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

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Abstract

Decisions where an individual lays their eggs are important, as the choice may affect their offspring's survival and lifetime reproductive success. Information produced by conspecifics can potentially be useful in decision-making as this "social information" may provide an energetically cheaper means of assessing oviposition site suitability rather than acquiring it personally. However, as not all public information may be equally beneficial, cues produced by kin may be especially valuable as they might signal suitable microenvironments, and are associated with other fitness advantages resulting from improved foraging success and/or a decreased risk of competition/cannibalism 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 environmentally-associated proxy cues, so we explored whether there were biases in how focal females interacted with cues from conspecifics that differed in both genetic relatedness, and environmental "familiarity." In a series of inter-connected assays, we examined the behaviour of focal females that interacted with a choice of potential egg-laying substrates that differed in the manner of their prior conspecific exposure, and counted the offspring that eclosed from these different substrates. Sites that had exhibited cues produced by conspecific demonstrators were visited more, and yielded more focal offspring compared to unexposed substrates. Furthermore, patterns of bias in offspring production were consistent with ovipositing females exhibiting sensitivity to the kinship status of the prior substrate's occupants. The basis of the kinship categorization by ovipositing females appears to be based on phenotypes that reflect true genetic relatedness, but the nature of the social information can be affected by other factors. These results further 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

Introduction

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

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

67 than unexposed females, presumably because they are better able to anticipate the forthcoming
68 availability of oviposition sites and the potential degree of future larval competition (Fowler et al.
69 2022). Mate choice can involve social information use as female fruit flies are more likely to mate
70 with males exhibiting similar characteristics as a male they've observed successfully mating with
71 another female (Mery et al. 2009, Monier et al. 2018, Nöbel et al. 2018, Nöbel et al. 2022).
72 While the robustness of this phenomenon is debatable (Belkina et al. 2021) it may still be
73 advantageous because mate choice is often costly to females (Reynolds and Gross 1990), and
74 copying the decisions made by conspecifics represents an economical adaptive strategy (Monier
75 et al. 2018). Important information about environmental conditions can also be obtained
76 indirectly by flies interacting with conspecifics. In a study by Kacsoh et al. (2015) female flies
77 decreased their oviposition rate in response to exposure to parasitic wasps, and this defensive
78 change in egg-laying behaviour was adopted by inexperienced flies housed with the wasp-
79 exposed females, with cues about environmental risk communicated visually using the
80 demonstrator flies' wings. Thus, while *D. melanogaster* has not been considered a "social" species
81 (Chen and Sokolowski 2022), it is evident that fruit flies can, and do, use social information to
82 inform their decisions.

83 One important context where social information seems to be used extensively by *D.*
84 *melanogaster* females is in selecting suitable oviposition sites (Battesti *et al.* 2012, Durisko et al. 2014,
85 Sarin and Dukas, 2009, Golden and Dukas 2014, Malek and Long 2020, Moreira-Soto et al.
86 2024). In the wild, there may be considerable heterogeneity in the availability and quality of
87 oviposition sites that are available to female *D. melanogaster* (Reaume and Sokolowki 2006,
88 Markow 2015), and the choice of oviposition environment can have profound consequences for
89 their offspring's fitness (Prasad et al. 2003, Klepsatel et al. 2020). *D. melanogaster* females may gain
90 insight on the suitability of oviposition sites through direct observation of other females

91 ovipositing (Sarin and Dukas, 2009 and Battesti *et al.* 2012), the presence of larvae (Golden and
92 Dukas, 2014) and/or the presence of chemical cues produced by conspecifics (Venu et al. 2014,
93 Lin et al. 2015, Duménil et al. 2016, Moreira-Soto et al. 2024). The reliance upon social cues by
94 ovipositing *D. melanogaster* is not without potential pitfalls. First, by outsourcing one's information
95 gathering to others, there is the risk of mimicking other's bad decisions. For instance, in a study
96 by Golden and Dukas (2014) when female flies were given a choice of laying their eggs on a site
97 where nutrition was poor but contained social cues (the presence of larvae) or a site where the
98 media contained 3 times more nutrition but lacked conspecific cues, the ovipositing females
99 treated them as being of equal quality, even though any offspring laid on the former patches did
100 not have access to sufficient nutrients to survive. Similarly, Duménil et al. (2016) observed that
101 the presence of a mated female demonstrator on yeast-free media made this undesirable
102 oviposition site equally-valued as yeasted media (that has not been exposed to conspecifics) to
103 later ovipositing females. In *D. melanogaster* the widespread use of social information may arise
104 because there can be significant benefits of laying one's eggs at sites already chosen by
105 conspecifics as offspring growth and development may be enhanced through communal digestion
106 via secreted enzymes (Gregg, et al. 1990, Sakaguchi and Suzuki 2013), through increased local
107 beneficial microbial activity (Gilbert 1980, Wertheim et al. 2002a, Wertheim et al. 2002b),
108 and/or via improved larval group foraging (Dombrovski et al. 2017, Khodaei et al. 2020, Shoot
109 et al. 2024). However, laying one's eggs near those of conspecifics can also pose risks arising from
110 the competition for limited resources (De Jong 1976, Joshi et al. 1996, Golden and Dukas 2014),
111 increased exposure to toxic waste products (Borash et al. 1998), and the threat of cannibalism at
112 many developmental stages (Vijendravarma et al. 2013, Ahmed et al. 2015, Yang 2018).
113 Crucially, some of these dangers may potentially be mediated if females are able to oviposit at
114 sites containing kin, as *D. melanogaster* larvae are more co-operative (Khodaei and Long 2019),

115 and less cannibalistic (Khodaei and Long 2020, Fisher et al. 2021) towards close relatives,
116 compared to how they engage with unrelated conspecifics. Additionally, as there can be extensive
117 intra-specific genetic variation *D. melanogaster* for nutritional preferences and dietary tolerances
118 (Toshima et al. 2014, Havula et al. 2022), preferentially laying eggs near those of close relatives
119 may increase the likelihood of selecting the best micro-habitat for the success of one's own
120 offspring. Thus, if females were able to differentiate social information produced by kin from
121 those generated by other demonstrators, they might be able to achieve greater fitness benefits
122 (and avoiding some of the risks) by selectively copying the choices made by relatives.

123 When considering the possibility that individuals might differentially use social cues
124 produced by relatives versus non-relatives, one must also consider what (if any) cues could
125 indicate kinship (or could be inferred to be reliable proxies of kinship). In *D. melanogaster* such
126 information could conceivably be perceived from cuticular hydrocarbons (CHCs) and/or gut
127 bacteria left behind by conspecifics. CHCs are long-chain hydrocarbons that are synthesized in
128 insect oenocyte cells and become part of the cuticle's waxy coating, where they help prevent
129 water loss, as well as also playing an important role in olfactory communication related to species
130 recognition and mate choice decisions (Singer 1998, Ferveur 2005, Scott et al. 2011, Richard
131 and Hunt 2013, Chung and Caroll 2015, Moreira-Soto et al. 2024). CHCs may provide accurate
132 information about kinship as there exists a substantial genetic variation underlying these
133 phenotypic traits within *D. melanogaster* populations (Foley et al. 2007, Dembeck et al. 2015, Heys
134 et al. 2018). Recent work has revealed that female flies transfer their own CHCs (along with
135 male-specific compounds acquired during mating) to their laid eggs (Moreira-Soto et al. 2024),
136 which could conceivably be used as signals of potential kinship by subsequent ovipositing females.

