was repeated using the same number of individuals (rather than vials) at the final timepoint, it was still evident that females in larger groups laid a higher proportion of their eggs in clusters ($F_{3, 41} = 6.40$, $p = 0.00117$) and that the number and size of clusters still increased with increasing social density (number: $F_{3, 134} = 199$, $p = 2.20 \times 10^{16}$; size: $F_{3, 102} = 25.1$, $p = 2.96 \times 10^{-12}$).

The only hypothesis not supported was of a fitness benefit for eggs laid in clusters in terms of increased egg-adult survival (H5; Fig 2c). There was no effect of social density (1, 2, 4, or 8 females) on the proportion of eggs that survived to adult stage ($F_{3, 99} = 0.292$, $p = 0.831$; Fig. S4) and we found no correlation between the proportion of eggs laid in clusters and egg-adult offspring viability in these densities ($t = -0.485$, d.f. = 101, $p = 0.629$; Fig. 2c).

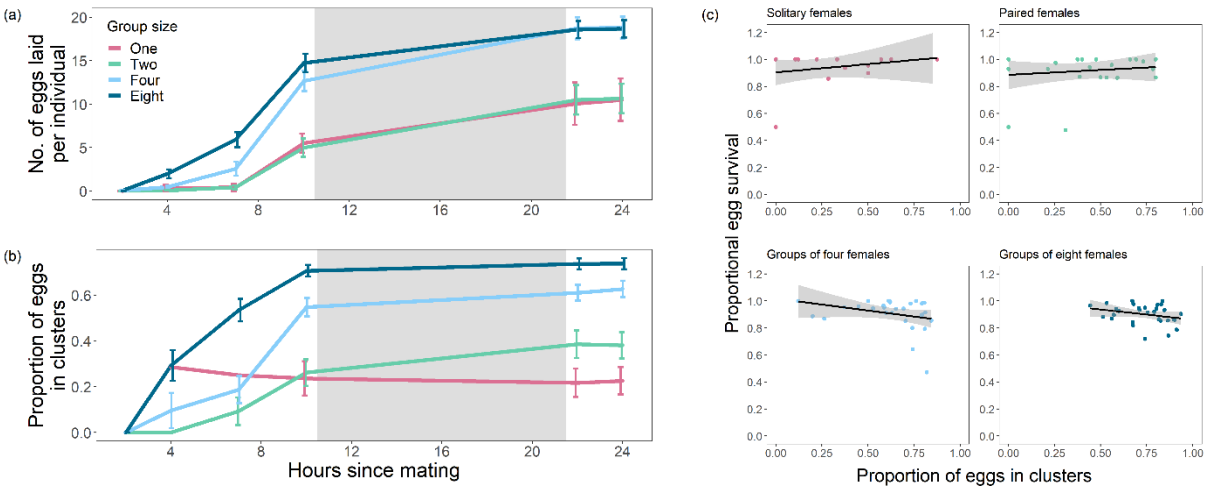


Figure 2. **Females held in larger social groups laid more eggs, more quickly, and in a higher proportion of clusters, although this did not impact egg-adult survival**. (a) Females housed in groups of four and eight were quicker to lay, and laid more eggs per female, than those kept in solitude or in pairs. Eggs laid per individual were calculated by dividing total number of observed eggs by the number of females in the vial. Social treatment means and standard errors are shown for the four housing densities at each observation time point. The grey box indicates period of dark (21:00 – 09:00 GMT). (b) Females housed in groups of four or eight laid a higher proportion of their eggs in clusters compared to those kept in solitude or in pairs. Proportion of eggs clustered was calculated by summing all cluster sizes and dividing by the total number of eggs counted. (c) There was no effect of the proportion of egg clustering on egg-adult viability (linear regressions with 95% confidence intervals).

Overall, the results supported hypotheses 2, 3 and 4 and show that females respond plastically to their social environment and lay eggs more quickly, lay more eggs and lay more eggs in clusters, as the social group size increases. However, there was no indication that eggs laid in clusters benefitted via increased egg-adult viability (H5).

Egg clusters are usually of mixed-maternity (H6)

In the groups of 4 females comprising 1 standard-fed focal wild type and 3 Sudan Black B dye-fed non-focals, we observed that of the focal eggs that had been laid in clusters, a mean of 79.5% of them were in mixed-maternity clusters. This supports the hypothesis (H6) that eggs in clusters are usually of mixed maternity.

Females prefer to lay their eggs within existing clusters rather than with singly laid eggs (H7)

In the final experiment in which we manipulated egg cluster sizes, we found that females were more likely to add at least one of their eggs to existing clusters of 4, 7, or 10 eggs, than they were to lay with a singly placed existing egg ($X^2 = 12.5$, d.f. = 156, $p = 4.18 \times 10^{-4}$; Fig. 3a). The total proportion of eggs laid that joined existing eggs was also higher when existing eggs were clustered ($F_{3, 154} = 4.75$, $p = 0.00340$; Fig. 3b). Proportions were calculated from the number of eggs females were able to lay within the 30 min observation window (between 1 – 19 eggs per vial). Post-hoc tests showed that egg cluster sizes of 4 and 10 were joined significantly more often than were single eggs (four: $F_{1, 71} = 13.0$, $p = 5.85 \times 10^{-4}$; ten: $F_{1, 78} = 8.76$, $p = 0.00407$), though this trend was marginally non-significant for a cluster size of 7 ($F_{1, 75} = 3.82$, $p = 0.0542$).

