On the use of kinship and familiarity associated social information in mediating *Drosophila melanogaster* oviposition decisions

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Abstract

Decisions about where to lay one's eggs are of great importance, as the specific conditions in one's offspring developmental environment may be critical to their subsequent survival and lifetime reproductive success. Information produced by conspecifics can potentially be useful to an individual's oviposition decision-making process as this "social information" may provide an energetically cheaper means of assessing site suitability rather than acquiring it personally. However, not all conspecific public information may be equally beneficial, and cues produced by kin may be especially valuable as they might signal uniquely suitable microenvironments, as well as suggesting other fitness advantages resulting from improved foraging success and/or a decreased risk of competition/cannibalism amongst kin compared to sites where unrelated conspecifics are located. Using the fruit fly, *Drosophila melanogaster*, we explored whether public information use is associated with kin-based egg-laying decisions. Kinship is potentially recognized in several ways, including cues produced by environmentally-associated gut microbiota of the egg-laying flies, and so we also explored whether there were biases in how focal females interacted with cues from individuals that differed in both their genetic relatedness, as well as their environmental "familiarity." In a series of inter-connected assays, we examined the egg-laying behaviour of females that interacted with a choice of potential egg-laying substrates that differed in the manner of their prior conspecific exposure. Not only do female flies lay more eggs on sites that had cues left behind by conspecifics compared to unexposed substrates, but they also exhibited strong biases in their oviposition patterns that was consistent with being sensitive to the kinship status of the prior substrate occupants. Furthermore, while females did not apparently discriminate between sites differing in prior egg abundance, they did exhibit bias in their egg-laying based on the population of origin of the demonstrator female. The basis of the kinship categorization by ovipositing females appears to be based on phenotypes that reflect true genetic relatedness that are neither mimicked nor disrupted by the conspecific's developmental environment. These results highlight the potential usefulness of *D. melanogaster* as a model to understand the evolution of social behaviour in the expression of decision-making.

***Keywords*:** Social information, *Drosophila melanogaster*, oviposition, conspecifics, kin recognition, microenvironments, behaviour, copying, public information

***Competing interests*:** The authors declare no competing interests.
***Ethics*:** This study did not require approval from an ethics committee.
***Supporting Data*:** All data analysed in this manuscript are archived in Borealis, the Canadian Dataverse Repository: https://doi.org/10.5683/SP3/N7XBOJ

48 ***Introduction***

49 In species where there is little-to-no post-natal parental care, decisions about where and
50 when an individual should lay their eggs are likely very important, as there are potentially dire
51 fitness outcomes for one's offspring if unsuitable oviposition sites are chosen (Thorpe 1945,
52 Heard 1994, Lihoreau et al. 2016). In order to make better decisions, using cues produced by
53 conspecifics (aka "social information" *sensu* Valone and Templeton 2002) may be of value to the
54 individual as they may provide meaningful information about potential egg-laying sites that can
55 be obtained at a lower energetic cost than having to explore and evaluate alternate options
56 oneself. Social information can be valuable in reducing an individual's uncertainty about their
57 environment, and may help them locate areas that are rich in important resources such as food,
58 shelter, desirable mates, increased foraging success, or are at a lower risk of predation/parasitism,
59 any of which may benefit the mimicking individual and/or their offspring (Sarin and Dukas
60 2009, Blanchet et al. 2010, Grüter and Leadbeater 2014). Furthermore, the energy savings of
61 using social information over private information can also be redirected into increased
62 provisioning and/or production of offspring, thereby enhancing the benefits of its use in making
63 decisions (Golden and Dukas 2014).

64 While fruit flies, *Drosophila melanogaster*, have a long and storied use as a model in studies of
65 genetics, development, behaviour and evolution (*see* Weiner 2000, Brookes 2001), they are also
66 emerging as a powerful model in which to study social learning and the use of social information
67 (Leadbeater 2009, Chen and Sokolowski 2022). *D. melanogaster* has been found to use the visual,
68 gustatory, tactile, and olfactory cues produced by conspecifics in a number of different decision-
69 making contexts. For instance, flies use social cues when engaged in spatial learning tasks to
70 improve the speed that a "safe zone" is detected (Foucaud et al. 2013). Virgin female flies that are
71 exposed to deposits left by conspecific demonstrators exhibit lower post-mating egg-laying rates

72 than unexposed females, presumably because they are better able to anticipate the forthcoming
73 availability of oviposition sites and the potential degree of future larval competition (Fowler et al.
74 2022). Mate choice can involve social information as female fruit flies are more likely to mate
75 with males exhibiting similar characteristics as a male they've observed successfully mating with
76 another female (Mery et al. 2009, Monier et al. 2018, Nöbel et al. 2018, Nöbel et al. 2022, *but see*
77 Belkina et al. 2021) presumably because mate choice is often costly to females (*see* Reynolds and
78 Gross 1990), and copying the decisions made by conspecifics represents an economical adaptive
79 strategy (Monier et al. 2018). Important information about environmental conditions can also be
80 obtained indirectly by flies interacting with conspecifics. In a study by Kacsoh et al. (2015) female
81 flies decreased their oviposition rate in response to exposure to parasitic wasps, and this defensive
82 change in egg-laying behaviour was adopted by inexperienced flies housed with the wasp-
83 exposed females, with cues about environmental risk communicated visually using the
84 demonstrator flies' wings. Thus, while *D. melanogaster* has not been considered a "social" species
85 (Chen and Sokolowski 2022), it is evident that fruit flies can, and do, use social information to
86 inform their decisions.

87 One important context where social information seems to be used extensively by *D.*
88 *melanogaster* females is in selecting suitable oviposition sites (Battesti *et al.* 2012, Durisko et al. 2014,
89 Sarin and Dukas, 2009, Golden and Dukas 2014, Malek and Long 2020, Moreira-Soto et al.
90 2024). In the wild, there may be considerable heterogeneity in the availability and quality of
91 oviposition sites that are available to female *D. melanogaster* (Reaume and Sokolowki 2006,
92 Markow 2015), and the choice of oviposition environment can have profound consequences for
93 their offspring's fitness (*e.g.* Prasad et al. 2003, Klepsatel et al. 2020). *D. melanogaster* females may
94 gain insight on the suitability of oviposition sites through direct observation of other females
95 ovipositing (*as in* Sarin and Dukas, 2009 and Battesti *et al.* 2012), the presence of larvae (*e.g.*

96 Golden and Dukas, 2014) and/or the presence of chemical cues produced by conspecifics (*e.g.*
97 Venu et al. 2014, Lin et al. 2015, Duménil et al. 2016, Moreira-Soto et al. 2024). Laying one's
98 eggs at sites already chosen by conspecifics may also be of benefit to the offspring's success due to
99 enhanced communal digestion via secreted enzymes (Gregg, et al. 1990, Sakaguchi and Suzuki
100 2013), increased beneficial microbial activity (Gilbert 1980, Wertheim et al. 2002a, Wertheim et
101 al. 2002b), and/or improved group foraging (Dombrovski et al. 2017, Khodaei et al. 2020, Shoot
102 et al. 2024). The reliance upon social cues by ovipositing *D. melanogaster* is not without potential
103 pitfalls. First, by outsourcing one's information gathering to others, there is the risk of mimicking
104 other's bad decisions. For instance, in a study by Golden and Dukas (2014) where females were
105 given a choice of laying their eggs on a site where nutrition was poor but contained social cues
106 (the presence of larvae) or a site where the media contained 3 times more nutrition but lacked
107 conspecific cues, the ovipositing females treated them as being of equal quality, even though any
108 offspring laid on the former patches did not have access to sufficient nutrients to survive.
109 Similarly, Duménil et al. (2016) observed that the presence of a mated female demonstrator on
110 yeast-free media made this undesirable oviposition site equally-valued as yeasted media (that has
111 not been exposed to conspecifics) to later ovipositing females. Furthermore, laying one's eggs
112 near those of others can have additional challenges arising from competition for limited resources
113 (De Jong 1976, Joshi et al. 1996, Golden and Dukas 2014), increased exposure to toxic waste
114 products (Borash et al. 1998), and the threat of cannibalism at many developmental stages
115 (Vijendravarma et al. 2013, Ahmed et al. 2015, Yang 2018). However, some of these risks may
116 be mediated if females are able to oviposit at sites containing kin, as *D. melanogaster* larvae are
117 more co-operative (Khodaei and Long 2019), and less cannibalistic (Khodaei and Long 2020,
118 Fisher et al. 2021) towards close relatives, compared to how they engage with unrelated
119 conspecifics. Thus, if females were able to differentiate social information produced by kin from

120 those generated by other demonstrators, they might be able to achieve greater fitness benefits by
121 selectively copying the choices made by relatives. Additionally, as there can be extensive intra-
122 specific genetic variation *D. melanogaster* for nutritional preferences and dietary tolerances (*e.g.*
123 Toshima et al. 2014, Havula et al. 2022), preferentially laying eggs near those of close relatives
124 may increase the likelihood of selecting the best micro-habitat for the success of one's own
125 offspring.

