1 **Evolution of reproductive isolation in a long-term evolution experiment with *Drosophila***
2 ***melanogaster*: 30 years of divergent life history selection.**
3

4 **ABSTRACT**
5

6   We ask if three decades and over 1 500 generations of divergent life history selection on
7 age at reproduction has resulted in the evolution of reproductive isolation (RI) between
8 laboratory populations of *Drosophila melanogaster*. We tested for premating, postmating-
9 prezygotic and postzygotic reproductive isolation between 3 replicate population pairs. Large,
10 evolved differences in body size between selection treatments suggested the potential for
11 prezygotic barriers driven by sexual selection or physical incompatibilities between the sexes.
12 Although a simple prediction would be preference for larger size, creating directional isolation,
13 our results from individual mate choice trials indicate that populations from both selection
14 treatments show a marked bias towards homotypic mate choice; indicative of prezygotic RI
15 driven by sexual selection or sexual conflict. Hybridization between the focal populations
16 resulted in the production of viable adult flies with intermediate size and developmental traits.
17 We observed a suggestive but statistically non-significant trend of fitness decline in the F2
18 generation of hybrids, but no significant evidence suggesting the evolution of postmating-
19 prezygotic or postzygotic RI. Our findings are in accord with extant literature that posits that
20 premating RI evolves before postmating forms of RI.

INTRODUCTION

Understanding the mechanisms underlying the evolution of reproductive isolation (RI) and the timing of their origination is central to the study of speciation. With the fossil record yielding limited information about this process on microevolutionary timescales, there has been a focus on extant incipient species, either where populations naturally intermingle in contact zones or through experimental crosses. Such populations, part way through the process of RI, can help to illuminate the nature of divergence, as indicated by premating isolation or hybrid inferiority due, for example, to antagonistic epistasis between incompatible alleles (Dobzhansky 1937; Muller 1942) and hence offer a snapshot of speciation in action. But what selective agents or historical contingencies have driven the divergence may or may not be apparent, particularly as replication and controls may be absent or limited, and partial isolation and changes in the environment can lead to reversals in the development of RI (Lackey & Boughman 2017), complicating the inference of sequence in the evolution of isolating mechanisms.

Here we focus on early stages of ecological speciation. Ecological speciation is the process through which reproductive isolation evolves as a consequence of local adaptation with limited gene flow. Divergent selection pressures can contribute to this process by acting directly, or through pleiotropy or tight linkage with genes that are directly responsible for reproductive isolation (Rice and Hostert 1993; Rundle and Nosil 2005). Divergent evolved responses between ecotypes may result in (1) changes in morphology, mating behaviours or physiological characters that hinder mating attempts mechanically or via mate choice (premating reproductive isolation); (2) changes in gametes/reproductive machinery that inhibit fertilization (postmating-prezygotic reproductive isolation); and/or (3) genetic incompatibilities that reduce survival or reproductive success of hybrid individuals (postzygotic reproductive isolation).

In addition to divergent natural selection in allopatric populations, divergent intersexual coevolution via sexual selection has long been recognized to have a potentially significant role in the evolution of reproductive isolation (Lande 1981; Maan and Seehausen 2011; Panhuis *et al*. 2001; Ritchie 2007; Singh and Singh 2014). Likewise, interlocus sexual conflict – where traits that increase the fitness of one sex directly cost the other – can result in rapid coevolution (an "evolutionary chase" or "arms race") between male and female reproductive traits (Rice & Holland 1997; Parker & Partridge 1998; Rice 1998) and potentially accelerate the process of speciation (Ritchie 2007; Gavrilets 2014; Syed *et al.* 2017); the evolution of female resistance to reduce the direct costs of mating is predicted to contribute to assortative mate choice (Gavrilets *et al.* 2001).