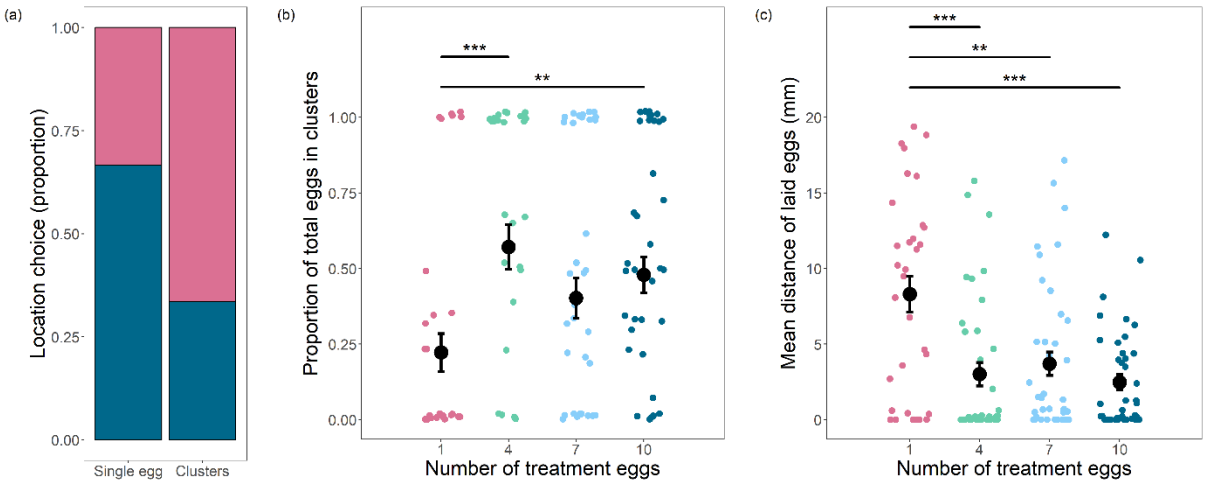


Figure 3. **Females lay more eggs within existing clusters and lay eggs closer to existing egg clusters** (a) A higher proportion of females clustered at least one of their eggs with existing eggs, when those existing eggs were in clusters compared to singly laid eggs. Blue = singly laid eggs; pink = clustered eggs. (b). Females laid a higher proportion of their eggs in clusters with existing eggs, when those existing eggs were already in a cluster (of any size) compared to a singly laid egg. Means and standard errors are shown for the four treatment egg cluster sizes. Significant differences between treatment egg cluster sizes are represented by an overarching bar; * indicates a significant difference between paired treatments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (c). Mean distance of laid eggs from existing treatment eggs was greater when the number of existing eggs was one, compared to egg clusters of any size.

In addition to scoring whether a laid egg was physically in contact with an existing egg (i.e. clustered), we also measured the distance between each laid egg and treatment eggs already present on the substrate. This is because there could be benefits of laying eggs near to other eggs without the need to be in direct contact, and so we wanted to ensure we did not exclude nearby eggs by only scoring physically touching eggs. Females laid their eggs closer to existing clusters (H6; $F_{3, 145} = 9.86$, $p = 5.86 \times 10^{-6}$; Fig. 3c) with all three cluster sizes having shorter inter-egg distances than eggs from females exposed to single eggs (four: $F_{1, 67} = 14.8$, $p = 2.68 \times 10^{-4}$; seven: $F_{1, 70} = 11.4$, $p = 0.00122$; ten: $F_{1, 70} = 23.8$, $p = 6.57 \times 10^{-6}$). Hence, laid eggs that did not strictly join a cluster were still laid closer to existing clusters than to single eggs. The results are consistent with the finding that females respond plastically to the egg clustering patterns of eggs already present in an environment and lay their eggs preferentially within existing egg clusters (H7).

455 **Discussion**

456 The main findings were that females responded plastically to their social environment and to
457 the presence of existing eggs within their oviposition environment. We found that egg laying
458 patterns were significantly non-random (H1), except when females were held in social
459 isolation. As the social group size of females increased, females laid eggs more quickly (H2),
460 laid more eggs (H3), and laid more of them in clusters (H4). However, there was no evidence
461 of a fitness benefit to clustered eggs in increased egg-adult viability (H5). Clustered eggs were
462 usually of mixed maternity (H6) and though females did not prefer to lay their eggs within
463 larger existing clusters in the environment, they did prefer egg laying in clusters overall (H7).
464 Therefore, the majority of the hypotheses tested were supported and show that females
465 show striking plasticity in their egg placement decisions, with a strong bias towards laying
466 eggs in mixed maternity clusters. However, the fitness benefits of such behaviour remain
467 elusive. These results are explored in more detail, below.

468 *Egg-clustering decisions are plastic and non-random (H1)*

469 When they are held in groups, we found that females were significantly more likely to cluster
470 eggs than was predicted by the null model, demonstrating that they clustered their eggs more
471 than expected by chance (H1). In contrast, solitary females laid eggs in patterns not
472 significantly different from random. This provides evidence that females make active
473 clustering decisions, and that egg-clustering responses are likely to be plastic and change in
474 response to varying social environments. Increased clustering has the potential to increase
475 local population density, especially at larval stages due to the limited mobility of this life stage
476 in comparison to that of adults. This has the potential to have cumulative effects, as even
477 relatively small-scale variation in density leading to increased competition may have
478 consequences for fitness (58).

479 *Presence of adult conspecifics increased the rate of oviposition and total number of eggs laid (H2,*
480 *H3)*

481 In larger groups, females laid more eggs and laid them more quickly, which is consistent with
482 existing research (32) (H2-3). Following raffle theory, producing more eggs when competition
483 is high ensures that mothers have a greater chance of at least some of their offspring surviving
484 to adulthood (46). With a greater number of conspecifics present in the environment, there

485 should also be a higher chance of copying behaviours arising due to an increased number of
486 potential 'initiators' (37, 38, 40). Increased copying could increase laying rates, benefitting
487 mothers as it may reduce sampling times needed to locate optimal oviposition sites and thus
488 any associated costs. As predicted, in addition to the overall egg laying rate being faster, the
489 latency to lay the first egg (a stage that is required to initiate any such copying behaviours)
490 was also shorter at higher adult densities. However, in situations in which larval density is
491 high and resources are limiting, it is likely that later-laid eggs will have reduced survival due
492 to resource degradation and cannibalisation (13, 32, 59). Thus, in high density environments,
493 mothers should ensure they begin laying quickly to avoid increased offspring lethality.