126 When considering the possibility that individuals might differentially use social cues
127 produced by relatives and non-relatives, one must also consider what (if any) cues could indicate
128 kinship (or could be inferred to be reliable proxies of kinship). In *D. melanogaster* such information
129 could conceivably be perceived from cuticular hydrocarbons (CHCs) and/or gut bacteria left
130 behind by conspecifics. CHCs are long-chain hydrocarbons that are synthesized in insect
131 oenocyte cells which become part of the cuticle's waxy coating along with lipids, aldehydes,
132 alcohols, esters and fatty acids which helps prevent water loss (*reviewed in* Richard and Hunt 2013,
133 Chung and Caroll 2015). These fat molecules also play an additional important role in olfactory
134 communication related to species recognition and mate choice decisions (*reviewed in* Singer 1998,
135 Ferveur 2005, Scott et al. 2011, Chung and Caroll 2015). CHCs may provide accurate
136 information about kinship, as in addition to differences between species (Moreira-Soto et al.
137 2024) there also exists substantial intra-specific genetic variation within *D. melanogaster* populations
138 for these phenotypes (Foley et al. 2007, Dembeck et al. 2015, Heys et al. 2018). It has been
139 suggested that assortative mating patterns may be mediated by the degree of similarity of CHC
140 chemical profiles of males and females (Haerty et al. 2002, Robinson et al. 2012). Recent work
141 has revealed that female flies transfer their own CHCs (along with male-specific compounds
142 acquired during mating) to their laid eggs (Moreira-Soto et al. 2024), which could conceivably be
143 used as signals of kinship by subsequent ovipositing females.

144 In addition to CHC chemical cues, olfaction-based decisions may be influenced by signals
145 associated with differences in an individual's bacterial community (Sharon et al. 2010, Najarro et
146 al. 2015). While the *D. melanogaster* microbiome is relatively simple compared to other hosts
147 (Broderick and Lemaitre 2012), there can be substantial variation between individuals/families
148 from the same population in the composition of their microbial communities (Wang and
149 Staubach 2018, Heys et al. 2018). Individuals acquire some of their microbial symbionts from
150 their mothers, as like CHCs, bacteria are transferred by ovipositing host mothers to the surface of
151 their eggs, and the subsequent consumption of the chorion by hatched larvae helps establish their
152 own gut flora (Bakula 1969, Guilhot et al. 2023). Thus, differences in cues produced by bacterial
153 communities could conceivably and independently be used to infer kinship (Lizé et al. 2014,
154 Lewis et al. 2014). Importantly, CHC phenotypes and/or microbiotic communities in *D.*
155 *melanogaster* can also be influenced by the flies' developmental environment (Sharon et al. 2010,
156 Fedina et al. 2012, Lizé et al. 2014, Venu et al. 2014, Heys et al. 2018, García-Roa et al. 2022,
157 Pavković-Lučić et al. 2022), raising the possibility that recognition of "kin" may alternately be the
158 recognition of similarity of environmental history ("familiarity"), or some combination of the two
159 factors. In a previous study that examined whether kinship mediated the magnitude and
160 consequences of male-male competition in adult flies, it was found that reductions in male-
161 induced harm were *only* seen in groups of males that were both closely related to each other *and*
162 'familiar' by having developed in the same larval environment (Le Page et al. 2017). Lizé et al.
163 (2014) described how females were more likely to mate with a familiar male, while males' ability
164 to strategically avoid investing into mates with a sibling was impeded if she had developed in the
165 same environment. Similarly, Heys et al. (2018) noted that the ability of fruit flies of both sexes to
166 perceive the kinship status of their mate appeared to be dependent on the state of the mate's
167 microbiome – with females only acting differently towards kin when those individuals had

168 developed in the same media type (and had an intact microbiota), while a male's increased
169 investment into mating with an unrelated female only occurred if she had been grown in a
170 different environment (or whose microbiota had been disrupted with the use of antibiotics). The
171 complicated nature of the interaction between developmental environment and relatedness was
172 also explored by García-Roa et al. (2022) who found that flies developing in different
173 environments exhibited both different CHC and microbial profiles, and that related flies had
174 microbiotic communities of similar diversity (but not composition), and that diversity was
175 correlated with CHC phenotypic profiles. Together these studies highlight that socially-available
176 kinship cues (or proxies thereof) may involve a number of independent and inter-related olfactory
177 signals, whose expression may depend not only on relatedness but also on the specific nature of
178 the developmental environment.

179 With these ideas and findings in mind, in this study we set out to examine whether female
180 flies showed bias in their use of social information cues left behind by conspecifics when selecting
181 ovipositing sites, and would respond differently to cues produced by kin vs non-kin.
182 Simultaneously, we also explored whether a female's response to potential kinship cues were
183 conditional on the conspecific's developmental environment. Overall, the goal of this research is
184 to better characterize copying behavior in *D. melanogaster* and to gain better insight into the factors
185 that influence these oviposition site decisions.

186

187 **Materials and Methods**

188 *Population Protocols and Fly Maintenance*

189 All fruit flies used in our assays were obtained from one of three associated laboratory *D.*
190 *melanogaster* populations: *Ives* (hereafter "IV"), "IV-*bw*", and "IV-*bwD*". The IV population was
191 derived from a sample of mated females collected in 1975 from Amherst MA, USA sample

192 population (Rose 1984). The IV-*bw* population was created by introgressing the brown-eye
193 recessive allele, bw^1, into the genetic background of the IV population via repeated rounds of
194 back-crossing, to create a competitor population (Khodaei and Long 2020). The IV-*bwD*
195 population was also created in the same fashion, by introducing the *bwD* allele (a dominant allele
196 that also produces a brown-eyed phenotype) into an IV genetic background. The IV-*bw* and IV-
197 *bwD* populations have been subject to period rounds of additional back-crossing to the IV
198 population in an attempt to minimize their divergence from the base population, with the last
199 session occurring approximately 2 years prior to the start of this experiment. These three
200 populations are each maintained at a census size of ~3500 adults per generation, incubated at
201 25°C incubation temperature with 60% humidity on a 12L:12D hour diurnal light cycle (Martin
202 and Long 2015). Flies are maintained in standard *Drosophila* vials (95mm H x 28.5mm OD)
203 containing 10mL of banana/agar/killed-yeast food, dusted with live yeast (Rose 1984, Shoot et
204 al. 2024). Populations are cultured on a 14-day cycle, wherein every fortnight adult flies are
205 removed from their natal vials, mixed *en masse* under light CO_2 anaesthesia and distributed into
206 vials containing new media for up to 18h, before being removed and the eggs laid are then
207 trimmed by hand to a density of approximately 100 eggs per vial (Martin and Long 2015).
208

209 *Experiment 1: Test of effect of conspecific relatedness and developmental environment on egg-laying behavioural*
210 *decisions.*

211 In our first experiment, we collected adult male and female flies as virgins (within 8h of
212 eclosion from their pupae) from the IV population, and housed them individually. We then
213 created 50 families by randomly combining single males and females into "mini-egg" chambers
214 (Kartell 733/4 polyethylene 20 mL sample vials; 74.5mm H x 24.8mm OD; *see* Figure S1a)
215 whose lid contained juice/agar media (Sullivan et al. 2000) and was used as a "dish" for

216 ovipositing. We left these chambers in the incubator for ~18h, before removing the adult flies
217 and counting the number of eggs that had been laid overnight. We then evenly split each family's
218 eggs among 2 different vials each containing 10ml of media, and then added a sufficient number
219 of similarly-aged eggs obtained from the IV-*bw* population so that each vial ultimately contained
220 ~100 eggs apiece. We returned these pairs of vials to the incubator where they were allowed to
221 develop for the next 9 days when we began collecting virgin females for use in the experiment.
222 For each pair of vials, we haphazardly designated one as the "focal" vial, from which we
223 collected 3 virgin females: a IV female ("the focal female"), a second IV female (the focal female's
224 sister, raised in the same vial), and one brown-eyed IV-*bw* female (an unrelated female, raised in
225 the same vial as the focal female). From the other (non-focal) vial in the pair, we collected 2 virgin
226 females: an IV female (the focal female's sister, raised in a different vial) and one brown-eyed IV-
227 *bw* female (an unrelated female, raised in a different vial than the focal female). We kept these
228 females individually for up to 48h in test-tubes containing media to further verify their virgin
229 status (by the absence of any fertilized eggs). Next, we introduced two similarly-aged adult IV
230 males into the focal female's vial (so that all her offspring would express the wild-type red-eye
231 phenotype). At the same time we transferred all other females into their own mini-egg chambers
232 whose dish lids contained standard banana media containing 3 IV-*bwD* males so that all offspring
233 produced by demonstrators would express a *brown-eyed* eye phenotype and to ensure that any
234 male-specific compounds transferred to the dish surface would be consistent across all dishes (Lin
235 et al. 2015, Moreira-Soto et al. 2024). Females and males were left overnight to ensure sufficient
236 time for mating to occur. On the following morning all non-focal flies were removed from the
237 mini-egg chambers, and sets of four dishes were affixed using adhesive putty to the bottom of a
238 larger chamber, (hereafter "arena"), which consisted of an inverted plastic box (KIS Omni box,
239 20.3 x 15.9 x 9.6cm) modified by the addition of mesh vent holes to box's outer edges (*see* Figure