137 In addition to CHC cues, olfaction-based decisions may be influenced by signals
138 associated with differences in an individual's bacterial community (Sharon et al. 2010, Najarro et

al. 2015). While the *D. melanogaster* microbiome is relatively simple compared to other hosts (Broderick and Lemaitre 2012), substantial variation exists between individuals/families from the same population in their microbial community composition (Wang and Staubach 2018, Heys et al. 2018). Individuals acquire some of their microbial symbionts from their mothers, who transfer bacteria to the surface of their eggs, and their larvae's subsequent consumption of their chorion establishes their offspring's own gut flora (Bakula 1969, Guilhot et al. 2023). Thus, differences in cues produced by bacterial communities could conceivably (and independently) be used to infer kinship (Lizé et al. 2014, Lewis et al. 2014). An important and potentially complicating factor is that CHC phenotypes and/or microbiotic communities in *D. melanogaster* can also be influenced by the flies' developmental environment (Sharon et al. 2010, Fedina et al. 2012, Lizé et al. 2014, Venu et al. 2014, Heys et al. 2018, García-Roa et al. 2022, Pavković-Lučić et al. 2022). This raises the possibility that the phenomenon of "kin recognition" may be based on inherited cues of relatedness, on the recognition of similarity of environmental histories ("familiarity"), or some combination of these two factors, and that furthermore under some circumstances that these cues might not provide contradictory or unreliable information about kinship. In a previous study that examined whether kinship mediated the magnitude and consequences of male-male competition in adult flies, it was found that reductions in male-induced harm were *only* seen in groups of males that were both closely related to each other *and* 'familiar' by having developed in the same larval environment (Le Page et al. 2017). Lizé et al. (2014) described how females were more likely to mate with a familiar male, while males' ability to strategically avoid investing into mates with a sibling was impeded if she had developed in the same environment. Similarly, Heys et al. (2018) noted that the ability of fruit flies of both sexes to perceive the kinship status of their mate appeared to be dependent on the state of the mate's microbiome – with females only acting differently towards kin when those individuals had developed in the same media type (and had an

intact microbiota), while a male's increased investment into mating with an unrelated female only occurred if she had been grown in a different environment (or whose microbiota had been disrupted with the use of antibiotics). The complicated nature of the interaction between developmental environment and relatedness was also explored by García-Roa et al. (2022) who found that flies developing in different environments exhibited both different CHC and microbial profiles, that related flies had microbiotic communities of similar diversity (but not composition), and that diversity was correlated with CHC phenotypic profiles. Together these studies highlight that socially-available kinship cues (or proxies thereof) may involve a number of independent and inter-related olfactory signals, whose expression may depend not only on relatedness but also on the specific nature of the developmental environment. As such, any study – such as ours – that explores the potential role of kin recognition in decision making, must explore how individuals respond to cues produced by demonstrators in which relatedness and familiarity are independently manipulated.

With these ideas and findings in mind, in this study we set out to examine whether female flies showed bias in their use of social information cues left behind by conspecifics when selecting ovipositing sites, and would differ their use of egg-laying substrates that had previously been in contact with either kin or non-kin demonstrator females. Simultaneously, we also explored whether a female's response to potential kinship cues were conditional on the conspecifics' "familiarity", by concurrently manipulating the developmental environment from which the demonstrator females were obtained. Overall, the goal of this research is to better characterize copying behavior in *D. melanogaster* and to gain better insight into the factors that influence these oviposition site decisions.

186 # Materials and Methods

187 ## Population Protocols and Fly Maintenance

188 All fruit flies used in our assays were obtained from one of three associated laboratory *D.*
189 *melanogaster* populations: *Ives* (hereafter "IV"), "IV-*bw*", and "IV-*bwD*". The IV population was
190 derived from a sample of mated females collected in 1975 from Amherst MA, USA sample
191 population (Rose 1984). The IV-*bw* population was created by introgressing the brown-eye
192 recessive allele, bw^1, into the genetic background of the IV population via 10 rounds of back-
193 crossing, to create a competitor population that has >99.9% of the IV genetic background
194 (Khodaei and Long 2020). The IV-*bwD* population was also created in the same fashion, by
195 introducing the *bwD* allele (a dominant allele that also produces a brown-eyed phenotype) into an
196 IV genetic background. The IV-*bw* and IV-*bwD* populations have been subject to periodic
197 rounds of additional back-crossing to the IV population in an attempt to minimize their
198 divergence from the base population, with the last session occurring approximately 50
199 generations prior to the start of this experiment. These three populations are each maintained at
200 a census size of ~3500 adults per generation, incubated at 25°C incubation temperature with
201 60% humidity on a 12L:12D hour diurnal light cycle (Martin and Long 2015). Flies are
202 maintained in standard *Drosophila* vials (95mm H x 28.5mm OD) containing 10mL of
203 banana/agar/killed-yeast food, dusted with live yeast (Rose 1984, Shoot et al. 2024). Populations
204 are cultured on a 14-day cycle, wherein every fortnight adult flies are removed from their natal
205 vials, mixed *en masse* under light CO_2 anaesthesia and distributed into vials containing new media
206 for up to 18h, before being removed and the eggs laid are then trimmed by hand to a density of
207 approximately 100 eggs per vial (Martin and Long 2015).
208

209 ## Experiment 1: Test of the effects of conspecific relatedness and developmental
210 environment on focal female behaviour and offspring production 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 an initial 50 fly "families" by randomly combining single pairs of males and females into
214 "mini-egg" chambers (Kartell 733/4 polyethylene 20 mL sample vials; 74.5mm H x 24.8mm
215 OD; Fig S1a) whose lid contained juice/agar media (Sullivan et al. 2000) and was used as a
216 "dish" for ovipositing. We left these chambers in the incubator for ~18h, before removing the
217 adult flies and counting the number of eggs that had been laid overnight. We then evenly split
218 each family's eggs among 2 different vials each containing 10ml of media, and then added a
219 sufficient number of similarly-aged eggs obtained from the IV-*bw* population so that each vial
220 ultimately contained ~100 eggs apiece. We returned these pairs of vials to the incubator where
221 they were allowed to develop for the next 9 days, at which point we began collecting virgin
222 females for use in the experiment. For each pair of vials, we haphazardly designated one vial as
223 the "focal" vial, from which we collected 3 virgin females: a IV female ("the focal female"), a
224 second IV female (the focal female's sister, raised in the same vial), and one brown-eyed IV-*bw*
225 female (an unrelated "familiar" female, who had developed in the same vial as the focal female).
226 From the other ("non-focal") vial in the pair, we collected 2 virgin females: an IV female (the
227 focal female's sister, raised in a different "unfamiliar" developmental environment) and one
228 brown-eyed IV-*bw* female (an unrelated female, raised in environment that was "unfamiliar" to
229 the focal female). In this way we were able to obtain all possible combinations of demonstrator
230 females that differed in their genetic relatedness and their potential environmental familiarity.
231 We kept these females individually for up to 48h in test-tubes containing media to further verify
232 their virgin status (by the absence of any fertilized eggs). Next, we introduced two similarly-aged
233 adult IV males into the focal female's vial (so that all her offspring would express the wild-type

234 red-eye phenotype). At the same time, we transferred all other females into their own mini-egg
235 chambers whose dish lids contained standard banana media containing 3 IV-*bwD* males so that
236 all offspring produced by demonstrators would express a *brown-eyed* eye phenotype and to ensure
237 that any male-specific compounds transferred to the dish surface would be consistent across all
238 dishes (Lin et al. 2015, Moreira-Soto et al. 2024). Females and males were left overnight to
239 ensure sufficient time for mating to occur, and ovipositing to begin. On the following morning all
240 non-focal flies were removed from the mini-egg chambers, and sets of four dishes were affixed
241 using adhesive putty to the bottom of a larger chamber, (hereafter "arena"), which consisted of
242 an inverted plastic box (KIS Omni box, 20.3 x 15.9 x 9.6cm) modified by the addition of mesh
243 vent holes to box's outer edges (Fig S1b, Young et al. 2018). A fifth dish (that had never been
244 exposed to flies) was also placed in the arena before it was sealed shut, and the (lightly
245 anesthetized) focal female was introduced via an access hole. Thus the five dishes inside each
246 arena represent available oviposition sites for the focal female that potentially differ in their social
247 information: The 'related/same' dish contains cues produced by a sister that developed in the
248 same vial environment as the focal IV female; the 'unrelated/same' dish contains cues from an
249 unrelated IV-*bw* female that developed in the same vial environment as the focal IV female; the
250 related/different' dish contains cues produced by a sister that developed in a different vial
251 environment than the focal IV female; the 'unrelated/different' dish contains cues from an
252 unrelated IV-*bw* female that developed in a different vial environment as the focal IV female; and
253 finally the 'control/unexposed' dish contains no social information, as it had no prior exposure to
254 flies (Fig S1c). In total we established 36 replicate arenas, which were housed in a quiet, well-lit
255 room. Arenas were haphazardly rotated when placed on the counter to randomize any potential
256 directional room effects, and were left undisturbed for 20 minutes following the introduction of
257 the focal female. The arenas were then observed every 20 min for ~4.5h (starting at 10:30am),

258 and for 4h on the next day (beginning at 9:30am) for a total of 26 observation sessions. During
259 each observation session, the arenas were watched for a 5s time period, allowing for the
260 identification of which of the 5 dishes (if any) the focal IV female was located. The identity of the
261 different treatment dishes were unknown to the observers. Once all observations were complete,
262 we unsealed the arena, discarded the focal female and transferred the media in each dish into
263 vials containing 10m of media. These labelled vials were sealed and returned to the incubator for
264 2 weeks. At that time, all eclosed adults were removed, and their eye colours were tallied. Red-
265 eyed flies were the offspring of the focal female, while brown eye-flies (*wt+/bwD* or bw_1/bwD)
266 were produced by one of the conspecific females. We used the number of eclosed adult offspring
267 as a proxy for the egg number laid, in this and subsequent experiments, as in the IV population
268 at moderate larval densities, there is very high egg-to-adult survivorship (Khodaei et al. 2020).
269

270 ## Experiment 2: Test of the effect of conspecific offspring abundance on focal female
271 offspring production decisions.