494 Previous research (32) showed that light can inhibit egg laying in *Drosophila*, an effect
495 that can be over-ridden by being in a group (five females). Intriguingly, our results show
496 remarkably similar laying rates for isolated and paired females (whereas groups of four or
497 eight females matched laying times reported in (32)), suggesting that exposure to only one
498 other gravid female does not counteract the light-induced inhibition of egg production. This
499 is surprising given that in male *Drosophila*, exposure to one other rival is enough to facilitate
500 reproductive behavioural responses (60-62). This suggests that there are sex differences in
501 responses to local population density.

502 *Females cluster more of their eggs in the presence of adult conspecifics (H4), and more commonly*
503 *form mixed-maternity clusters (H6)*

504 As social density increased, proportion of eggs laid in clusters increased. Although there was
505 a higher chance of clustering when there are more eggs present in the environment, this
506 increase in clustering with increasing social density did not depend upon egg number (in
507 agreement with the null model comparison). It was not possible to determine the maternity
508 of the clusters in grouped treatments in our initial experiment, but our later experiment
509 (testing H6) showed that the clusters are often of mixed maternity. Clusters containing eggs
510 from multiple mothers could contain more diverse nutrients and microbiomes (63, 64) and
511 have a reduced risk of consisting of only cheaters under a potential public goods system (29,
512 30). These benefits would not be available to isolated females laying their eggs in single
513 maternity clusters, and they would only incur increased risks of sibling competition if food is
514 limited. Females may be opting for a bet-hedging strategy of laying in multiple sites thus

515 minimising predation/parasitism risk both by reducing transmission and egg visibility (65),
516 whilst adding to existing clusters to reap the benefits of any diffusible compounds present –
517 e.g., anticannibalism pheromones (28). It is not yet known what other compounds *Drosophila*
518 eggs may contain, but it is possible that they are also coated with compounds that minimise
519 transfer of pathogens such as antimicrobials (as seen in medfly (26)). If true, this could be a
520 further benefit of clustering decisions.

521 *Females preferred to lay their eggs in existing egg clusters (H7)*

522 To understand if females had a preference for existing cluster size, we manipulated exposure
523 to egg clusters via manual transfer of eggs. Our results were not consistent with the
524 hypothesis that females would show a preference for adding their eggs to larger clusters
525 compared to smaller clusters. Instead, our results suggest that females showed a strong
526 preference for laying with clusters of any size compared to with single eggs, by choosing to
527 cluster their eggs with existing clusters more frequently and by laying their eggs closer to
528 those clusters. The absence of a preference for adding eggs to larger clusters could be because
529 (i) there is no benefit to increasing cluster size (i.e., benefits to joining clusters are not
530 additive), or (ii) there is some trade-off to the benefits of clusters of these sizes. For example,
531 with larger clusters of eggs, there could be a higher chance that at least some eggs are older,
532 and given that eggs laid later are at a higher risk of cannibalisation (28, 59) the risk/cost of
533 this increases as cluster size increases.

534 We have shown that clusters are very often of mixed-maternity (at least 79%)
535 suggesting that there are greater benefits to females if eggs in an existing cluster have been
536 laid by more than one other female. It is possible that mothers could identify maternity of egg
537 clusters due to pheromones used to identify conspecific presence (41, 66, 67), and thus do
538 not rely on cluster size for information regarding maternity. Even if this were not true,
539 increased cluster size would not be a mixed maternity benefit in our experiment as in our
540 cluster size manipulations we ensured that all clusters were of mixed-maternity. Another
541 reason that we did not detect additive effects, could be that the cluster sizes chosen were not
542 sufficiently large enough to elicit benefits of larger cluster sizes. More work needs to be
543 completed to investigate whether females have a preference for egg clusters of any particular
544 size or if their choices are based upon other characteristics.

The strong aversion to isolated eggs that we observed raises the question of how clusters form if females are only willing to lay eggs with existing clusters. It is possible that when copying laying behaviours, females are searching for evidence of a repeated decision – i.e., at least two eggs at the same site. Otherwise, a solitary egg could be present only because the mother could no longer retain it, despite not finding an optimal site. It is not known if females can detect whether an egg is infertile (although if laid by a virgin female pheromonal cues differ (41)). However, if females cannot detect fertility, an isolated egg could be infertile and thus could provide fewer clustering benefits. This result might suggest that in order for clusters to form, the first two eggs need to be laid by the same mother before other gravid females are induced to add to a cluster. However, the fact that clusters of two eggs laid by different mothers were observed in our mixed-maternity investigation suggests further studies on clustering initiation are required.

Contrary to our hypothesis of fitness benefits (H7), egg-adult viability did not increase with increasing cluster proportions. It is not clear why we did not observe fitness effects, given costs of maintaining plasticity (68, 69). It is possible that as mothers were able to choose a clustering strategy, all individuals chose the optimal strategy and thus there were no negative consequences associated with the differing clustering proportions. To test this, future work could force mothers into making an incorrect choice via a mismatch of environments. It is also possible that the *ad libitum* food provided obscured any costs of choosing the 'wrong' egg laying strategy. It was necessary to remove females from the egg laying environments before they had oviposited all fertile eggs, as eggs needed to be counted prior to larvae hatching which takes place after 22 – 24 hours at 25°C (70, 71). Thus, it is also possible that differences in egg-adult viability could have been detected if females were able to continue laying if the benefit only occurs in clusters of mixed-age eggs.

Conclusion

Overall, we demonstrated that *D. melanogaster* mothers express non-random plasticity in egg-clustering decisions and respond to fluctuations in social density from multiple life stages. Exposure to increased adult social density resulted in females laying more eggs, at a faster rate, and laying a higher proportion of those eggs in clusters. Exposure to existing egg clusters also led to a higher frequency of clustering decisions, regardless of existing cluster size.

However, the potential costs and benefits of this plasticity and its overall ecological importance, remain to be discovered. These results demonstrate a clear importance of egg location decisions as well as the overall quantity of eggs produced. This could have strong relevance in nature given that spatial distribution has significant ecological importance in wild-living populations.

References

1. West-Eberhard MJ. Phenotypic plasticity and the origins of diversity. Annual Review of Ecology and Systematics. 1989;20(1):249-78.