240 S1b, Young et al. 2018). A fifth dish (that had never been exposed to flies) was also placed in the
241 arena before it was sealed shut, and the (lightly anesthetized) focal female was introduced via an
242 access hole. Thus the five dishes inside each arena represent available oviposition sites for the
243 focal female that potentially differ in their social information: The 'related/same' dish contains
244 cues produced by a sister that developed in the same vial environment as the focal IV female; the
245 'unrelated/same' dish contains cues from an unrelated IV-*bw* female that developed in the same
246 vial environment as the focal IV female; the related/different' dish contains cues produced by a
247 sister that developed in a different vial environment than the focal IV female; the
248 'unrelated/different' dish contains cues from an unrelated IV-*bw* female that developed in a
249 different vial environment as the focal IV female; and finally the 'control/unexposed' dish
250 contains no social information, as it had no prior exposure to flies (*see* Figure S1c). The arenas
251 were housed in a quiet, well-lit room, and were left undisturbed for 20 minutes following the
252 introduction of the focal female. The arenas were then observed every 20 min for ~4.5h (starting
253 at 10:30am), and for 4h on the next day (beginning at 9:30am) for a total of 26 observation
254 sessions. During each observation session, the arenas were watched for a 5s time period, allowing
255 for the identification of which of the 5 dishes (if any) the focal IV female was located. The identity
256 of the different treatments was unknown to the observers. One all observations were complete,
257 we unsealed the arena, discarded the focal female and transferred the media in each dish into
258 vials containing 10m of media. These labelled vials were sealed and returned to the incubator for
259 2 weeks. At that time, all eclosed adults were removed, and their eye colours were tallied. Red-
260 eyed flies were the offspring of the focal female, while brown eye-flies (*wt+/bwD* or bw_1/bwD)
261 were produced by one of the conspecific females.
262
263 *Experiment 2: Test of effect of conspecific egg production on focal female egg-laying behavioural decisions.*

264 Based on the egg-laying patterns observed in Experiment 1, we performed a series of
265 complementary assays designed to test alternative hypotheses for the focal flies' oviposition
266 biases. Thus, in this experiment we set out to examine whether the number of eggs laid by
267 conspecifics on a substrate was associated with the number of eggs laid by a focal female. We
268 began by collecting mated IV-*bw* female flies from the lab's stock population and placed either 1,
269 2, or 0 individuals overnight into mini-egg chambers containing media in their dish lid. The next
270 morning, one of each type of the dishes was affixed to the bottom of an arena (*see* Figure S1d).
271 Next, we added a single mated IV female to each arena and left them undisturbed for 22h,
272 before transferring the media in each dish to a vial containing 10ml of media. We returned those
273 vials to the incubator and counted the number of wild-type and brown-eye offspring that had
274 eclosed 12 days later.
275
276 *Experiment 3: Test of egg-laying biases of focal females towards cues from flies originating from the IV versus the*
277 *IV-bw populations.*
278 In our third experiment we explored the alternate hypothesis that the egg-laying patterns
279 observed in Experiment 1 might reflect preferences of the IV-originating focal female to lay eggs
280 on substrates that had come into contact with any other IV flies (regardless of their degree of
281 immediate genetic consanguinity) and/or the avoidance of substrates that had come in contact
282 with any IV-*bw* flies. For this assay we set up replicate vials containing 5 mated IV and 5 mated
283 IV-*bw* females, that were allowed to oviposit for 18h before being discarded, and the number of
284 eggs laid standardized to 100 apiece. These vials were placed in the incubator, and we collected a
285 virgin red-eyed and a virgin brown-eyed female from each vial as they eclosed (thus both flies had
286 experienced the same developmental environment). Each female was then placed into their own
287 mini egg chamber with 2 IV-*bwD* males overnight before being discarded, and the dish affixed to

288 the inside of an arena, along with an unexposed/control dish (*see* Figure S1e). An individual
289 mated IV female obtained from the lab stock population was introduced to each of the arenas,
290 and her location in the arena was periodically observed every 20 minutes over the course of 5h
291 (for a total of 15 sessions). The arena was left undisturbed overnight, and the following morning
292 media was removed from the dishes and transferred to vials containing 10ml of media. These
293 vials were incubated for 12 days, and the number of offspring with different eye colours was
294 recorded.
295
296 *Experiment 4: Revisiting the effect of conspecific relatedness on egg-laying behavioural decisions.*
297 Based on the outcomes of our second and third experiments, we set about directly
298 comparing the egg-laying behaviour of focal females towards substrates that had been exposed to
299 their sibling or to a entirely unrelated IV-derived female. As in experiment 1 we collected the
300 focal female's parents from the IV population, mated them, placed them into mini-egg chambers
301 overnight, then counted and split their eggs amongst 2 vials, with additional similarly-aged IV-bw
302 eggs added to bring the density to a total of 100 eggs apiece. From each of the pairs of vials we
303 collected virgin females as they eclosed and kept them individually. One of the vials was
304 arbitrarily designated as that which yielded the focal female (who was then housed with 2 IV
305 males from the stock population overnight), and a female from the other vial (*i.e.* her sister raised
306 in a different environment) was housed with 2 IV-*bwD* males overnight in a mini egg chamber.
307 We also haphazardly chose one of the IV females collected from one of the other pairs of vials
308 (thus an unrelated female, raised in a different environment) and housed her 2 IV-*bwD* males
309 overnight in a mini egg chamber. These dishes (along with a control/unexposed dish) were
310 placed into an arena (*see* Figure S1f), into which we added the mated focal females. Her location
311 was periodically observed every 20 minutes over the course of 5h (for a total of 15 sessions). Next,

312 the chamber was left undisturbed overnight and the following morning, we transferred the media
313 from each dish into a new vial containing 10ml of media which were incubated for an additional
314 12 days. The eye colour of all adult flies present in these vials were tallied and recorded.
315
316 *Statistical Analyses*
317 All data analyses were conducted in the R statistical computing environment (version 4.0.3, R
318 Core Team, 2020). In all of our experiments we compared the total number of visits by focal
319 females to the five different lids in each arena using generalized linear models (GLMs), with
320 quasipoisson error distributions, where the independent factor was the lid treatment category.
321 We determined whether the treatment means were significantly different from each other with
322 likelihood-ratio chi-square tests using the *Anova* function in the *car* package (Fox and Weisberg
323 2011), followed by Tukey HSD test executed by the *glht* function in the *multcomp* package
324 (Hothorn et al. 2008) if necessary to determine the specific location of differences between
325 treatments. For experiment 1 we also set out to determine if there was any difference in patch
326 visitation that varied with relatedness and/or familiarity treatments, by using the same response
327 data (excluding observations made on the control lid, as this treatment by definition had received
328 no prior conspecific contact) which was analyzed using a 2-way GLM with quasipoisson errors.
329 The degree of kinship, the source, and their interaction were the independent factors in this
330 model. The significance of these effects was determined using a likelihood-ratio chi-square test
331 using the *Anova* function.
332 For all of our experiments we (separately) analyzed the total number of red-eyed and brown-
333 eyed adults that successfully eclosed from the different lids in each arena using GLMs, with
334 quasipoisson error distributions, where the independent factor was the lid treatment category.
335 For each we determined whether the treatment means were significantly different from each

336 other using likelihood-ratio chi-square tests implemented using the *Anova* function followed by
337 Tukey HSD tests if necessary to determine the specific location of differences between
338 treatments. As above for experiment 1 data we also set out to determine if there was any
339 difference in offspring number associated with relatedness and/or familiarity treatments, by
340 excluding data collected from the control lid treatment, and analyzing the rest of the data set
341 using a 2-way GLM with quasipoisson errors. The degree of kinship, the vial source, and their
342 interaction were the independent factors in this model. The significance of these effects was
343 determined using a likelihood-ratio chi-square test using the *Anova* function.
344

345 **RESULTS**

346 *Experiment 1: Test of effect of conspecific relatedness and developmental environment on egg-laying behavioural*
347 *decisions.*

348 In our first experiment, we compared the cumulative number of visits that *D. melanogaster*
349 focal females made to five different dishes, as well as the number of offspring that eclosed as
350 adults from each of those dishes. Our analysis found that those females exhibited heterogeneity in
351 their dish associations (GLM: LRχ^2 =17.8, df=4, p=0.001), and while they visited the
352 control/unexposed dishes less frequently than the demonstrator dishes, within these four
353 treatment groups, they did not express any significant bias (Figure S2). Offspring production
354 patterns indicated that the females' oviposition patterns were also heterogeneous (GLM: LRχ^2
355 =110.08, df=4, p=4.91x10^{-23}), with significantly more red-eyed focal offspring eclosing from
356 those dishes that had previously been in contact with a related demonstrator individual than from
357 either the control/unexposed dish or either of the dishes whose prior occupant was an unrelated
358 individual (Figure 1). Our focused two-way GLM confirmed that the amount of oviposition