272 Based on the offspring production patterns observed in Experiment 1, we performed a
273 series of complementary assays designed to test alternative hypotheses for the focal flies' potential
274 oviposition biases. Thus, in this experiment, we set out to examine whether the number of
275 offspring produced by conspecifics on a substrate was associated with the number offspring
276 subsequently producd on the same dish by a focal female. We began by collecting mated IV-*bw*
277 female flies from the lab's stock population and placed either 1, 2, or 0 individuals overnight into
278 mini-egg chambers containing media in their dish lid. The next morning, one of each type of the
279 dishes was affixed to the bottom of an arena (Fig S1d). Next, we added a single mated IV female
280 to each of the 48 replicate arenas and left them undisturbed for 22h, before transferring the
281 media in each dish to a vial containing 10ml of media. We returned those vials to the incubator
282 and counted the number of wild-type and brown-eye offspring that had eclosed 12 days later.

283

284 ## Experiment 3: Test of offspring production and behavioural biases of focal females
285 towards cues from flies originating from the IV versus the IV-bw populations.

286 In our third experiment we explored the alternate hypothesis that the offspring
287 production patterns observed in Experiment 1 might reflect preferences of the IV-originating
288 focal female for substrates that had been in contact with any other IV flies (regardless of their
289 degree of immediate genetic consanguinity) and/or the avoidance of substrates that had come in
290 contact with any IV-*bw* flies. For this assay we set up replicate vials containing 5 mated IV and 5
291 mated IV-*bw* females, that were allowed to oviposit for 18h before being discarded, and the
292 number of eggs laid standardized to 100 apiece. These vials were placed in the incubator, and we
293 collected a virgin red-eyed and a virgin brown-eyed female from each vial as they eclosed (thus
294 both flies had experienced the same developmental environment). Each female was then placed
295 into their own mini egg chamber with 2 IV-*bwD* males overnight before being discarded, and the
296 dish affixed to the inside of an arena, along with an unexposed/control dish (Fig S1e). An
297 individual mated IV female obtained from the lab stock population was introduced to each of the
298 arenas, and her location in the arena was periodically observed every 20 minutes over the course
299 of 5h (for a total of 15 sessions). In total we established 72 replicate arenas, which were left
300 undisturbed overnight, and the following morning media was removed from the dishes and
301 transferred to vials containing 10ml of media. These vials were incubated for 12 days, and the
302 number of offspring with different eye colours was recorded.

303

304 ## Experiment 4: Revisiting the effect of conspecific relatedness on offspring production and
305 behavioural decisions.

306 Based on the outcomes of our second and third experiments, we set about to directly
307 compare the behaviour and offspring production of focal females towards substrates that had
308 been exposed to their sibling or to an unrelated IV-derived female. As in experiment 1 we

309 collected the focal female's parents from the IV population, mated them, placed them into mini-
310 egg chambers overnight, then counted and split their eggs amongst 2 vials, with additional
311 similarly-aged IV-*bw* eggs added to bring the density to a total of 100 eggs apiece. From each of
312 the pairs of vials we collected virgin females as they eclosed and kept them individually. One of
313 the vials was arbitrarily designated as that which would yield the focal female (who was collected
314 as a virgin, then housed with 2 IV males from the stock population overnight), and virgin IV
315 female from the other vial (*i.e.* the focal female's sister raised in a different environment) was
316 housed with 2 IV-*bwD* males overnight in a mini egg chamber. We also haphazardly chose one of
317 the virgin IV females collected from one of the other sets of vials (thus an unrelated female, raised
318 in a different environment) and housed her with 2 IV-*bwD* males overnight in a mini egg
319 chamber. These dishes (along with a control/unexposed dish) were placed into an arena (Fig
320 S1f), into which we added the mated focal females. In each of the 84 replicate arenas, the
321 location of the focal female was periodically observed every 20 minutes over the course of 5h (for
322 a total of 15 sessions). Next, the chamber was left undisturbed overnight and the following
323 morning, we transferred the media from each dish into a new vial containing 10ml of media
324 which were incubated for an additional 12 days. The eye colour of all adult flies present in these
325 vials were tallied and recorded.
326

327 ## Statistical Analyses

328 All data analyses were conducted in the R statistical computing environment (version 4.0.3, R
329 Core Team, 2020). In all of our experiments we compared the frequencies of visits by focal
330 females to the five different lids in each arena. As these data were zero-inflated (determined using
331 diagnostic functions in the *DHARMa* package (Hartig 2022)), we constructed zero-inflated
332 binomial mixed models using the *glmmTMB* function in the *glmmTMB* package (Brooks 2017)

333 where the independent fixed factor was the dish treatment category, and the arena that
334 contained them was included as a random effect. We determined whether the treatment means
335 were significantly different from each other with likelihood-ratio chi-square tests using the *Anova*
336 function in the *car* package (Fox and Weisberg 2011), which was followed, if necessary, by post-
337 hoc tests with Tukey method p-value adjustments executed using the *emmeans* function in the
338 *emmeans* package (Lenth 2022) to determine the specific location of differences between
339 treatments. For experiment 1 we also used a two-way, zero-inflated *glmmTMB* model to
340 determine if there was any difference in patch visitation that varied with relatedness and/or
341 familiarity treatments, by using the same response data (excluding observations made on the
342 control dish, as this treatment by definition had received no prior conspecific contact). The
343 demonstrator female's degree of kinship, their environmental origin, and the interaction between
344 these two factors were the independent fixed factors in this GLMM model, and arena was the
345 random effect. The significance of these effects was determined using a likelihood-ratio chi-
346 square test using the *Anova* function.

347 For all of our experiments we (separately) analyzed the total number of red-eyed and brown-
348 eyed adults that successfully eclosed from the different lids in each arena using zero-inflated
349 *glmmTMB* GLMMs, with poisson error distributions, where the independent fixed factor was the
350 dish treatment category, and arena was the random effect. For each model we determined
351 whether the treatment means were significantly different from each other using likelihood-ratio
352 chi-square tests implemented using the *Anova* function, and post-hoc tests were executed, if
353 necessary, using the *emmeans* function, with Tukey method p-value adjustments, to determine the
354 specific location of differences between treatments. As above for experiment 1 data, we also set
355 out to determine if there was any difference in offspring number associated with relatedness
356 and/or familiarity treatments, by excluding data collected from the control dish treatment, and

357 analyzing the rest of the data set using a 2-way zero-inflated *glmmTMB* GLMM with poisson
358 errors. The demonstrator female's degree of kinship, their environmental origin, and the
359 interaction between these two factors were the independent fixed factors in this model, and arena
360 was included as a random effects. The significance of these effects was determined using a
361 likelihood-ratio chi-square test using the *Anova* function, and, if necessary, post-hoc tests were
362 conducted using the *emmeans* function, with Tukey method p-value adjustments, to determine the
363 specific location of differences between treatments.

364 In experiment 2 we also compared the frequencies of dishes that yielded zero focal offspring
365 using the *chisq.test* function to conduct a Pearson's Chi-squared test, then examined the specific
366 differences between cells in the contingency table using the *chisq.posthoc.test* function in the
367 *chisq.posthoc.test* package (Ebbert, 2019).

368

369 # Results

370 ## Experiment 1: Test of the effects of conspecific relatedness and developmental
371 environment on focal female behaviour and offspring production decisions.