2. Komers PE. Behavioural plasticity in variable environments. Canadian Journal of Zoology. 1997;75(2):161-9.

3. Birkhead T, Møller A. Female control of paternity. Trends in Ecology & Evolution. 1993;8(3):100-4.

4. Rodriguez-Enriquez CL, Tadeo E, Rull J. Elucidating the function of ejaculate expulsion and consumption after copulation by female *Euxesta bilimeki*. Behavioral Ecology and Sociobiology. 2013;67(6):937-46.

5. Ward PI. Females influence sperm storage and use in the yellow dung fly *Scathophaga stercoraria*. Behavioral Ecology and Sociobiology. 1993;32(5):313-9.

6. Elgar MA, Schneider JM, Herberstein ME. Female control of paternity in the sexually cannibalistic spider *Argiope keyserlingi*. Proceedings of the Royal Society of London Series B: Biological Sciences. 2000;267(1460):2439-43.

7. Passera L, Aron S, Vargo EL, Keller L. Queen control of sex ratio in fire ants. Science. 2001;293(5533):1308.

8. Sato A, Karino K. Female control of offspring sex ratios based on male attractiveness in the guppy. Ethology. 2010;116(6):524-34.

9. Churchill ER, Dytham C, Bridle JR, Thom MDF. Social and physical environment independently affect oviposition decisions in *Drosophila*. Behavioral Ecology. 2021.

10. Fowler EK, Leigh S, Rostant WG, Thomas A, Bretman A, Chapman T. Memory of social experience affects female fecundity via perception of fly deposits. BMC Biology. 2022;20(1):244.

11. Takasuka A, Yoneda M, Oozeki Y. Disentangling density-dependent effects on egg production and survival from egg to recruitment in fish. Fish and Fisheries. 2019;20(5):870-87.

12. Matsushima N, Kawata M. The choice of oviposition site and the effects of density and oviposition timing on survivorship in *Rana japonica*. Ecological Research. 2005;20(1):81-6.

13. Khodaei L, Long TAF. Kin recognition and egg cannibalism by *Drosophila melanogaster* larvae. Journal of Insect Behavior. 2020;33(1):20-9.

14. Perry JC, Roitberg BD. Games among cannibals: Competition to cannibalize and parent-offspring conflict lead to increased sibling cannibalism. Journal of Evolutionary Biology. 2005;18(6):1523-33.

15. Kestemont P, Jourdan S, Houbart M, Mélard C, Paspatis M, Fontaine P, et al. Size heterogeneity, cannibalism and competition in cultured predatory fish larvae: Biotic and abiotic influences. Aquaculture. 2003;227(1):333-56.

16. Novčić I, Aleksić D, Vidović Z. Copying of diving behavior in the Eurasian Coot *Fulica atra*. Journal of Ornithology. 2022;163(2):523-9.

17. Cocroft RB. Vibrational communication facilitates cooperative foraging in a phloem-feeding insect. Proceedings of the Royal Society B: Biological Sciences. 2005;272(1567):1023-9.

18. Fitzgerald TD, Peterson SCCFpdJ. Cooperative foraging and communication in caterpillars. BioScience. 1988;38(1):20-5.

19. Smith CC, Sargent RC. Female fitness declines with increasing female density but not male harassment in the western mosquitofish, *Gambusia affinis*. Animal Behaviour. 2006;71(2):401-7.

20. Zhao L, Wang X, Zhan J. Effects of female and male density on their mating performance and female post-mating reproductive fitness in *Plagiodera versicolora* (Laicharting) (Coleoptera: Chrysomelidae). The Coleopterists Bulletin. 2022;76(1):73-81.

21. Welsh DP, Fuller RC. Where to place your eggs: The effects of conspecific eggs and water depth on oviposition decisions in bluefin killifish. Journal of Zoology. 2011;284(3):192-7.

22. Riehl C. Evolutionary routes to non-kin cooperative breeding in birds. Proceedings of the Royal Society B: Biological Sciences. 2013;280(1772):20132245.

23. Doody JS, Freedberg S, Keogh JS. Communal egg-laying in reptiles and amphibians: Evolutionary patterns and hypotheses. The Quarterly Review of Biology. 2009;84(3):229-52.

24. Courtney SP. The evolution of egg clustering by butterflies and other insects. The American Naturalist. 1984;123(2):276-81.

25. Ioannou C. Grouping and predation. In: Shackelford TK, Weekes-Shackelford VA, editors. Encyclopedia of Evolutionary Psychological Science. Cham: Springer International Publishing; 2017. p. 1-6.

26. Marchini D, Marri L, Rosetto M, Manetti AG, Dallai R. Presence of antibacterial peptides on the laid egg chorion of the medfly *Ceratitis capitata*. Biochemical and biophysical research communications. 1997;240(3):657-63.

27. Eisner T, Eisner M, Rossini C, Iyengar VK, Roach BL, Benedikt E, et al. Chemical defense against predation in an insect egg. Proceedings of the National Academy of Sciences. 2000;97(4):1634-9.

28. Narasimha S, Nagornov KO, Menin L, Mucciolo A, Rohwedder A, Humbel BM, et al. *Drosophila melanogaster* cloak their eggs with pheromones, which prevents cannibalism. PLOS Biology. 2019;17(1):e2006012.

29. Olson M. The theory of collective action: Public goods and the theory of groups. Harvard UniversityPress, Cambridge. 1965.

30. Archetti M. Cooperation as a volunteer's dilemma and the strategy of conflict in public goods games. Journal of Evolutionary Biology. 2009;22(11):2192-200.

31. Archetti M, Scheuring I, Yu D. The non-tragedy of the non-linear commons. Preprints: Preprints; 2020.

32. Bailly TPM, Kohlmeier P, Etienne RS, Wertheim B, Billeter J-C. Social modulation of oogenesis and egg-laying in *Drosophila melanogaster*. Current Biology. 2023;33(14):2865-77.e4.