359 activity on a dish was significantly associated with the demonstrator's kinship ($LR\chi^2$ =78.94,
360 df=1, p=6.40×10^{-19}), and there was no significant effect of developmental environment ($LR\chi^2$
361 =1.97, df=1, p=0.16), and the interaction between kinship and developmental environment was
362 also not significant ($LR\chi^2$ =0.176, df=1, p=0.67). We also noted that the number of
363 demonstrator offspring were not homogeneous across the four treatment groups, with more
364 brown-eyed offspring eclosing from vials whose prior occupant was an unrelated female (IV-*bw*,
365 mated to a IV-*bwD* male) than those that had previously been exposed to an unrelated female.
366
367 *Experiment 2: Test of effect of conspecific egg production on focal female egg-laying behavioural decisions.*
368 In our second experiment, we set out to test the alternate hypothesis that the patterns of
369 *red-eyed* offspring laid by our focal females was influenced by the abundance of *brown-eyed* eggs
370 already present on the dishes. In this assay either zero, one or two demonstrator females was
371 housed on a dish prior to being offered as a potential oviposition site for a mated focal female.
372 More demonstrators on a dish yielded more *brown-eyed* offspring: zero *brown-eyed* offspring eclosed
373 from the control dishes, and two demonstrator females produced, on average more *brown-eyed*
374 offspring than a single demonstrator female (GLM: $LR\chi^2$ =19.97, df=1, p=7.88×10^{-16}; Figure
375 2a). Despite the dramatic differences in the number of demonstrator eggs between the three
376 dishes, there was no significant difference in the number of *red-eyed* focal offspring that eclosed
377 from the three dishes (GLM: $LR\chi^2$ =3.69, df=2, p=0.157; Figure 2b), but there was a trend
378 towards *more* offspring eclosing from those dishes that had greater amounts of demonstrator
379 exposure/offspring.
380

381 *Experiment 3: Test of egg-laying biases of focal females towards cues from flies originating from the IV versus the*
382 *IV-bw populations.*

383 In our third experiment, we set out to test the alternate hypothesis that the patterns of *red-*
384 *eyed* offspring laid by our focal females was influenced by the population of origin of the
385 demonstrator females, and not their degree of kinship. This involved comparing the cumulative
386 number of visits that *D. melanogaster* focal females made to three different dishes (a
387 control/unexposed dish, a dish whose demonstrator was an unrelated IV female or a dish whose
388 demonstrator was an unrelated IV-*bw* female), as well as the number of offspring that eclosed as
389 adults from each of those dishes. Our analysis found that focal females exhibited heterogeneity in
390 their dish associations (GLM: LRχ^2 =92.27, df=2, p=9.17 x10^{-21}) with flies associating with the
391 demonstrator dishes more frequently than with the control/unexposed dishes; but no significant
392 difference in the mean number of visits to the two demonstrator dishes (Figure S3). The
393 distribution of focal female offspring was heterogenous across the three dish types (GLM: LRχ^2
394 =96.1, df=2, p=1.39 x10^{-21}). Focal females laid more eggs on the unrelated IV dish than they did
395 either on the control/unexposed dish, or the unrelated *IV-bw* dish. The mean number of *red-eyed*
396 offspring that were collected from the *IV-bw* dish was not significantly different from the number
397 collected from the control/unexposed dish (Figure 3b). We also noted that the number of
398 demonstrator offspring were significantly different from each other, with more brown-eyed
399 offspring eclosing from vials whose prior occupant was an unrelated IV female (mated to a IV-
400 *bwD* male) than those that that had previously been exposed to an unrelated IV-*bw* female (also
401 mated to a IV-*bwD* male) (GLM: LRχ^2 =366.14, df=2, p=3.11 x10^{-80}; Figure 3a).
402

403 *Experiment 4: Revisiting the effect of conspecific relatedness on egg-laying behavioural decisions.*

404 In our fourth experiment, we set out (again) to compare the patterns of dish visitation and
405 oviposition by focal females on three different dishes (a control/unexposed dish, a dish whose
406 demonstrator was an unrelated IV female, or a dish whose demonstrator was an unrelated IV
407 female). The focal females exhibited bias on their dish visitation rates (GLM: $LR\chi^2 = 12.86$, df=2,
408 p=0.0016), as they were more frequent visitors to the two types of demonstrator dishes than the
409 control/unexposed dishes (but did not differ significantly between these two treatment types;
410 Figure S4). The distribution of focal female offspring was heterogenous across the three dish
411 types (GLM: $LR\chi^2 = 180.24$, df=2, $p=7.26\times10^{-40}$). Focal females laid significantly more eggs on
412 the related IV dish than they on the unrelated IV dish, and both demonstrator dishes yielded
413 more focal female offspring than did the control/unexposed dishes (Figure 4). There was no
414 significant difference in the number of demonstrator offspring eclosing from the related IV dishes
415 than from the unrelated IV dishes (GLM: $LR\chi^2 = 2.59$, df=1, p=0.11; Figure S5).
416

417 **DISCUSSION**

418 The decision where to oviposit may be very important, especially in these species where
419 there is no post-laying parental care (Prasad et al. 2003, Klepsatel et al. 2020). Fruit flies,
420 *Drosophila melanogaster*, like many other species, use social information in their decision-making
421 (Leadbeater 2009, Chen and Sokolowski 2022). While there are benefits of laying one's eggs in
422 proximity to those of others, there are also risks – which might be mediated by the degree of
423 kinship between conspecifics. In this study, we set out to examine the possibility that female flies
424 might selectively use cues produced by close relatives, presumably to increase their offspring's
425 chance of success. In doing so, we simultaneously explored the factors that might influence how
426 flies might infer kinship, as well as investigate alternate hypotheses about social information

427 usage. Our assays confirm the extensive use of social cues by *D. melanogaster* females, and
428 furthermore collectively suggest that they are capable to kinship discrimination and are capable
429 of distinguishing between conspecifics from different populations. These results add to the
430 growing body of knowledge about the sophisticated social information use by this model species
431 (Chen and Sokolowski 2022).

432 In all assays focal females associated more with those substrates that had come into
433 contact with conspecifics than with the unexposed/control dishes (Figures S2, S3 & S4), clearly
434 illustrating the importance of social cues in their decision-making, and consistent with numerous
435 previous studies (*e.g.* Battesti *et al.* 2012, Duménil et al. 2016, Malek and Long 2020). However,
436 not all conspecific cues elicited the same responses. In the first assay, we compared the egg-laying
437 patterns of focal females on dishes exposed to a range of different demonstrators. By splitting sets
438 of full sibling eggs between two different vials to develop, and collecting both adult relatives and
439 non-relatives from each of the pairs of vials, we set out to test if and how *D. melanogaster* females
440 might distinguish kin cues. The focal females laid significantly more eggs on those dishes where
441 the demonstrator female was a sister, but did not appear to differentiate between the cues of
442 demonstrator sisters originating from the same or a different vial environment (Figure 1a). These
443 results suggest not only that ovipositing females selectively use social information, and favour cues
444 produced by their relatives, but furthermore that kinship cues were not adopted by non-kin
445 demonstrators developing in the same vial, nor disrupted in those kin developing in a different
446 vial. Determining the specific cues being used by focal females is beyond the scope of this study as
447 demonstrator females transfer their gut bacteria, CHCs (and male-specific compounds acquired
448 during copulation) to the surface of their eggs and the media (Bakula 1969, Guilhot et al. 2023,
449 Moreira-Soto et al. 2024). The expression these olfactory cues can be influenced by both an
450 individual's genotype and/or their developmental environment (Sharon et al. 2010, Fedina et al.

451 2012, Lizé et al. 2013, Venu et al. 2014, Heys et al. 2018, García-Roa et al. 2022, Pavković-
452 Lučić et al. 2022). While the absence of significant differences associated with the developmental
453 environment (Figure 1b), could suggest that "familiarity" (and by extension the microbiotic cues
454 that could be used as proxies of kinship) are not as important as "relatedness", it should be noted
455 that in our experimental design, the conditions of the two vial environments were similar (same
456 media, same larval density), which may have minimized the potential for the development of
457 differences in these cues. Interestingly, Malek and Long (2020) observed that when focal females
458 developed in a protein-rich vial environment, they oviposited more on dishes where the
459 demonstrator from the same population had also developed on similar media, but when the focal
460 females developed on standard media, they laid more on those dishes where the demonstrator
461 came from a different *D. melanogaster* population but had also developed on standard media than
462 they did on dishes where the demonstrator was from the same population but had developed on
463 high-protein media. Thus, one should not necessarily conclude that our results indicate that
464 CHCs are the only way in which egg kinship was/can be recognized by the focal females in this
465 species, and future studies exploring this idea across a range of divergent environments are likely
466 to expand our understanding of how social information is produced and interpreted in *D.*
467 *melanogaster*.