372 In our first experiment, we compared the cumulative number of visits that *D. melanogaster*
373 focal females made to five different dishes, as well as the number of offspring that eclosed as
374 adults from each of those dishes. Our analysis found that those females exhibited heterogeneity in
375 their dish associations ($LR\chi^2$ =20.12, df=4, p=0.0005), and while they visited the
376 control/unexposed dishes less frequently than the demonstrator dishes, within these four
377 treatment groups, they did not express any significant bias (Fig S2). Offspring production
378 patterns indicated that the females' oviposition patterns were also heterogeneous (Fig 1a; $LR\chi^2$
379 =123.92, df=4, p=7.77×10^{-26}), with significantly more red-eyed focal offspring eclosing from
380 those dishes that had previously been in contact with a related demonstrator individual than from

381 either the control/unexposed dish or either of the dishes whose prior occupant was an unrelated
382 individual, and the greatest number of offspring eclosing from those dishes that had been exposed
383 to a related conspecific, raised in a different vial. Our focused two-way GLMM confirmed that
384 the amount of oviposition activity on a dish was significantly associated with the demonstrator's
385 kinship ($LR\chi^2$ =84.01, df=1, p=4.91x10^{-20}) with more offspring eclosing from 'related' dishes
386 ($\bar{x}$=4.97; 95% CI: 3.57, 6.92) than 'unrelated' dishes ($\bar{x}$=0.19; 95% CI: 0.08, 0.42), there was a
387 small but significant effect of developmental environment ($LR\chi^2$ =9.38, df=1, p=0.0021), with
388 slightly more offspring eclosing from 'different' dishes ($\bar{x}$=1.08; 95% CI: 0.57, 1.84) than 'same'
389 dishes ($\bar{x}$=0.93; 95% CI: 0.52, 1.67), while the interaction between kinship and developmental
390 environment was not significant ($LR\chi^2$ =1.20, df=1, p=0.27). We also noted that the number of
391 demonstrator offspring were not homogeneous across the four treatment groups (Fig 1b), with
392 more brown-eyed offspring eclosing from dishes whose prior occupant was a unrelated female
393 (IV-*bw*, mated to a IV-*bwD* male; $\bar{x}$=14.49; 95% CI: 13.03, 16.10) than those that had previously
394 been exposed to a related female (IV, mated to a IV-*bwD* male; $\bar{x}$=6.83; 95% CI: 5.95, 7.83;
395 $LR\chi^2$ =147.93, df=1, p=4.90x10^{-34}), and fewer offspring eclosing from vials where the
396 demonstrator originated from the same vial ($\bar{x}$=8.55; 95% CI: 7.53, 9.71) than if they were
397 collected from a different vial ($\bar{x}$=11.57; 95% CI: 10.28, 13.02; $LR\chi^2$ =30.83, df=1, p=2.82x10^{-}
398 ^{8}), yet there was no significant interaction between these two factors ($LR\chi^2$ =0.001, df=1,
399 p=0.975).
400

401 Experiment 2: Test of the effect of conspecific offspring abundance production on focal
402 female offspring production decisions.
403 In our second experiment, we set out to test the alternate hypothesis that the biased
404 patterns of *red-eyed* offspring produced by our focal females was influenced by the abundance of

405 *brown-eyed* conspecific offspring already present on the dishes. In this assay either zero, one or two
406 demonstrator females were exposed to a dish prior to being offered as a potential oviposition site
407 to a mated focal female. More demonstrators on a dish yielded more *brown-eyed* offspring: zero
408 *brown-eyed* offspring eclosed from the control dishes, and two demonstrator females produced, on
409 average more *brown-eyed* offspring ($\bar{x}$=13.53; 95% CI: 11.2, 16.3) than a single demonstrator
410 female ($\bar{x}$ =8.68; 95% CI: 7.0, 10.8; LRχ^2 =36.14, df=1, p=1.84x10^{-9}; Fig 2a). Despite the
411 dramatic differences in the number of demonstrator offspring that eclosed between the three
412 dishes, there was no significant difference in the number of *red-eyed* focal offspring that eclosed
413 from the three dishes (LRχ^2 =1.99, df=2, p=0.371; Fig 2b). Interestingly, dishes where there had
414 been two demonstrator females has been present were significantly *less* likely to yield zero focal
415 female offspring (8/48) than those that had been exposed to one demonstrator females (25/48) or
416 those control dishes that lacked any demonstrator exposure (32/48; χ^2 =13.43, df=2, p=0.0012).
417

418 ## Experiment 3: Test of offspring production and behavioural biases of focal females
419 towards cues from flies originating from the IV versus the IV-bw populations.

420 In our third experiment, we set out to test the alternate hypothesis that the patterns of *red-*
421 *eyed* offspring produced by our focal females was influenced by the population of origin of the
422 demonstrator females, and not their degree of kinship. This involved comparing the cumulative
423 number of visits that *D. melanogaster* focal females made to three different dishes (a
424 control/unexposed dish, a dish where the demonstrator was an unrelated IV female, or a dish
425 whose demonstrator was an unrelated IV-*bw* female), as well as the number of offspring that
426 eclosed as adults from each of those dishes. Our analysis found that focal females exhibited
427 heterogeneity in their dish associations (LRχ^2 =144.8, df=2, p=3.61 x10^{-32}) with the
428 control/unexposed dishes receiving significantly fewer visits ($\bar{x}$=0.05; 95% CI: 0.03, 0.07), than

429 either the dishes that had been exposed to the unrelated IV demonstrator ($\bar{x}$=0.31; 95% CI: 0.25,
430 0.36) or the dishes that had been exposed to the unrelated IV-*bw* demonstrator ($\bar{x}$=0.25; 95% CI:
431 0.21, 0.31), with no significant difference in visitation rates to the two demonstrator dishes
432 (t=21.96, df=1, p=0.07; Fig S3). The distribution of focal female offspring was also heterogenous
433 across the three dish types (Fig 3b; LRχ^2 =139.27, df=2, p=5.72 x10^{-31}). More focal females
434 offspring eclosed from the unrelated IV dishes ($\bar{x}$=8.84; 95% CI: 7.45, 10.49) than they from
435 either the control/unexposed dishes ($\bar{x}$=2.32; 95% CI: 1.49, 3.61), or the unrelated *IV-bw* dishes
436 ($\bar{x}$=3.09; 95% CI: 2.44, 3.91). The mean number of *red-eyed* offspring that were collected from the
437 *IV-bw* dish was not significantly different from the number collected from the control/unexposed
438 dish (t=1.31, df=1, p=0.39). We also noted that the number of eclosed demonstrator offspring
439 were significantly different between dish types (Fig 3a; LRχ^2 =89.42, df=1, p=3.82 x10^{-20}). with
440 more brown-eyed offspring eclosing from vials whose prior occupant was an unrelated IV female
441 (mated to a IV-*bwD* male; $\bar{x}$=12.33; 95% CI: 10.83, 14.05) than those that that had previously
442 been exposed to an unrelated IV-*bw* female (also mated to a IV-*bwD* male; $\bar{x}$=6.53; 95% CI:
443 5.55, 7.69).
444

445 ## Experiment 4: Revisiting the effect of conspecific relatedness on offspring production and
446 behavioural decisions.

447 In our fourth experiment, we set out (again) to compare the patterns of dish visitation and
448 offspring production by focal females on three different dishes (a control/unexposed dish, a dish
449 whose demonstrator was a related IV female, or a dish where the demonstrator was an unrelated
450 IV female). The focal females exhibited bias on their dish visitation rates (Fig S4; LRχ^2 =12.81,
451 df=2, p=0.0017), as they were significantly fewer visitations to the control/unexposed dishes
452 ($\bar{x}$=0.05; 95% CI: 0.03, 0.07), than to related IV demonstrator dishes ($\bar{x}$=0.08; 95% CI: 0.06,
453 0.12) or to the unrelated IV dishes ($\bar{x}$=0.11; 95% CI: 0.08, 0.14). There was no significant

454 difference in visitation rates between the two demonstrator groups (t=1.33, df=1, p=0.38). The
455 distribution of focal female offspring was heterogenous across the three dish types (Fig 4; GLM:
456 LRχ^2 =368.28, df=2, p=1.07x10^{-80}). Significantly more focal female offspring eclosed from the
457 related IV dishes ($\bar{x}$=11.46; 95% CI: 9.96, 13.19) than from the unrelated IV dishes ($\bar{x}$=7.47;
458 95% CI: 6.43, 8.68), and both demonstrator dishes yielded more focal female offspring than did
459 the control/unexposed dishes ($\bar{x}$=0.48; 95% CI: 0.33, 0.69). There was a small, but statistically
460 significant difference in the number of demonstrator offspring eclosing from the related IV dishes
461 ($\bar{x}$=11.3; 95% CI: 10.32, 12.4) and from the unrelated IV dishes ($\bar{x}$=10.1; 95% CI: 9.14, 11.1),
462 LRχ^2 = 6.59, df=1, p=0.01; Fig S5).
463

464 ## Discussion

465 The decision where to oviposit may be very important, especially in those species where
466 there is no post-laying parental care (Prasad et al. 2003, Klepsatel et al. 2020). Fruit flies,
467 *Drosophila melanogaster*, like many other species, use social information in their decision-making
468 (Leadbeater 2009, Chen and Sokolowski 2022). While there are benefits of laying one's eggs in
469 proximity to those of others, there are also risks – both of which might be mediated by the degree
470 of kinship between conspecifics. In this study, we set out to examine the possibility that female
471 flies might selectively use cues produced by close relatives, presumably to increase their
472 offspring's chance of success. In doing so, we simultaneously explored the factors that might
473 influence how flies might infer kinship, as well as investigate alternate hypotheses about social
474 information usage. Our assays confirm the extensive use of social cues by *D. melanogaster* females,
475 and furthermore collectively suggest that they are capable of kinship discrimination and are able
476 of distinguishing between conspecifics from different populations. These results add to the

477 growing body of knowledge about the sophisticated social information use by this model species
478 (Chen and Sokolowski 2022).