33. Soto-Yéber L, Soto-Ortiz J, Godoy P, Godoy-Herrera R. The behavior of adult *Drosophila* in the wild. PLOS ONE. 2018;13(12):e0209917.

34. Schwartz NU, Zhong L, Bellemer A, Tracey WD. Egg laying decisions in *Drosophila* are consistent with foraging costs of larval progeny. PLOS ONE. 2012;7(5):e37910.

35. Lihoreau M, Clarke IM, Buhl J, Sumpter DJT, Simpson SJ. Collective selection of food patches in *Drosophila*. The Journal of Experimental Biology. 2016;219(5):668.

36. Zhang L, Sun H, Grosse-Wilde E, Zhang L, Hansson BS, Dweck HKM. Cross-generation pheromonal communication drives *Drosophila* oviposition site choice. Current Biology. 2023;33(10):2095-103.e3.

37. Golden S, Dukas R. The value of patch-choice copying in fruit flies. PLOS ONE. 2014;9(11):e112381.

38. Malek HL, Long TAF. On the use of private versus social information in oviposition site choice decisions by *Drosophila melanogaster* females. Behavioral Ecology. 2020.

39. Battesti M, Moreno C, Joly D, Mery F. Spread of social information and dynamics of social transmission within *Drosophila* groups. Current Biology. 2012;22(4):309-13.

40. Sarin S, Dukas R. Social learning about egg-laying substrates in fruitflies. Proceedings of the Royal Society B: Biological Sciences. 2009;276(1677):4323-8.

41. Duménil C, Woud D, Pinto F, Alkema JT, Jansen I, Van Der Geest AM, et al. Pheromonal cues deposited by mated females convey social information about egg-laying sites in *Drosophila melanogaster*. Journal of Chemical Ecology. 2016;42(3):259-69.

42. Dombrovski M, Poussard L, Moalem K, Kmecova L, Hogan N, Schott E, et al. Cooperative behavior emerges among *Drosophila* larvae. Current Biology. 2017;27(18):2821-6.e2.

43. Fowler EK, Leigh S, Bretman A, Chapman T. Plastic responses of males and females interact to determine mating behavior. Evolution. 2022;76(9):2116-29.

44. Yang C-h, Belawat P, Hafen E, Jan LY, Jan Y-N. *Drosophila*; Egg-laying site selection as a system to study simple decision-making processes. Science. 2008;319(5870):1679.

45. Vijayan V, Wang Z, Chandra V, Chakravorty A, Li R, Sarbanes SL, et al. An internal expectation guides *Drosophila* egg-laying decisions. Science Advances.8(43):eabn3852.

46. Parker GA. Sperm competition games: Raffles and roles. Proceedings of the Royal Society B: Biological Sciences. 1990;242(1304):120-6.

47. Chess KF, Ringo JM. Oviposition site selection by *Drosophila melanogaster* and *Drosophila simulans*. Evolution. 1985;39(4):869-77.

48. Moore JA. Competition between *Drosophila melanogaster* and *Drosophila simulans.* I. Population cage experiments. Evolution. 1952;6(4):407-20.

49. Wolfram Research Inc. Mathematica. Champaign, Illinois: Wolfram Research, Inc.; 2023.

50. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nature methods. 2012;9(7):671-5.

51. R Core Team. R: A language and environment for statistical computing. 2019.

52. Wickham H. ggplot2: Elegant graphics for data analysis: Springer-Verlag New York; 2016.

53. Wilke CO. cowplot: Streamlined plot theme and plot annotations for 'ggplot2': R package version 1.1.1; 2020.

54. Hope RM. Rmisc: Ryan miscellaneous: R package version 1.5.1; 2022.

55. Kleiber C, Zeileis A. Applied econometrics with R: Springer-Verlag New York; 2008.

56. Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models using lme4. Journal of Statistical Software; Vol 1, Issue 1 (2015). 2015.

57. Kuznetsova A, Brockhoff PB, Christensen RHB. lmerTest Package: Tests in Linear Mixed Effects Models. Journal of Statistical Software. 2017;1(13).

58. Churchill ER, Bridle JR, Thom MDF. Spatially clustered resources increase male aggregation and mating duration in *Drosophila melanogaster*. Animal Behaviour. 2020;169:45-50.

59. Vijendravarma RK, Narasimha S, Kawecki TJ. Predatory cannibalism in *Drosophila melanogaster* larvae. Nature Communications. 2013;4(1):1789.

60. Bretman A, Fricke C, Chapman T. Plastic responses of male *Drosophila melanogaster* to the level of sperm competition increase male reproductive fitness. Proceedings of the Royal Society B: Biological Sciences. 2009;276(1662):1705-11.

61. Moatt JP, Dytham C, Thom MDCP. Exposure to sperm competition risk improves survival of virgin males. Biol Lett. 2013;9(2):20121188.

62. Bretman A, Westmancoat JD, Chapman T. Male control of mating duration following exposure to rivals in fruitflies. Journal of Insect Physiology. 2013;59(8):824-7.

63. Guilhot R, Xuéreb A, Lagmairi A, Olazcuaga L, Fellous S. Microbiota acquisition and transmission in *Drosophila* flies. iScience. 2023;26(9):107656.

64. Bakula M. The persistence of a microbial flora during postembryogenesis of *Drosophila melanogaster*. Journal of Invertebrate Pathology. 1969;14(3):365-74.

65. Turner GF, Pitcher TJ. Attack abatement: A model for group protection by combined avoidance and dilution. The American Naturalist. 1986;128(2):228-40.

66. Bartelt RJ, Schaner AM, Jackson LL. cis-Vaccenyl acetate as an aggregation pheromone in *Drosophila melanogaster*. Journal of Chemical Ecology. 1985;11(12):1747-56.

67. Verschut TA, Ng R, Doubovetzky NP, Le Calvez G, Sneep JL, Minnaard AJ, et al. Aggregation pheromones have a non-linear effect on oviposition behavior in *Drosophila melanogaster*. Nature Communications. 2023;14(1):1544.