468 While the observed pattern of focal female oviposition is consistent with kin-associated
469 biases, other processes could also have resulted in the differential egg-laying outcomes, and so in
470 order to validate or rule-out these alternate hypotheses we conducted several complementary
471 assays. We had been initially struck with the distinctive pattern of offspring laid by the
472 demonstrator females (Figure 1b), wherein there were more *brown-eyed* conspecific offspring
473 collected from dishes where the demonstrator was unrelated than when the demonstrator was
474 related. Perhaps our focal females weren't deciding to lay their eggs on dishes that contained

475 their kin's offspring, but instead were avoiding dishes where there were already lots of eggs (and a
476 heightened risk of competition and/or cannibalism)? We explored this possibility in our second
477 assay, where focal females could choose between dishes with dramatically different number of
478 eggs (Figure 2a). If females were avoiding dishes where there were too many conspecific eggs
479 already present, we would expect to see more eggs laid on the "one female" treatment than on
480 the "two females" treatment. In fact, we observed no significant difference in focal female
481 oviposition bias between the two groups (and if anything, they often laid *more* eggs on the latter
482 group's dishes; Figure 2b). Previous work by Golden and Dukas (2014) found that female flies
483 given a choice between a control and a demonstrator dish (which had been exposed to 5 or 20
484 larvae), did not appear to discriminate against the demonstrator dishes that possessed cues
485 indicating greater competition during offspring development. Thus, we are fairly confident that
486 the differences in focal female egg-laying patterns observed in experiment one were not due to
487 differences in the quantity of demonstrator cues present on a dish. As a side note, we suggest that
488 the differences in the number of demonstrator offspring observed in that assay is a side effect of
489 the way in which our demonstrator females were collected (*see* Figure S6 *caption for details*).
490 The second alternate hypothesis we explored was that the biased pattern of oviposition
491 made by the focal females in the first experiment on the two "related" dishes, was not due to the
492 perceived kinship of the demonstrators, but instead was because those demonstrators originated
493 from the same population (IV), and the demonstrators for the two "unrelated" dishes were from a
494 different population (IV-*bw*). While IV and IV-*bw* are maintained following the same protocols,
495 and IV-*bw* is periodically back-crossed to IV, perceptible differences in the cues present in these
496 isolated populations (or their microbiotic communities) may have arisen via drift and/or
497 selection. Thus in our third experiment, we compared egg-laying behaviour for focal females
498 exposed to (unrelated) IV or IV-*bw* demonstrator females. We found that focal females laid more

499 eggs on the former dishes than they did on the latter (and virtually none on the
500 control/unexposed dishes). This suggests that at least some of the patterns of oviposition seen in
501 our first experiment may not necessarily be the result of recognition of kinship *per se*, but instead
502 with cue(s) that differ between the IV and IV-*bw* populations. Thus, based on the insights gained
503 in our third assay, we set out to re-examine the social information use of *D. melanogaster* females in
504 our fourth assay where females were given a choice of oviposition sites that either had no prior
505 exposure (control), a demonstrator sister (raised in a different vial), or an unrelated demonstrator
506 female (also from the IV population and raised in a different vial). We observed that focal females
507 still biased their egg-laying towards the dishes that had previously been in contact with their
508 sisters, albeit to a lesser extent than seen in experiment 1. This provides additional support to the
509 hypothesis that female *D. melanogaster* are both capable of distinguishing between cues produced
510 by kin and non-kin, and that these cues are used in their social decision-making. In doing so, they
511 may be trying to obtain the potential benefits of developing in a group (Gilbert 1980, Gregg, et
512 al. 1990, Sakaguchi and Suzuki 2013, Wertheim et al. 2002a, Wertheim et al. 2002b,
513 Dombrovski et al. 2017, Shoot et al. 2024), while reducing the potential risks from conspecifics
514 (Khodaei and Long 2019, Khodaei and Long 2020, Fisher et al. 2021).

515 Collectively, our results illustrate the subtle and plastic nature of social information use by
516 female *D. melanogaster*: Given a choice, a mated female *D. melanogaster* will lay more eggs on a dish
517 that has previously been in contact with a sister than one exposed to a mated female from the
518 same population; will lay more eggs on a dish if it has been previously exposed to an unrelated
519 mated female from the same population than one exposed to a mated female from a different
520 population; and will lay more eggs on a dish where the previous occupant was a mated female
521 from the same population (and raised in a similar environment) than on a previously unoccupied
522 dish (i.e. the unexposed/control dish). At the same time, a mated female *D. melanogaster* does not

523 appear to show any bias in their egg laying towards dishes that differ in the number of eggs (and
524 their associated biochemical cues) present, nor do they show any bias between dishes that had
525 previously been occupied by a sister raised in the same environment or a different vial. Together,
526 these pattens illuminate that *D. melanogaster* females are sensitive to differences in the cues
527 produced by conspecifics and add to the growing appreciation of social decision-making in this
528 model species.

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734 **ACKNOWLEDGEMENTS**

735 We would like to thank Simran Mann, Lukas Ghiglione, and Harleen Taneja of the #DrosLife
736 Lab for their fly-pushing, behavioral observations and camaraderie. Natasha B. Gallo provided
737 helpful feed-back and constructive comments. Ruby Lindsay and Michael Steeleworthy of the
738 Wilfrid Laurier University Library are thanked for their help with data archiving. T.A.F.L. was
739 funded with a Natural Sciences and Engineering Research Council Discovery grants (RGPIN-
740 2016-06133 and RGPIN-2022-03988). This work was conducted at Wilfrid Laurier University,
741 which exists on the traditional territory of the Neutral, Anishnawbe, and Haudenosaunee
742 peoples.

743 **FIGURES**

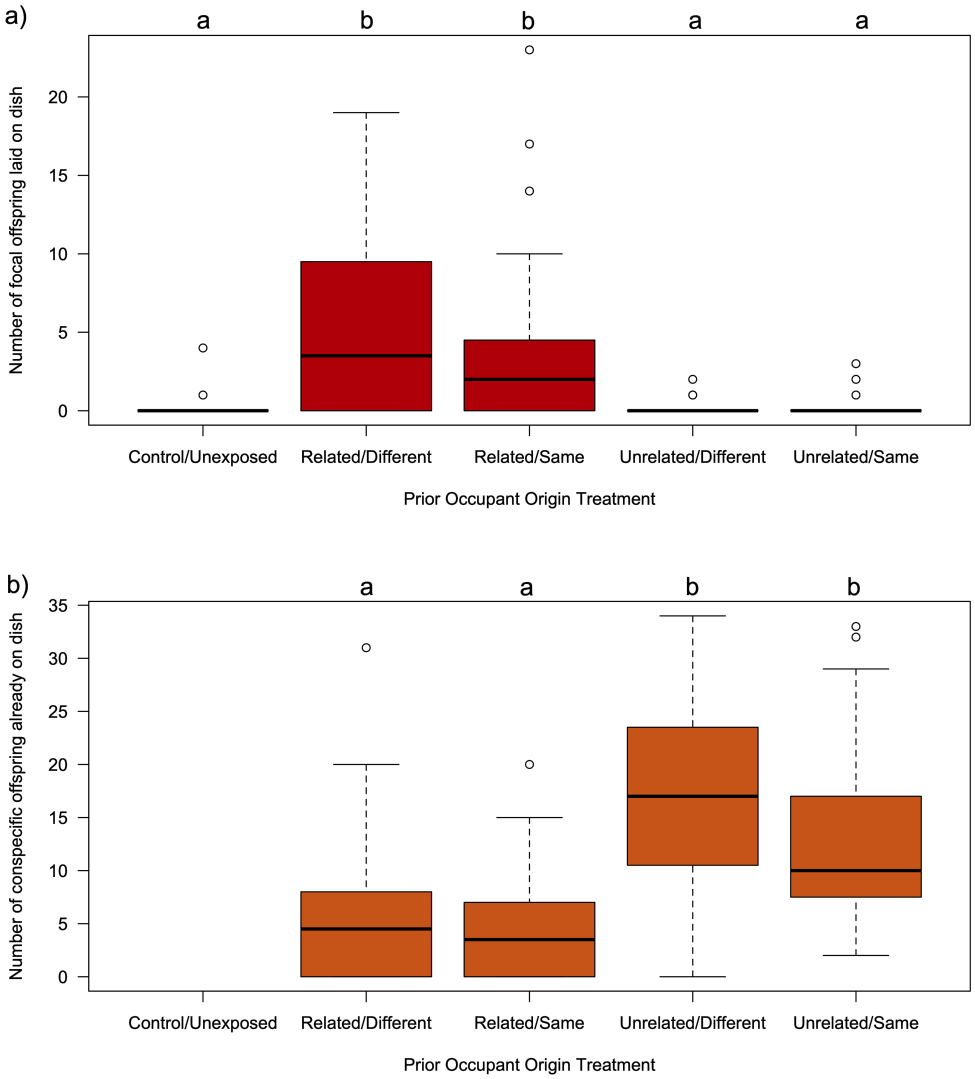

Figure 1. Boxplots illustrating data collected in the first experiment of a) the total number of wild-type (*red-eyed*) adult flies that eclosed from eggs laid by the focal *D. melanogaster* females on 5 different media dishes, b) the total number of *brown-eyed* adult flies that eclosed from those same 5 media dishes, which had been laid by the prior conspecific occupants on the dish. The boxes enclose the middle 50% of data (the inter-quartile range, IQR), with the thick horizontal line representing the location of median. Data points > ±1.5*IQR are designated as outliers. Whiskers extend to largest/smallest values that are not outliers, indicated as closed circles. The results of a Tukey HSD post-hoc tests comparing group mean is indicated by letters, where groups that do not share the same letter are considered statistically different at the $\alpha=0.05$ level. The control/unexposed dishes by definition had not had any prior exposure to a female capable of producing *brown-eyed* offspring, and thus those dishes never yielded any offspring with that phenotype.