479 In all assays focal females associated more with those substrates that had come into
480 contact with conspecifics than with the unexposed/control dishes (Figs S2, S3 and S4), clearly
481 illustrating the importance of social cues in their decision-making, and consistent with numerous
482 previous studies (*e.g.* Battesti *et al.* 2012, Duménil et al. 2016, Malek and Long 2020). However,
483 not all conspecific cues elicited the same responses. In the first assay, we compared the offspring
484 production patterns of focal females on dishes exposed to a range of different demonstrators. By
485 splitting sets of full sibling eggs between two different vials to develop, and collecting both adult
486 relatives and non-relatives from each of the pairs of vials, we set out to test if and how *D.*
487 *melanogaster* females might distinguish kin cues. We observed that dishes that had been in previous
488 contact with a related demonstrator female yielded considerably more focal female offspring that
489 those where the demonstrator was non kin. Interestingly, dishes where the demonstrator came
490 from the same vial as the focal vial produced fewer focal female offspring than those when the
491 demonstrator had developed trh same vial, however the size of this difference was much smaller,
492 and there was no significant interaction between demonstrator kinship and origin factors (Fig 1a).
493 These results suggest that ovipositing females are selectively use social information, and largely
494 favouring cues produced by their relatives, while perceived environmental familiarity does not
495 enhance oviposition site use. Determining the specific cues being used by focal females is beyond
496 the scope of this study as demonstrator females transfer their gut bacteria, CHCs (and male-
497 specific compounds acquired during copulation) to the surface of their eggs and the media
498 (Bakula 1969, Guilhot et al. 2023, Moreira-Soto et al. 2024). The expression of these olfactory
499 cues can be influenced by both an individual's genotype and/or their developmental
500 environment (Sharon et al. 2010, Fedina et al. 2012, Lizé et al. 2013, Venu et al. 2014, Heys et

501 al. 2018, García-Roa et al. 2022, Pavković-Lučić et al. 2022). While the pattern of significant
502 differences associated with the developmental environment (Fig 1a) could suggest that
503 "familiarity" (and by extension the microbiotic cues that could be used as proxies of kinship) are
504 unimportant, and potentially misleading, cues of "relatedness", it should be noted that in our
505 experimental design, the conditions of the two vial environments were similar (same media, same
506 larval density), which may have minimized the potential for the development of differences in
507 these cues. Interestingly, Malek and Long (2020) observed that a focal female's offspring
508 production patterns depended on the developmental environment of *both* the focal female and the
509 demonstrator. In one assay, they observed focal females who developed in a protein-rich vial
510 environment, oviposited more on dishes where the demonstrator females (that came from the
511 same population) who had also developed on similar protein-rich media. However, in a second
512 assay when the focal females developed on standard media, these flies laid fewer eggs on dishes
513 where the demonstrator came from the same population, but had instead developed on protein-
514 rich media, than they did on dishes where the demonstrator came from a different *D. melanogaster*
515 population but had *also* developed on standard media. Thus, one should not necessarily conclude
516 that our results indicate that inherited cues are the only way in which egg kinship was/can be
517 recognized by the focal females in this species, and future studies exploring this idea across a
518 range of divergent environments are likely to expand our understanding of how social
519 information is produced and interpreted in *D. melanogaster*.

520 While the observed pattern of focal female offspring production is consistent with kin-
521 associated biases, other processes could also have resulted in the differential outcomes, and so in
522 order to validate or rule-out these alternate hypotheses we conducted several complementary
523 assays. We had been initially struck with the distinctive pattern of offspring production by the
524 demonstrator females (Fig 1b), wherein there were more *brown-eyed* conspecific offspring collected

525 from dishes where the demonstrator was unrelated than when the demonstrator was related.
526 Perhaps our focal females weren't deciding to lay their eggs on dishes that contained their kin's
527 offspring, but instead were avoiding dishes where there were already lots of eggs (and a
528 heightened risk of competition and/or cannibalism)? We explored this possibility in our second
529 assay, where focal females could choose between dishes with dramatically different number of
530 demonstrator offspring (Fig 2a). If females were avoiding dishes where there were too many
531 conspecific eggs already present, we would expect to see more focal offspring eclosing on the
532 "one female" treatment than on the "two females" treatment. In fact, we observed no significant
533 difference in focal female offspring production between the two groups (and if anything, there
534 were fewer cases where we observed *zero* focal female offspring produced when there were more
535 demonstrators; Fig 2b). Previous work by Golden and Dukas (2014) found that female flies given
536 a choice between a control and a demonstrator dish (which had been exposed to 5 or 20 larvae),
537 did not appear to discriminate against the demonstrator dishes that possessed cues indicating
538 greater competition during offspring development. Thus, we are fairly confident that the
539 differences in focal female offspring production patterns observed in experiment one were not
540 due to differences in the quantity of demonstrator cues present on a dish. As a side note, we
541 suggest that the differences in the number of demonstrator offspring observed in that assay is a
542 side effect of the way in which our demonstrator females were collected (*see* Fig S6 *caption for*
543 *details*).

544 The second alternate hypothesis we explored was that the biased pattern of oviposition
545 made by the focal females in the first experiment on the two "related" dishes, was not due to the
546 perceived kinship of the demonstrators, but instead was because those demonstrators originated
547 from the same population (IV), and the demonstrators for the two "unrelated" dishes were from a
548 different population (IV-*bw*). While IV and IV-*bw* are maintained following the same protocols,

549 and IV-*bw* is periodically back-crossed to IV, perceptible differences in the cues present in these
550 isolated populations (or their microbiotic communities) may have arisen via drift and/or
551 selection. Thus in our third experiment, we compared the behaviour and offspring production of
552 focal females exposed to (unrelated) IV or IV-*bw* demonstrator females. We found that focal
553 females produced more offspring on the former dishes than they did on the latter (and virtually
554 none on the control/unexposed dishes). This suggests that at least some of the patterns of
555 offspring production seen in our first experiment may not necessarily be the result of recognition
556 of kinship *per se*, but instead with other cue(s) that differ between the IV and IV-*bw* populations.
557 Thus, based on the insights gained in our third assay, we set out to re-examine the social
558 information use of *D. melanogaster* females in our fourth assay where females were given a choice
559 of oviposition sites that either had no prior exposure (control), a demonstrator sister (raised in a
560 different vial), or an unrelated demonstrator female (also from the IV population and raised in a
561 different vial). We observed that focal females still biased their offspring towards the dishes that
562 had previously been in contact with their sisters, albeit to a lesser extent than seen in experiment
563 1. This provides additional support to the hypothesis that female *D. melanogaster* are both capable
564 of distinguishing between cues produced by kin and non-kin, and that these cues are used in their
565 social decision-making. In doing so, they may be trying to obtain the potential benefits of
566 developing in a group (Gilbert 1980, Gregg, et al. 1990, Sakaguchi and Suzuki 2013, Wertheim
567 et al. 2002a, Wertheim et al. 2002b, Dombrovski et al. 2017, Shoot et al. 2024), while reducing
568 the potential risks from conspecifics (Khodaei and Long 2019, Khodaei and Long 2020, Fisher et
569 al. 2021).

570 Collectively, our results illustrate the subtle and plastic nature of social information use by
571 female *D. melanogaster*: Given a choice, a mated female *D. melanogaster* will be more likely to
572 produce offspring on a dish that has previously been in contact with any conspecific, compared

573 to an socially unexposed surface, there will be more offspring production associated with
574 substrates that have been in previous contact with an unrelated mated female from the same
575 population than one exposed to a mated female from a different population, and that focal if a
576 substrate has been exposed to a mated sister, that site will yield more focal offspring than if the
577 demonstrator is an unrelated individual. At the same time, mated female *D. melanogaster* do not
578 appear to show any bias in their egg production against dishes that differ in the number of
579 conspecific eggs (and their associated biochemical cues) present. Together, these pattens
580 illuminate that *D. melanogaster* females are sensitive to differences in the cues produced by
581 conspecifics and add to the growing appreciation of social decision-making in this model species.