68. Auld JR, Agrawal AA, Relyea RA. Re-evaluating the costs and limits of adaptive phenotypic plasticity. Proceedings of the Royal Society B: Biological Sciences. 2009;277(1681):503-11.

69. DeWitt TJ. Costs and limits of phenotypic plasticity: Tests with predator-induced morphology and life history in a freshwater snail. Journal of Evolutionary Biology. 1998;11(4):465-80.

740 70. Markow TA, Beall S, Matzkin LM. Egg size, embryonic development time and
741 ovoviviparity in *Drosophila* species. Journal of Evolutionary Biology. 2009;22(2):430-4.

742 71. Fernández-Moreno MA, Farr CL, Kaguni LS, Garesse R. *Drosophila melanogaster* as a
743 model system to study mitochondrial biology. Methods in molecular biology (Clifton, NJ).
744 2007;372:33-49.

745

746

Funding

This study was funded by NERC (NE/T007133/1).

Author Contributions

Conceptualisation: Emily R. Churchill, Emily K. Fowler, Lucy A. Friend, Marco Archetti, Douglas W. Yu, Andrew F.G. Bourke, Tracey Chapman, and Amanda Bretman. *Data curation*: Emily R. Churchill, Emily K. Fowler, and Lucy A. Friend. *Funding acquisition*: Emily K. Fowler, Marco Archetti, Douglas W. Yu, Andrew F.G. Bourke, Tracey Chapman, and Amanda Bretman. *Investigation*: Emily R. Churchill, Emily K. Fowler, Lucy A. Friend, and Marco Archetti. *Methodology*: Emily R. Churchill, Emily K. Fowler, Lucy A. Friend, and Marco Archetti. *Project administration*: Emily R. Churchill, Emily K. Fowler, Lucy A. Friend, Tracey Chapman, and Amanda Bretman. *Resources*: Tracey Chapman and Amanda Bretman. *Software*: Emily R. Churchill and Marco Archetti. *Visualisation*: Emily R. Churchill and Marco Archetti. *Writing – original draft*: Emily R. Churchill. *Writing – review and editing*: Emily R. Churchill, Emily K. Fowler, Lucy A. Friend, Marco Archetti, Douglas W. Yu, Andrew F.G. Bourke, Tracey Chapman, and Amanda Bretman. All authors gave final approval for publication.

Supplementary Information

Methods

Effects of adult social density on egg-clustering decisions (H1-5)

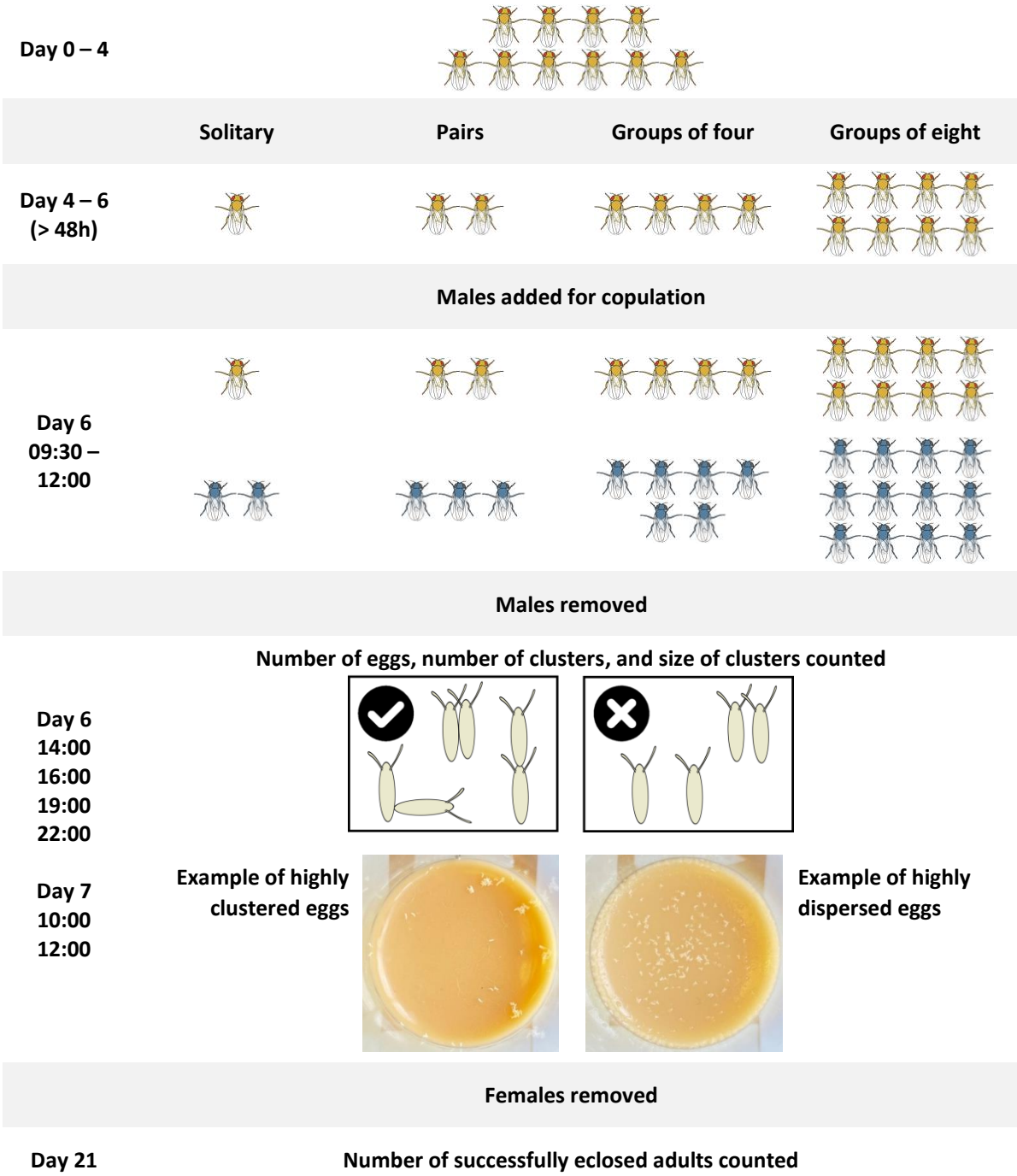


Figure S1. The experimental set up for testing the effects of social densities on egg laying. Day 0 – 4: males and females housed in same-sex groups of 10. Day 5 – 6: females transferred to one of four social treatments (whilst males remained in groups of 10). Day 6: males were added to female treatment vials in a ratio of 3:2 (2:1 for isolated females) and given 2h to mate. Day 6 – 7: number of eggs, number of clusters and size of clusters were

771 counted at the six listed timepoints. Shown is the strategy for defining egg clusters (contact between the main
772 body of eggs).