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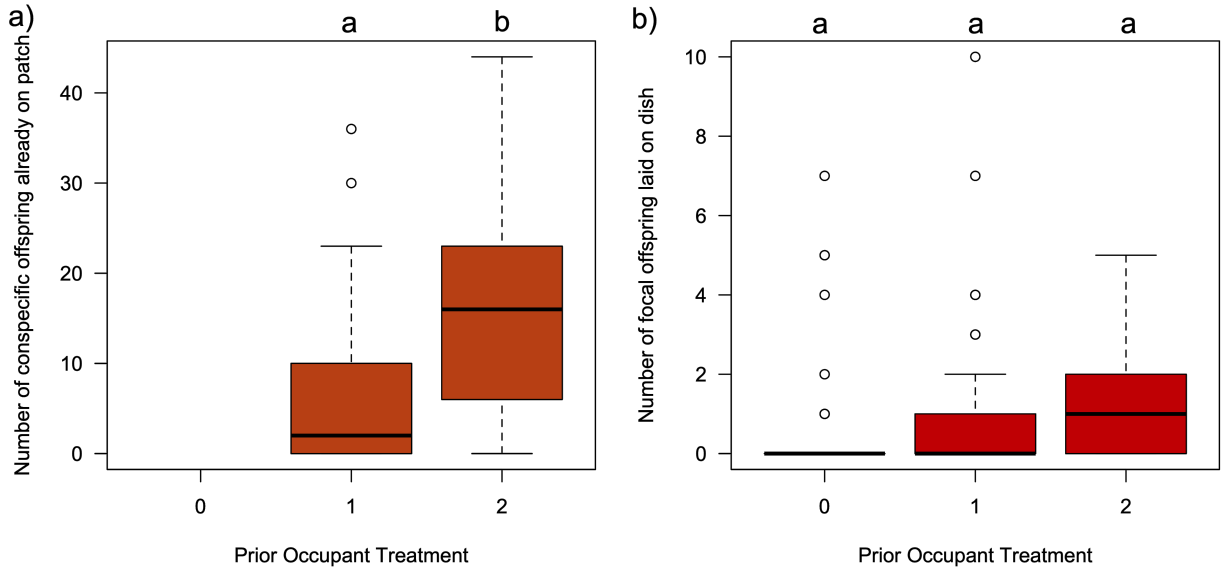

745 **Figure 2.** Boxplots illustrating data collected in the second experiment of a) the number of
746 *brown-eyed* adult flies that eclosed from dishes that had been exposed to 0, 1 or 2 conspecific
747 females, b) the number of *wild-type* (red-eyed) adult flies that eclosed from the same dishes that
748 had been laid by the focal female *D. melanogaster.* Boxplot components as in Figure 1. The results
749 of a Tukey HSD post-hoc test comparing group mean is indicated by letters, where groups that
750 do not share the same letter are considered statistically different at the α=0.05 level. The
751 control/unexposed dishes by definition had not had any prior exposure to a female capable of
752 producing *brown-eyed* offspring, and thus those dishes never yielded any offspring with that
753 phenotype
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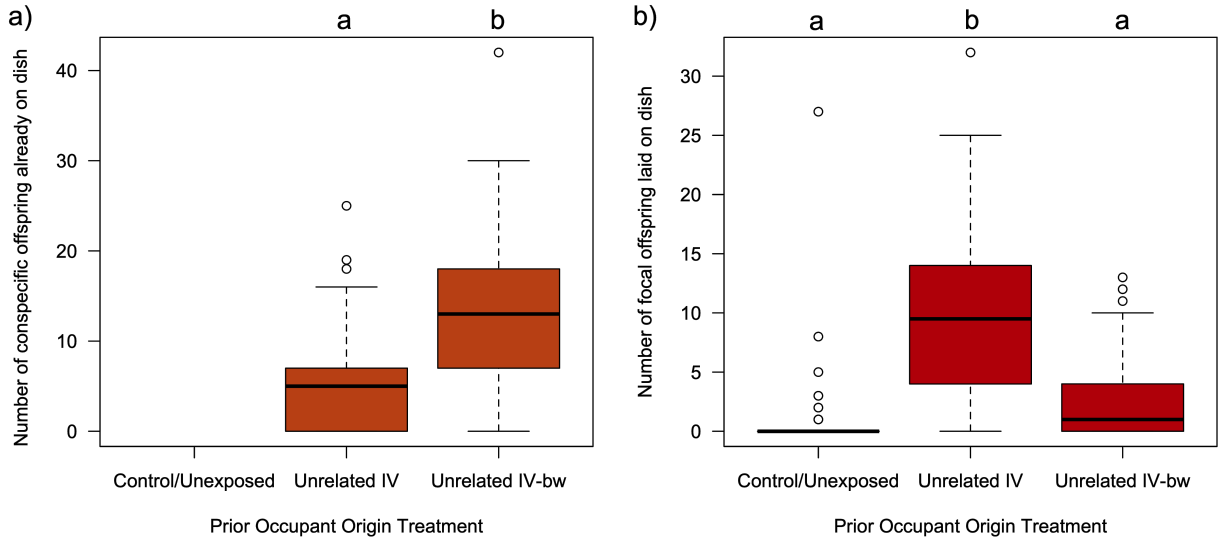

Figure 3. Boxplots illustrating data collected in third experiment of a) the number of *brown-eyed* adult flies that eclosed from dishes that had been exposed to either an unrelated IV female (mated to a IV-*bwD* male), a unrelated IV-*bw* female (mated to a IV-*bwD* male), or neither b) the number of *wild-type* (red-eyed) adult flies that eclosed from the same dishes that had been laid by the focal female *D. melanogaster.* Boxplot components as in Figure 1. The control/unexposed dishes by definition had not had any prior exposure to a female capable of producing *brown-eyed* offspring, and thus those dishes never yielded any offspring with that phenotype. The results of a Tukey HSD post-hoc test comparing group mean is indicated by letters, where groups that do not share the same letter are considered statistically different at the α=0.05 level.

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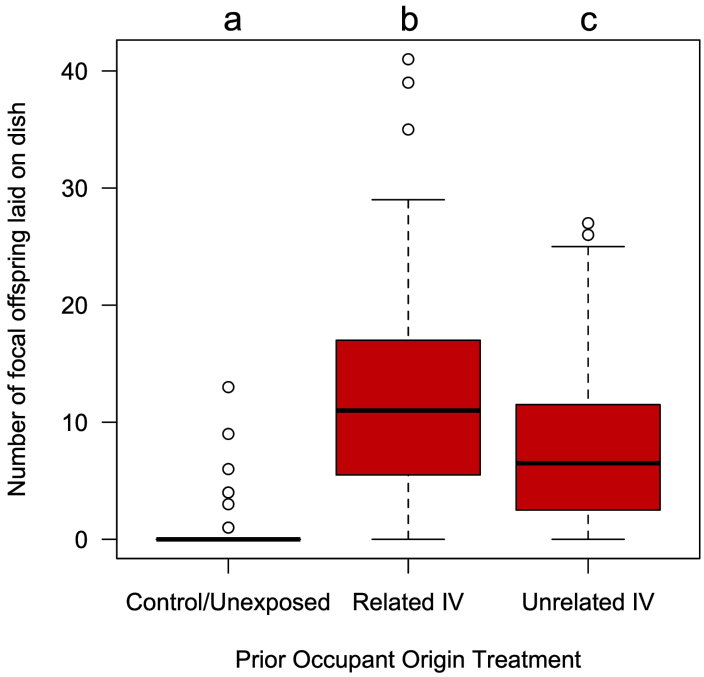

Figure 4. Boxplots illustrating data collected in the fourth experiment of the number of *wild-type* (red-eyed) adult flies that had been laid by the focal female *D. melanogaster* that eclosed from dishes that had been exposed to either a related IV female (mated to a IV-*bwD* male), an unrelated IV female (mated to a IV-*bwD* male), or neither. Boxplot components as in Figure 1. The results of a Tukey HSD post-hoc test comparing group mean is indicated by letters, where groups that do not share the same letter are considered statistically different at the $\alpha=0.05$ level.