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Acknowledgements

We would like to thank Simran Mann, Lukas Ghiglione, and Harleen Taneja of the #DrosLife Lab for their fly-pushing, behavioral observations and camaraderie. Natasha B. Gallo and two anonymous reviewers provided helpful feed-back and constructive comments. Ruby Lindsay and Michael Steeleworthy of the Wilfrid Laurier University Library are thanked for their help with data archiving. This work was conducted at Wilfrid Laurier University, which exists on the traditional territory of the Neutral, Anishnawbe, and Haudenosaunee peoples.

804 **FIGURES**

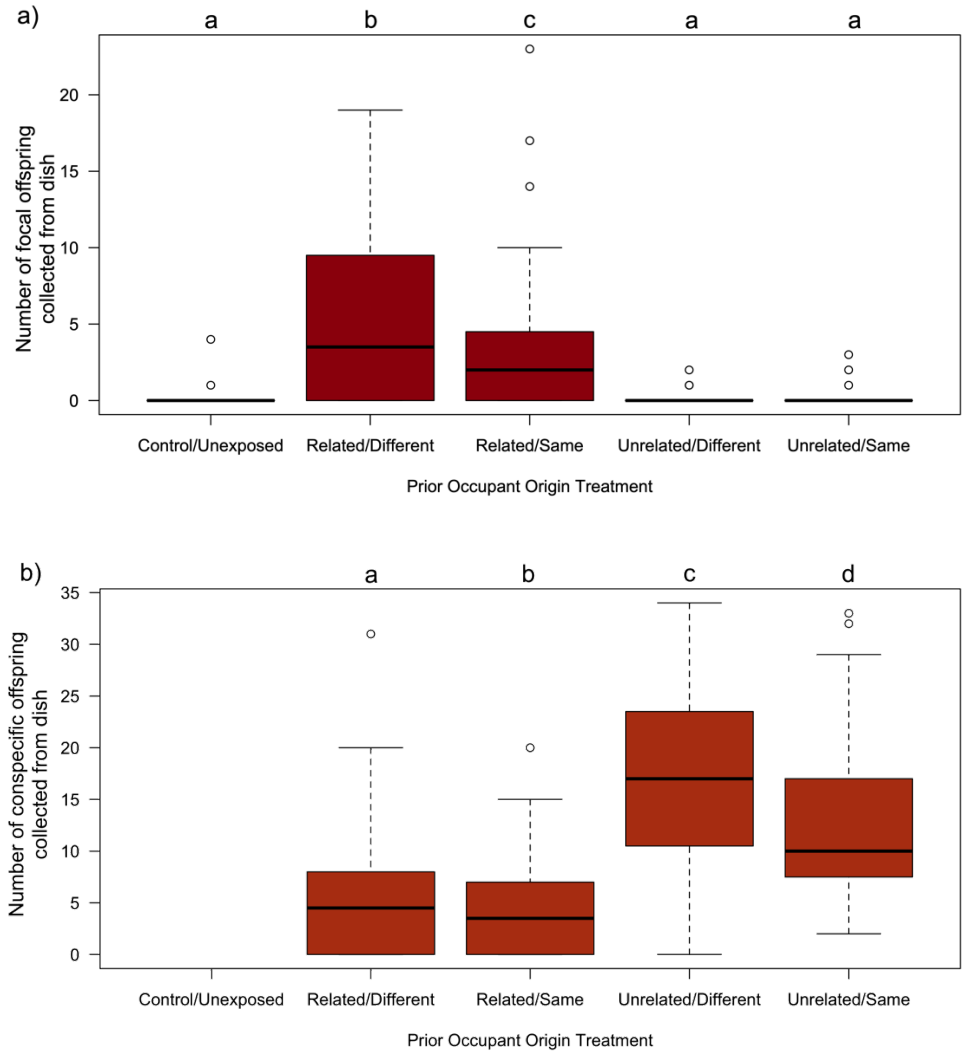

Figure 1. More focal female offspring produced on dishes where the prior occupant was a related female, while more conspecific offspring were produced on dishes where the female was unrelated. Boxplots illustrating data collected in from the 36 replicate arenas in the first experiment of a) the total 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 Tukey HSD post-hoc tests comparing group mean are 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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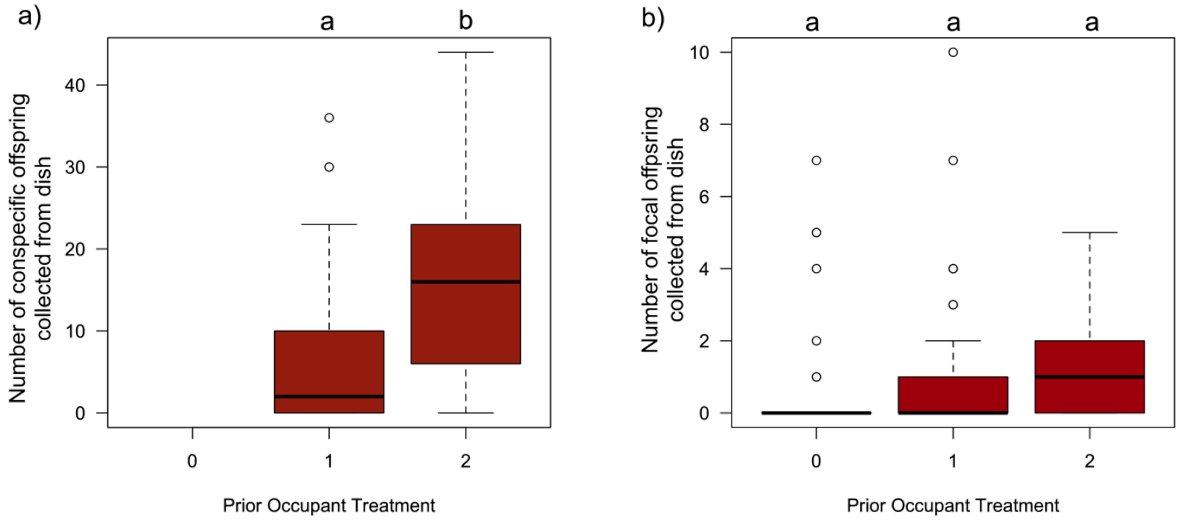

806 **Figure 2. No evidence that focal female offspring production on dishes is inhibited**
807 **by increased abundance of conspecific offspring.** Boxplots illustrating the data collected
808 from the 48 replicate arenas in the second experiment of a) the number of *brown-eyed* adult flies
809 that eclosed from dishes that had been exposed to 0, 1 or 2 conspecific females, b) the number of
810 *wild-type* (red-eyed) adult flies that eclosed from the same dishes that had been laid by the focal
811 female *D. melanogaster.* Boxplot components as in Figure 1. The results of Tukey HSD post-hoc
812 tests comparing group mean are indicated by letters, where groups that do not share the same
813 letter are considered statistically different at the α=0.05 level. The control/unexposed dishes by
814 definition had not had any prior exposure to a female capable of producing *brown-eyed* offspring,
815 and thus those dishes never yielded any offspring with that phenotype.

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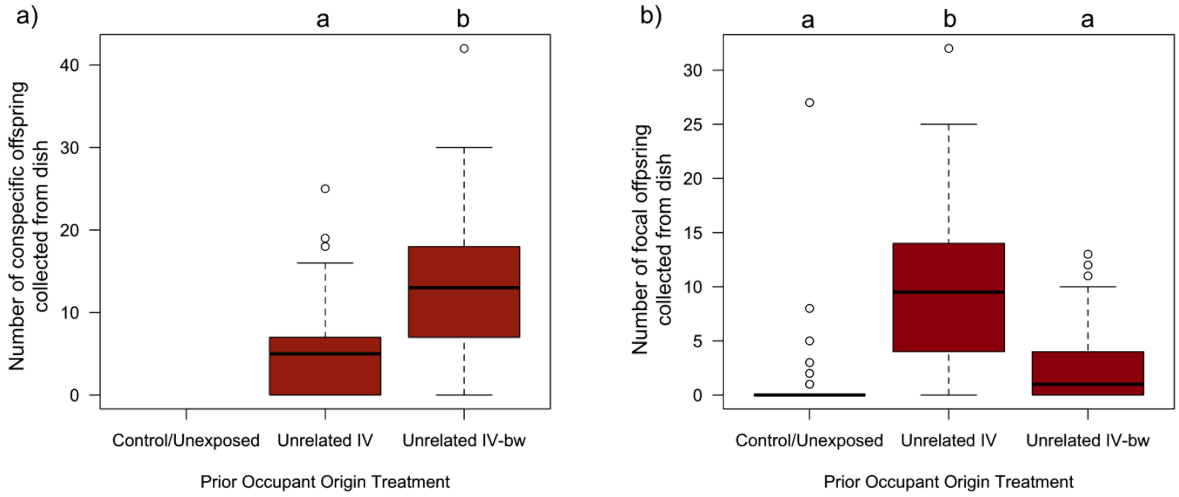