773 *Effects of existing egg location on egg-clustering decisions*

774 *(i) Set up of egg locations and adult density treatments*

775 The rationale was to test isolated versus grouped females laying eggs in 3 types of 'egg
776 treatment' vials: no egg present (control), and 1 egg either placed in the centre or at the edge
777 of the vial. The non-focal eggs used to create the egg treatment vials were laid by groups of
778 10 gravid females allowed to lay overnight on Petri dishes filled with 40ml of SYA medium.
779 Only solitary eggs were chosen and transferred, using a mounted needle, to make the
780 experimental egg treatment vials (to reduce variation in uncontrolled factors). To enable
781 transfer of eggs without damage to the SYA medium, standard plastic vials were cut in half,
782 and the top half reattached after egg transfer using Sellotape. Test flies were collected from
783 standard density vials (as described above) and females were housed in solitude, and males
784 in groups of four. After six days, one male was transferred into the vial with each female, and
785 pairs were watched to ensure matings occurred. We then transferred females either alone or
786 in groups of 4 into vials containing the egg treatments (no egg, 1 edge egg, or 1 central egg)
787 described above (N = 15 - 42 vials per treatment).

788 *(ii) Laying latency and egg location decisions*

789 We then checked vials every 30 minutes until 21:30 (one count after lights off in the constant
790 environment room) and removed the female(s) if they had laid eggs. If they did not lay, they
791 were kept overnight, and egg counts resumed at 09:30 for three further hours, until 24 hours
792 after females were introduced to the vials. We calculated egg laying latencies and the number
793 of eggs laid by the focal females. We also noted whether eggs were laid in a cluster (defined
794 in Fig. S1) alongside the existing egg.

795

796 *Results*

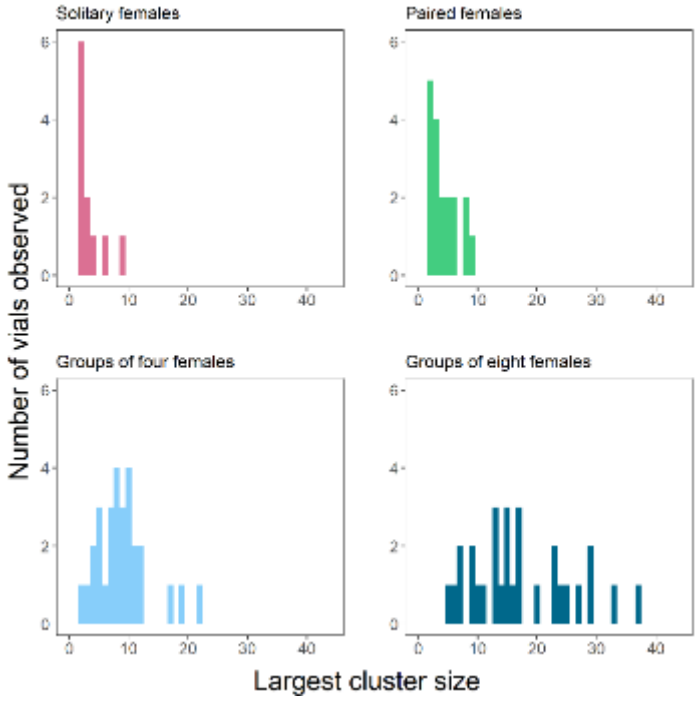
797 *Egg laying patterns of females were significantly non-random (H1)*

798 Table S1. Comparison of the clustering proportion model outputs and empirical egg laying patterns observed in
799 *D. melanogaster*. Shown are Kolmogorov-Smirnov P value outputs for the comparison of empirical data to
800 expected clustering preference values (K = 0 to K = 1, in 0.1 increments) rounded to two decimal places. Values
801 that show the most likely clustering preference are highlighted in bold.

Social treatment	**K values**										
	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Solitary N = 20 vials	0.09	0.34	0.44	**0.48**	0.46	0.29	0.17	0.06	0.02	0.03	0.01
Paired N = 25 vials	0.01	0.20	0.34	0.43	**0.43**	0.38	0.22	0.15	0.07	0.04	0.01
Groups of four N = 29 vials	0.00	0.08	0.16	0.25	**0.29**	0.29	0.23	0.13	0.05	0.02	0.00
Groups of eight N = 30 vials	0.00	0.00	0.07	0.15	0.20	**0.21**	0.15	0.08	0.03	0.01	0.00

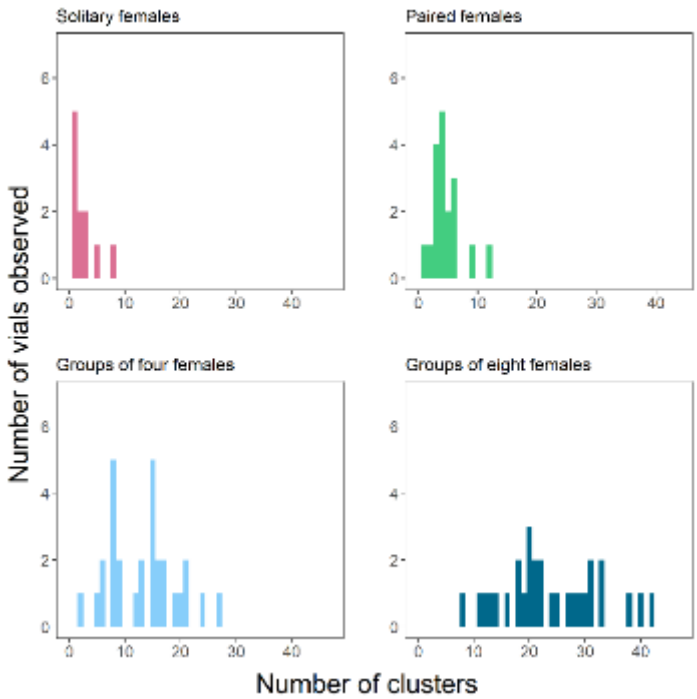
802

803 *Females in larger social groups laid significantly more of their eggs in clusters (H4)*