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760 **SUPPLEMENTARY FIGURES**

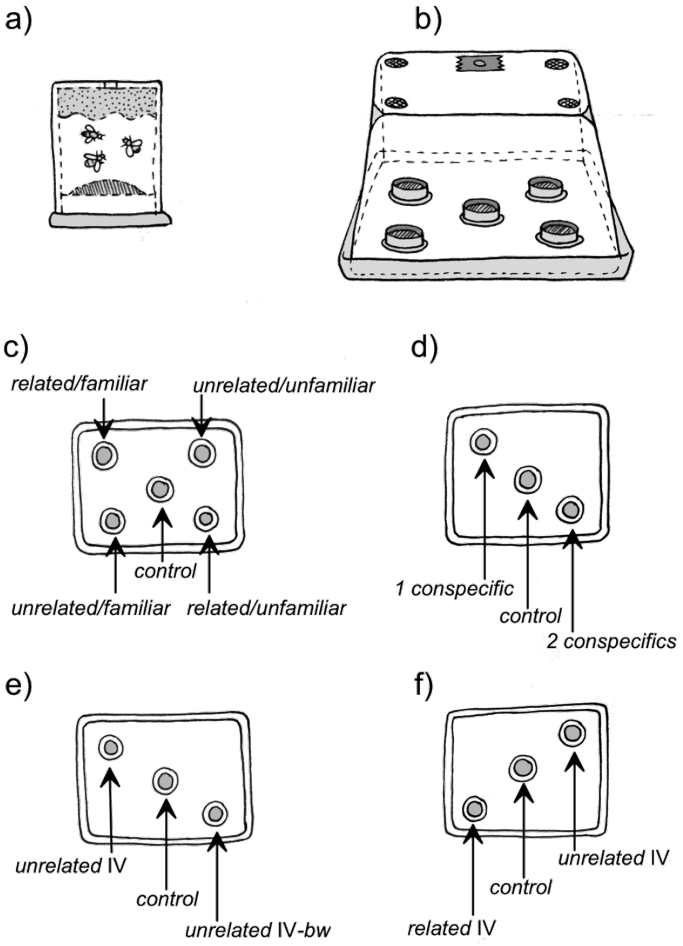

Figure S1. Drawings illustrating **a**) the "mini-egg" chambers used to collect eggs and/or create media that exhibited social cues. **b**) an observation arena into which the focal ovipositing female was introduced and monitored. At the bottom of the chamber the lids that exhibited different kinds of social information were affixed. **c**) the arrangement of dishes in the first experiment. In this experiment there were four lids that exhibited social information and the 'control/unexposed' dish that contained no social information (as it had no prior exposure to flies). **d**) the arrangement of the two dishes with social information and the control dish in the second experiment. **e**) the arrangement of the two dishes with social information and the control dish in the third experiment. **f**) the arrangement of the two dishes with social information and the control dish in the fourth experiment.

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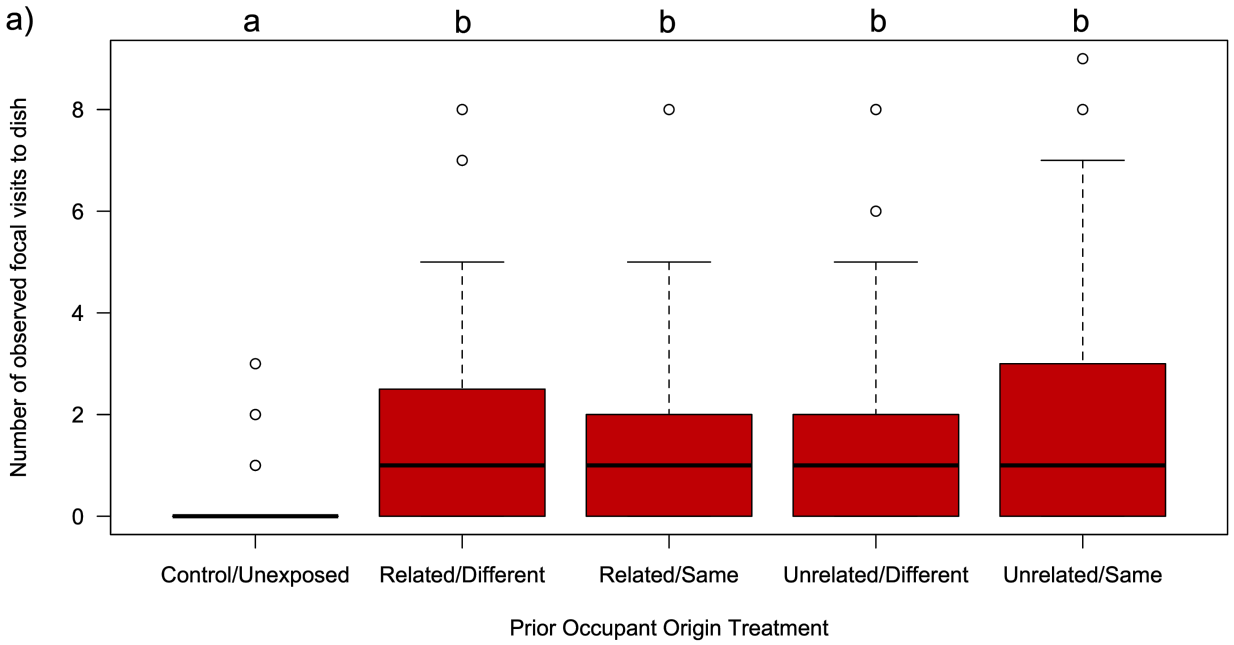

Figure S2. Boxplots illustrating the cumulative number of observations made across 26 sessions in the first experiment in which a focal female *D. melanogaster* was observed on the surface of one of 5 different media dishes present in the arena in the first experiment. The boxes enclose the middle 50% of data (the inter-quartile range, IQR), with the thick horizontal line representing the location of median. Data points > ±1.5*IQR are designated as outliers. Whiskers extend to largest/smallest values that are not outliers, indicated as closed circles. The results of a Tukey HSD post-hoc test comparing group mean is indicated by letters, where groups that do not share the same letter are considered statistically different at the $\alpha=0.05$ level.

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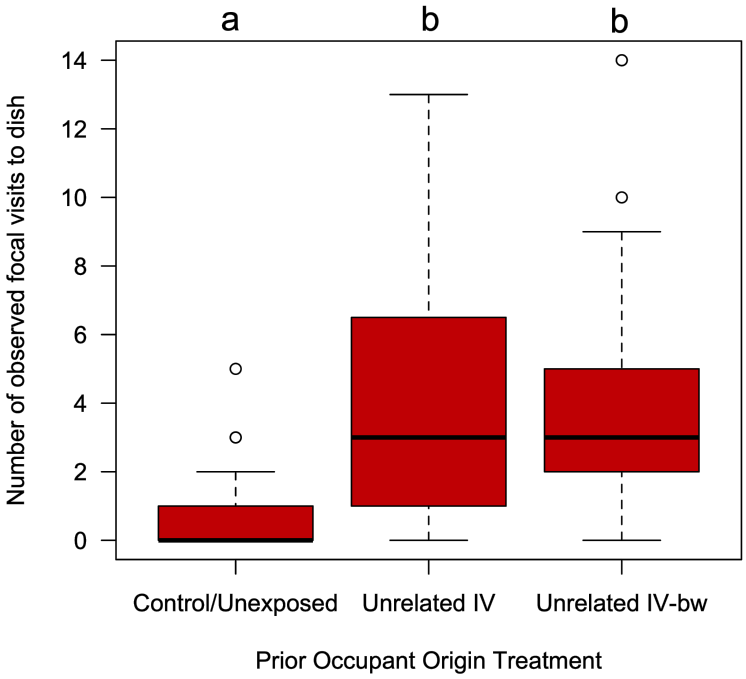

Figure S3. Boxplots illustrating the cumulative number of observations made across 26 sessions in the third experiment in which a focal female *D. melanogaster* was observed on the surface of one of 3 different media dishes present in the arena that had previously been exposed to either an unrelated IV female (mated to a IV-*bwD* male), an unrelated IV-*bw* female (mated to a IV-*bwD* male), or neither. Boxplot components as in Figure S2. The results of a Tukey HSD post-hoc test comparing group mean is indicated by letters, where groups that do not share the same letter are considered statistically different at the $\alpha=0.05$ level.