Figure 3. Focal female offspring production is higher on dishes where the unrelated demonstrator originates from the same (IV) population than a different (IV-bw) population. Boxplots illustrating data collected from the 72 replicate arenas in third experiment of a) the number of *brown-eyed* adult flies 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 Tukey HSD post-hoc tests comparing group mean are 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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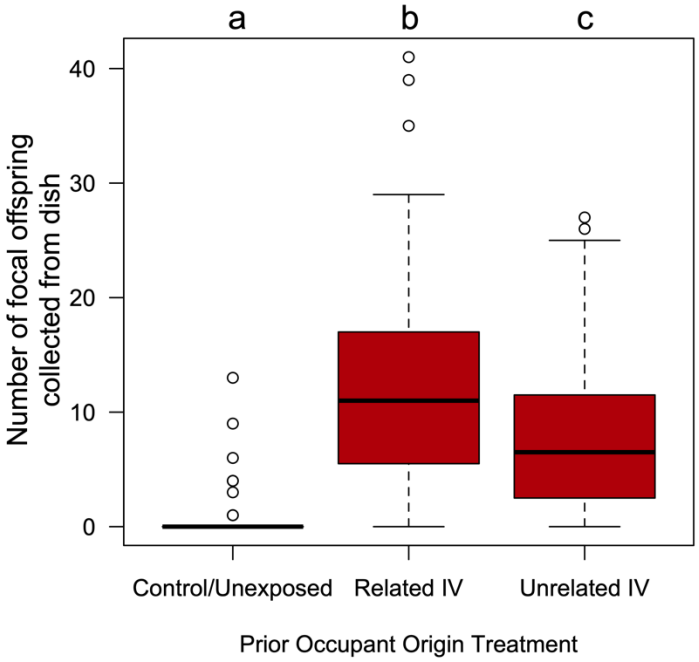

Figure 4. More focal female offspring produced on dishes where the prior (IV-originating) occupant was a related female. Boxplot illustrating data collected from the 84 replicate arenas in the fourth experiment of the number of *wild-type* (red-eyed) adult flies that had been laid by the focal female *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 α=0.05 level.

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822 **SUPPORTING INFORMATION**

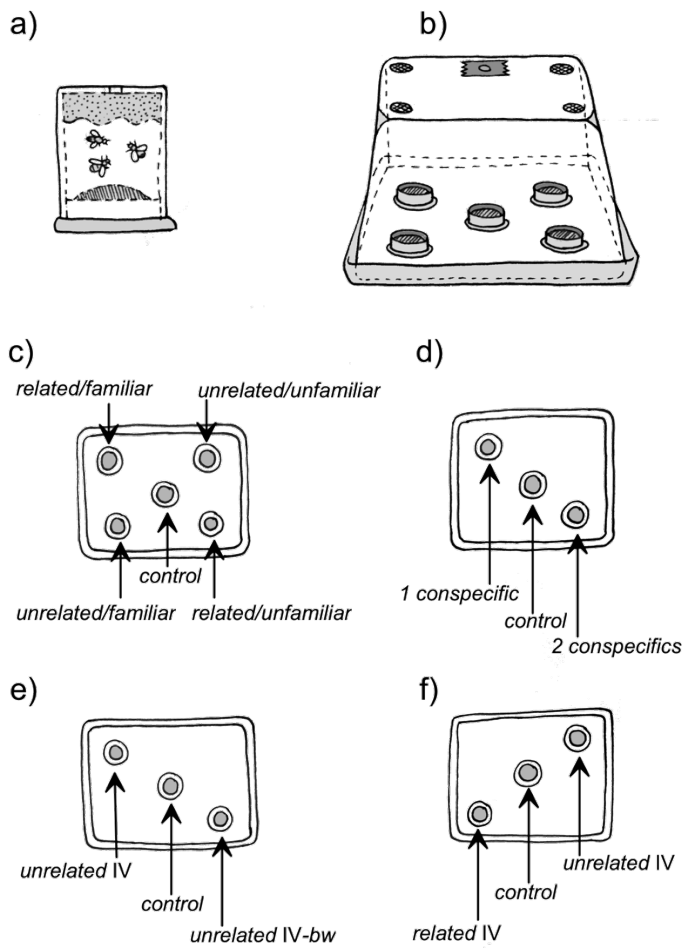

Figure S1. Drawings illustrating **a**) the "mini-egg" chambers used to collect eggs and/or create media that exhibited social cues. **b**) an observation arena into which the focal ovipositing female was introduced and monitored. At the bottom of the chamber the lids that exhibited different kinds of 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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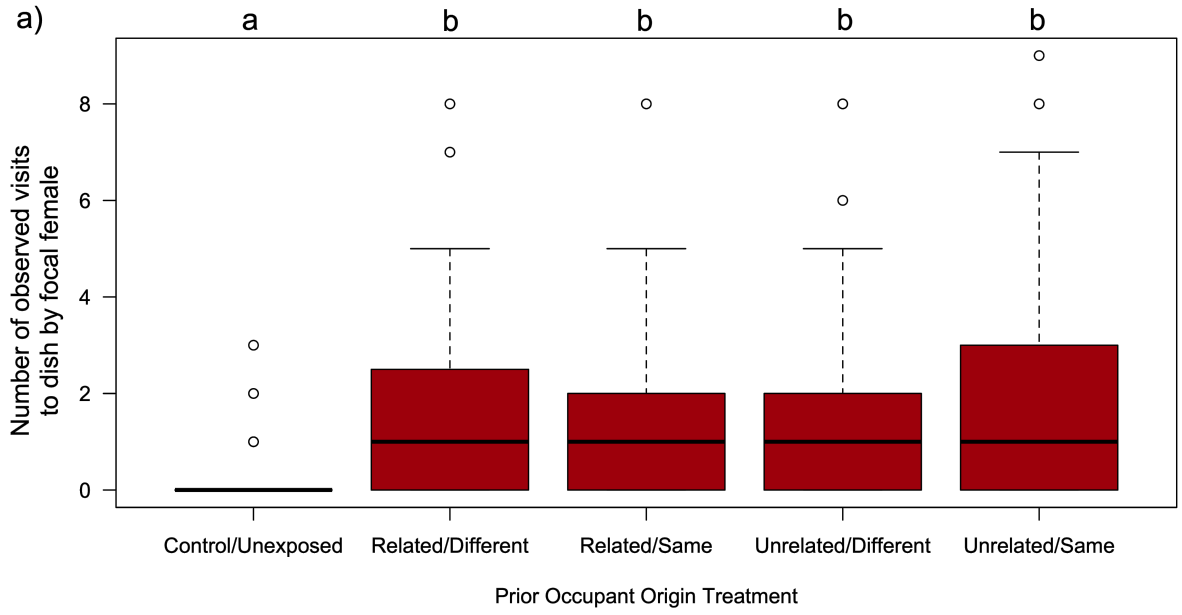

Figure S2. Focal females visited demonstrator-exposed dishes more frequently than to control/unexposed dishes. Boxplots illustrating the cumulative number of observations made across 26 sessions in the first experiment in which a focal female *D. melanogaster* was observed on the surface of one of 5 different media dishes present in the 36 replicate arenas. 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 α=0.05 level.

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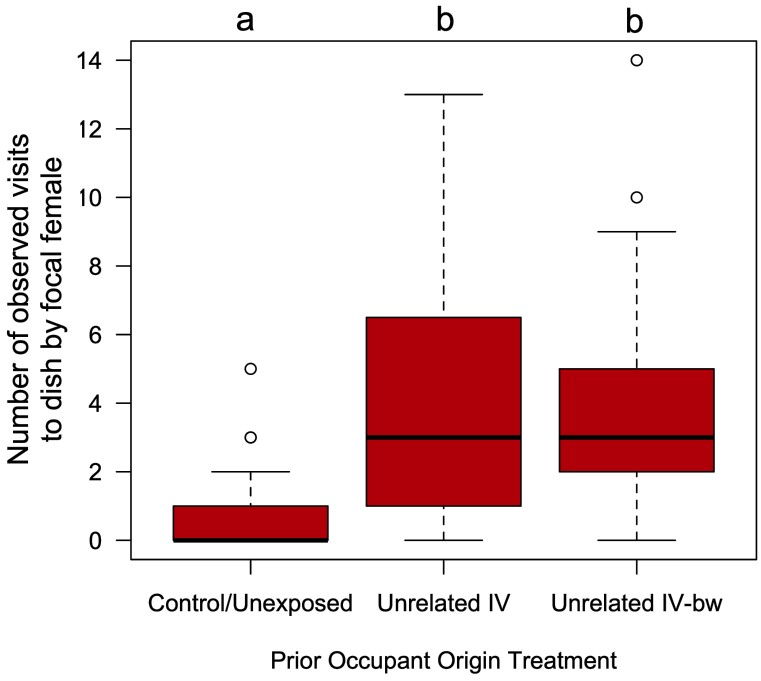

Figure S3. Focal females visited demonstrator-exposed dishes more frequently than to control/unexposed dishes. Boxplots illustrating the cumulative number of observations made across 26 sessions in the third experiment in which a focal female *D. melanogaster* was observed on the surface of one of 3 different media dishes present in the 72 replicate arenas 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 α=0.05 level.