804

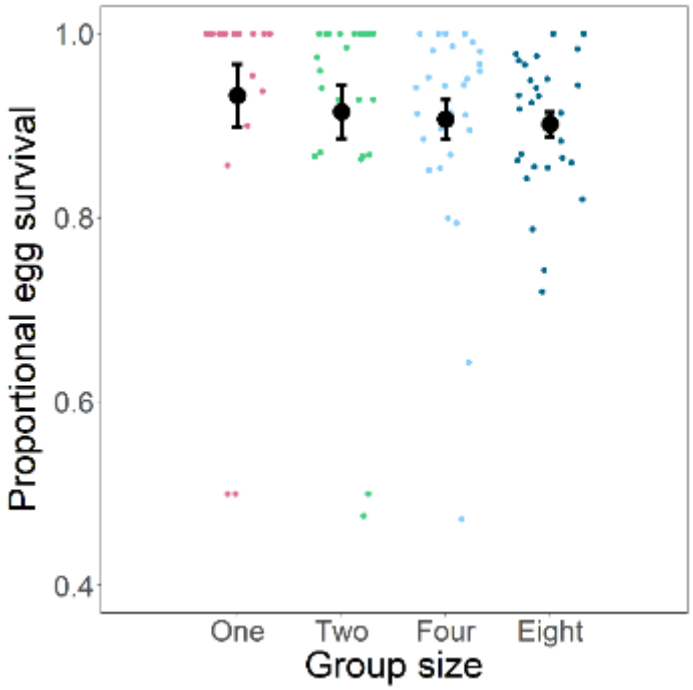
805 Figure S2. Maximum cluster size increased with increasing female social density. Total number of vials with each
806 cluster size observed is displayed for each of the four social treatments.



807

808 Figure S3. A greater number of clusters were observed with increasing female social density. Total number of
809 clusters per vial is displayed for each of the four social treatments.

810 *Fitness effects of social environment (H5)*



811

812 Figure S4. There was no effect of social group size on egg-adult viability. Means (large dot) and standard errors
813 are shown in black.

814 *Edge effect egg laying decisions were not overridden by adult social group size or existing egg*
815 *locations*

816 There was a strong preference to lay at the edge of the vial, and there was no effect of existing
817 egg location (X^2 = 1.32, d.f. = 113, p = 0.517) or group size (X^2 = 3.80, d.f. = 112, p = 0.0513) on
818 where in the environment females laid. There was also no effect of either egg location or
819 group size on whether females chose to cluster their eggs as no females clustered their eggs
820 (Table S2).

821

822 Table S2. Effect of social density and previous egg laying patterns on female fecundity and egg clustering. The
823 proportion of vials where eggs laid were observed to be clustered with the existing egg (main bodies in direct
824 contact) and laid at the edge of the vial (within 0.5cm from the vial circumference).

		Proportion of eggs laid at the edge (%)	Proportion of eggs clustered (%)
Social treatment	Egg location		
Solitary females	None (N = 33 vials)	100	-
	Edge (N = 22 vials)	100	0
	Centre (N = 15 vials)	100	0
Groups of four	None (N = 17 vials)	94.1	-
	Edge (N = 17 vials)	93.8	0
	Centre (N = 13 vials)	100	0

825

826 As before, we found that females housed in solitude took longer to initiate laying than those
827 in groups of four (X^2 = 4.38, d.f. = 126, p = 0.001; Fig. S5). There was no effect of existing egg
828 location on laying latency (X^2 = 1.30, d.f. = 125, p = 0.139; Fig. S5).

829

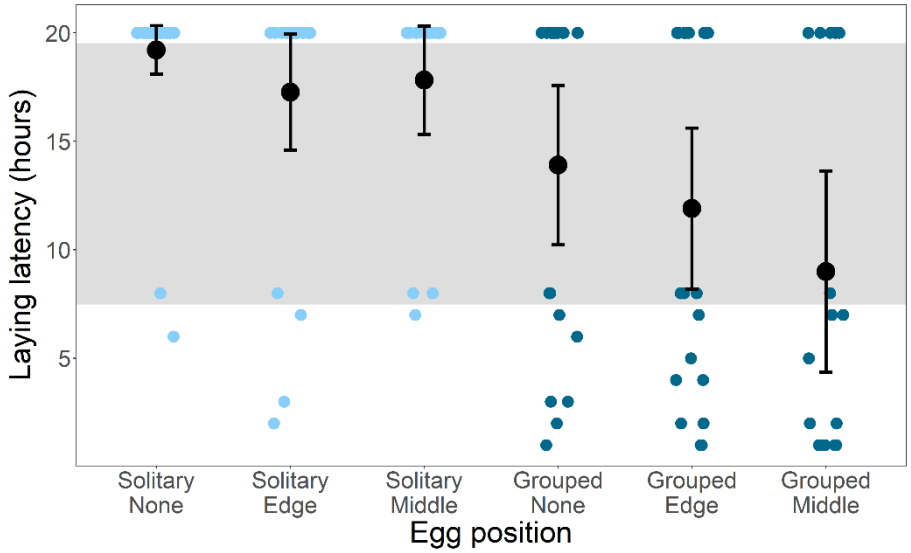


Figure S5. Females housed in solitude were slower to start laying than those housed in groups of 4, but there was no effect of existing egg location on laying latency. Means and standard errors are shown for the six treatments. Grey box indicates period of dark (21:00 – 09:00 GMT).