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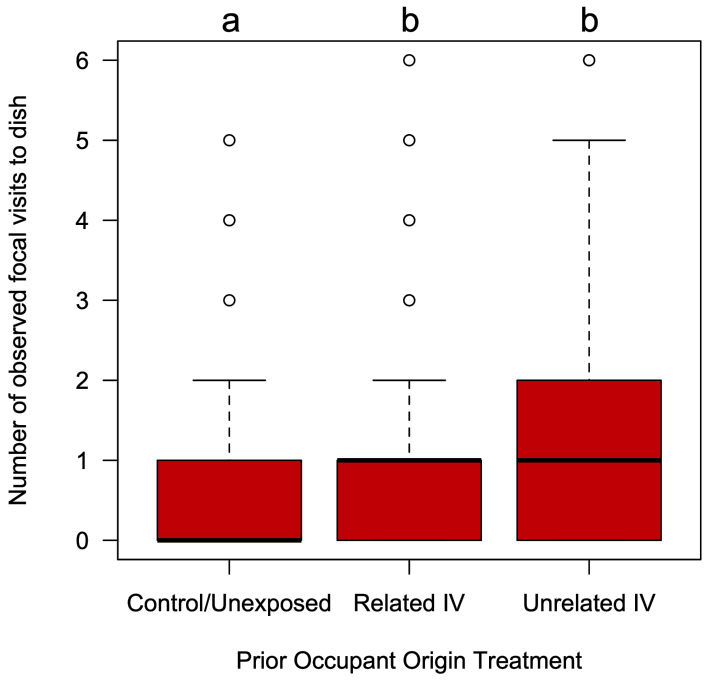

Figure S4. Boxplots illustrating the cumulative number of observations made across 26 sessions in the fourth experiment in which a focal female *D. melanogaster* was observed on the surface of one of 3 different media dishes present in the arena that had previously been exposed to either a related IV female (mated to a IV-*bwD* male), an unrelated IV female (mated to a IV-*bwD* male), or neither. Boxplot components as in Figure S2. The results of a Tukey HSD post-hoc test comparing group mean is indicated by letters, where groups that do not share the same letter are considered statistically different at the α=0.05 level.

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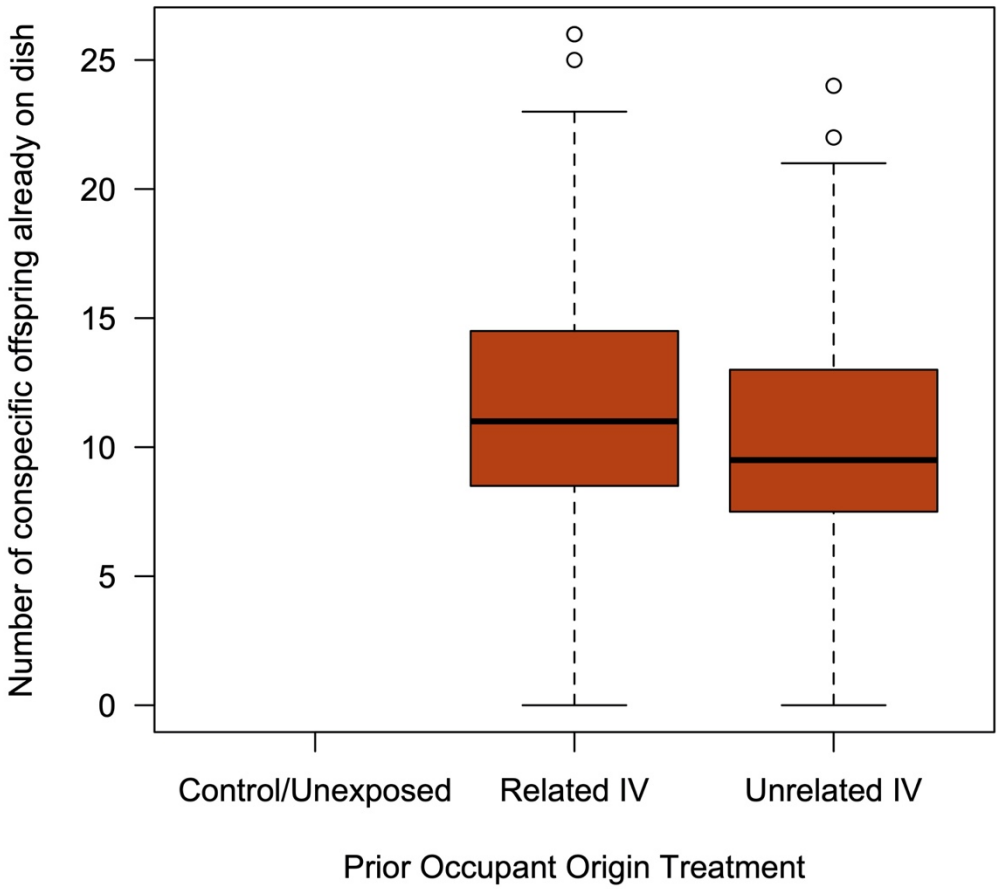

Figure S5. Boxplots illustrating the number of *brown-eyed* adult flies that eclosed from dishes that had been exposed to either an related IV female (mated to a IV-*bwD* male), a unrelated IV female (mated to a IV-*bwD* male), or neither in the fourth experiment. Boxplot components as in Figure S2. The control/unexposed dishes my definition had not had any prior exposure to a female capable of producing *brown-eyed* offspring, and thus those dishes never yielded any offspring with that phenotype.

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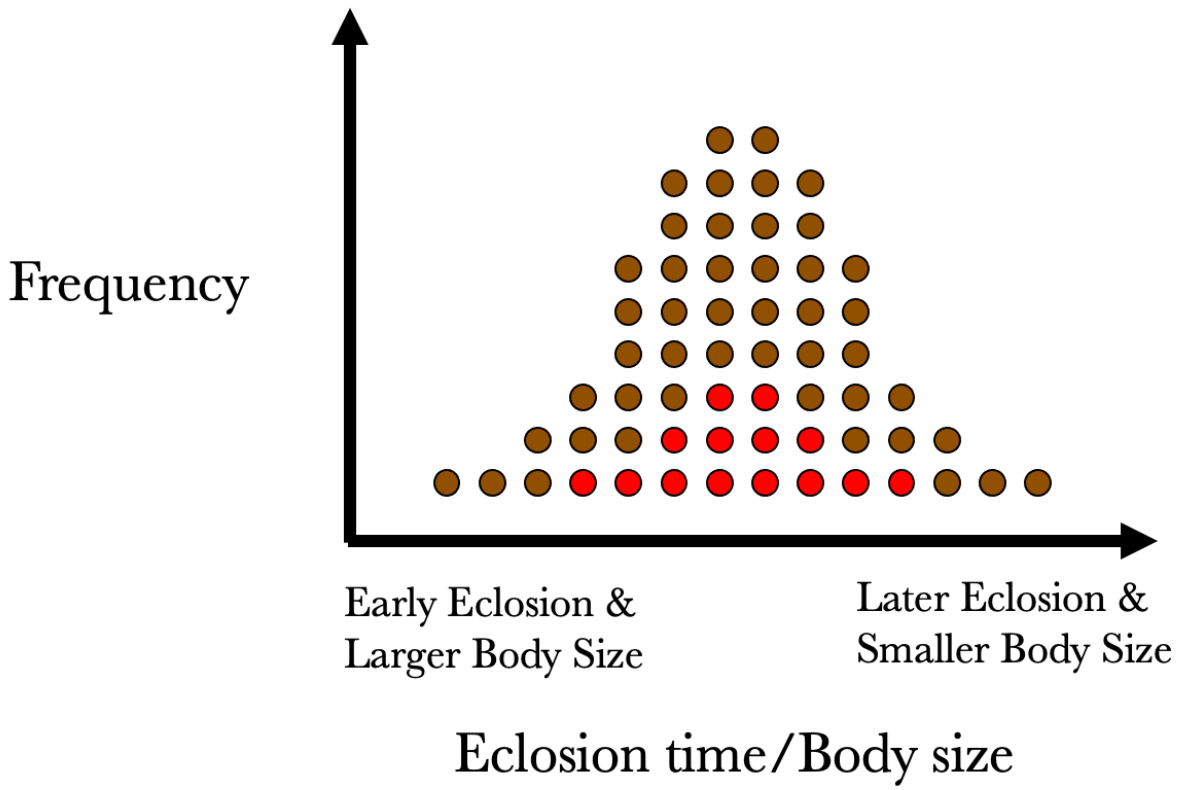

Figure S6. Schematic diagram depicting hypothetical distribution of female *D. melanogaster* eclosion times in a mixture of eggs collected at the same time from the IV and the IV-*bw* populations (where we assume are randomly sampled, and that both populations have identical distributions, means and variances). While both subset means are the same, as there is a greater number of IV-*bw* eggs in the vial than IV eggs, the larger sample size of the former will likely contain individuals exhibiting a wider range of phenotypes than the latter. The unanticipated consequence of this being that when sampling for virgin female flies, there is a greater probability of colleting a suitable IV-*bw* before encountering a suitable IV female. As body size is negatively correlated with eclosion time (Partridge et al. 1987), and positively correlated with female fecundity (Lefranc and Bundgaard 2000, Long et al. 2009), it is conceivable that the IV-*bw* and the IV females collected and used as demonstrators differed in their capacity to produce offspring, resulting in the conspecific patterns seen in Figure 1b and 3b. It is worth noting that in our fourth experiment, where only *red-eyed* females were collected from vials containing both IV and IV-*bw* eggs that there was no significant difference in the number of demonstrator offspring that were collected (Figure S5), which is consistent with the hypothesis described above.

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