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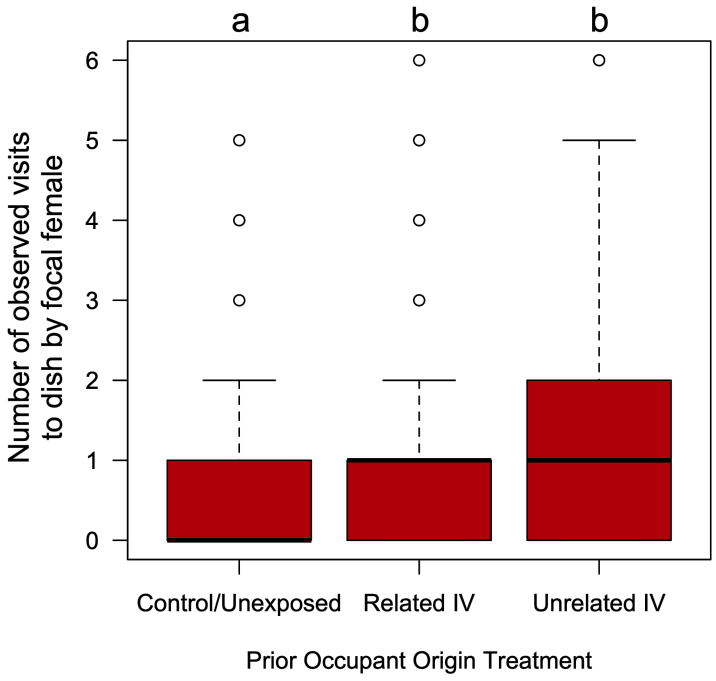

Figure S4. Focal females visited demonstrator-exposed dishes more frequently than to control/unexposed dishes. Boxplots illustrating the cumulative number of observations made across 26 sessions in the fourth experiment in which a focal female *D. melanogaster* was observed on the surface of one of 3 different media dishes present in the 84 replicate arenas 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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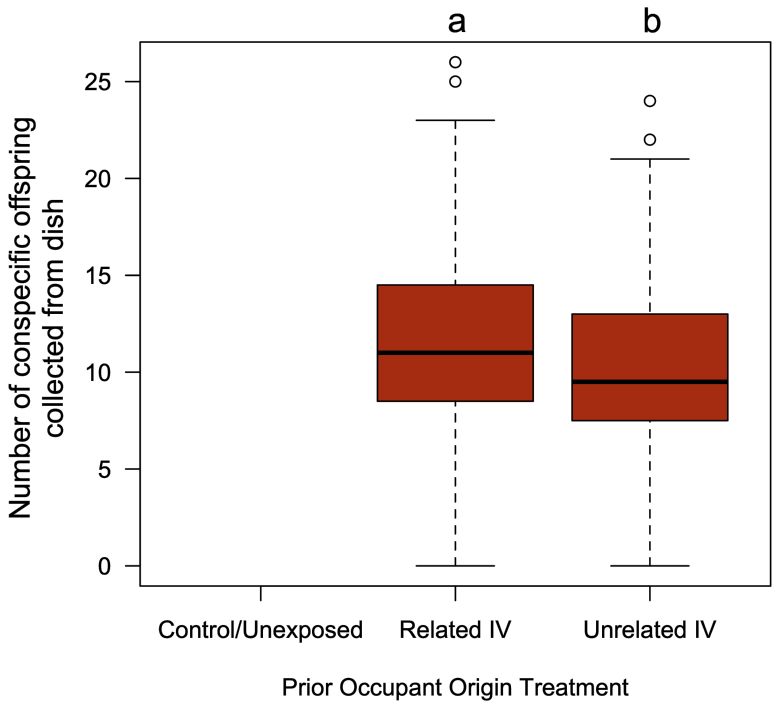

Figure S5. Number of conspecific offspring collected from demonstrator-exposed dishes. Boxplots illustrating the number of *brown-eyed* adult flies that eclosed from dishes that had been exposed to either a related IV female (mated to a IV-*bwD* male), an unrelated IV female (mated to a IV-*bwD* male), or neither in the fourth experiment. There was a small (Cliff's Delta effect size=0.15) but statistically significant difference in the mean number of offspring collected from these dishes. 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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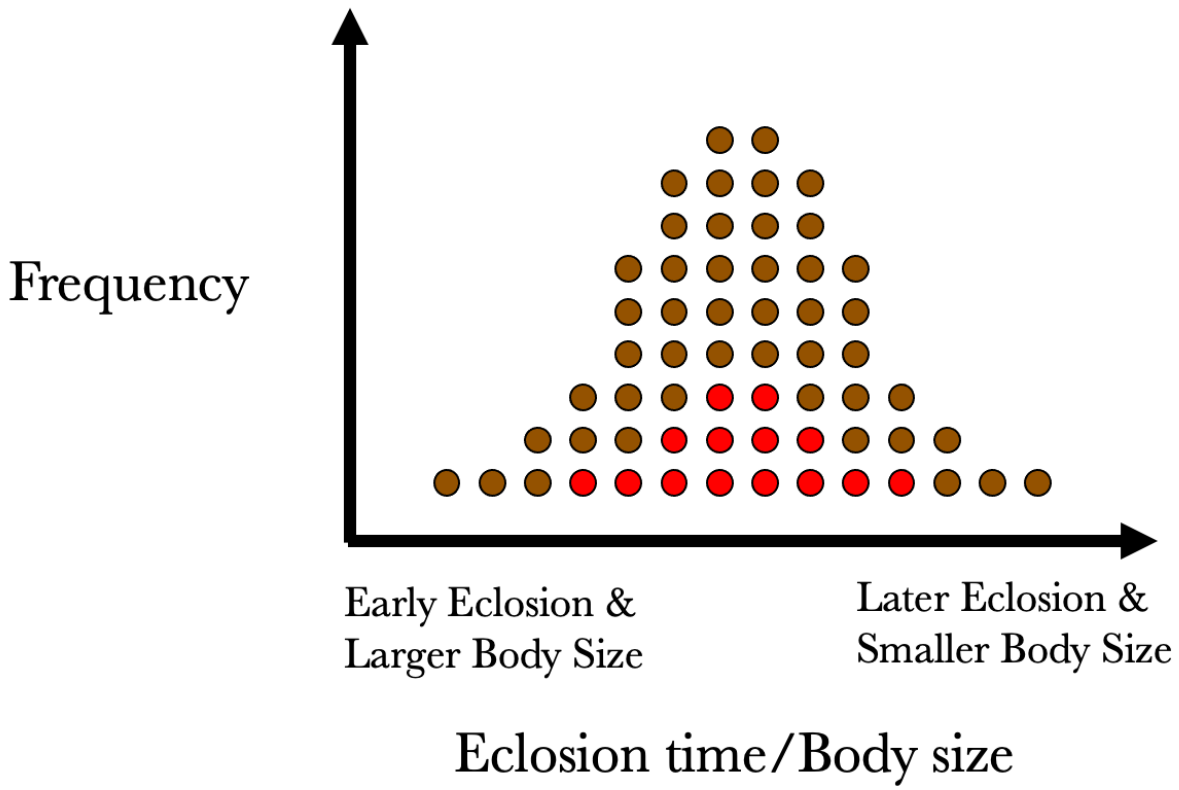

Figure S6. Schematic diagram depicting hypothetical distribution of female *D. melanogaster* eclosion times in a mixture of eggs collected at the same time from the IV and the IV-*bw* populations (where we assume are randomly sampled, and that both populations have identical distributions, means and 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 only a small difference in the number of demonstrator offspring that were collected (Figure S5), which is consistent with the hypothesis described above.

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