While natural systems such as hybrid zones have advanced our understanding of the evolution of RI immensely, researchers are frequently limited to indirect inferences, such as using estimates of gene flow and clines to infer reproductive isolation. Beyond knowledge of the evolutionary history, these systems limit the number RI barriers that can be assessed, and

60   generally lack replication at the population-pair level. Notable exceptions include stickleback
61   fishes, where repeated invasion of freshwater from marine ancestors, and subsequent
62   differentiation into limnetic-benthic and lake-stream ecotypes provide extensive replication
63   (Lackey & Boughman 2017); and storm-petrels, where repeated separation of populations by
64   breeding time (allochrony) provides replicated evidence of reproductive isolation in sympatry
65   (Wallace *et al.* 2017). Experimental evolution can be a valuable tool for more direct
66   investigation, allowing us to probe potential drivers of speciation between populations with well
67   understood evolutionary histories. Experimental studies in speciation have attempted to test if (1)
68   non-ecological drivers like drift, (2) negative selection against hybridization between ecotypes,
69   (3) divergent selection on populations or (4) sexual conflict produced reproductive isolation
70   (reviewed in Rice and Hostert 1993; Coyne and Orr 2004; Fry 2009; White *et al.* 2020). An
71   obvious limitation to experimental evolutionary studies of speciation is time: research projects
72   rarely exceed granting cycles and few organisms lend themselves to experimental evolution. It is
73   little surprise then that *Drosophila* is far and away the genus most investigated in the laboratory.

74   Early divergent selection studies produced seemingly equivocal results with respect to
75   creating reproductive isolation. A closer examination suggested that divergent selection was
76   highly effective, given a pre-existent tendency to mate assortatively on the basis of the trait
77   selected upon (Rice and Hostert 1993; Fry 2009). Divergent selection upon behavioural traits
78   resulted in the correlated evolution of reproductive isolation (del Solar 1966; Soans *et al.* 1974;
79   Hurd and Eisenberg 1975; Lofdahl *et al.* 1992) while divergent selection on other traits like
80   bristle number (Barker and Cummins 1969; Santibañez and Waddington 1958) produced
81   negative results. More recently, experimental evolution studies have focused on selection
82   regimes that do not directly involve characters that are known to influence intra-population
83   assortment. For example, a number of studies have investigated the importance of local
84   adaptation to distinct nutrition sources for mating assortment. Divergent selection regimes with
85   different nutritional environments result in premating isolation through (1) the evolution of
86   signalling traits and mating preferences in *Drosophila serrata* (Rundle et al. 2005), (2) the
87   evolution of symbiotic microbiota in inbred strains of *D. melanogaster* (Najarro *et al.* 2015;
88   Sharon *et al.* 2010), (3) competitive ability differences between controls and selected individuals
89   in *D. melanogaster* (Belkina *et al.* 2018), (4) positive assortative mating preferences in *D.*
90   *melanogaster* (albeit unstable) (Nash *et al.* 2019) and in *Saccharomyces cerevisiae* (Mahilkar *et*
91   *al.* 2023); and (5) postzygotic reproductive isolation through genetic incompatibilities in
92   *Saccharomyces cerevisiae* (Dettman *et al.* 2007) and *Neurospora* (Dettman *et al.* 2008).

93   Life history traits are less frequently studied in the context of reproductive isolation, but
94   have also received some attention – predominantly in studies of allochronic speciation. Powell *et*
95   *al.* (2020) note how adaptation to differences in seasonal timing has been shown to contribute to
96   reproductive isolation in a wide variety of organisms including plants (Savolainen *et al.* 2006;
97   Lowry *et al.* 2008), insects (Tauber *et al.* 1977; Wood & Guttman 1982; Horner *et al.*1999;
98   Eubanks *et al.* 2003; Abbot & Withgott 2004; Forbes *et al.* 2009; Ording *et al.* 2009; Wadsworth

99   *et al.* 2013; Hood *et al.* 2015) and vertebrates (Friesen *et al.* 2007). Speciation through other life-
100   history traits and correlated changes in intersexual coevolution has received less attention, due to
101   the myriad challenges associated with studying these systems in nature. Divergent selection on
102   pre-adult development time has been shown to produce pre-mating isolation through (1)
103   disturbances in circadian rhythm resulting in changes in phases of mating in the melon fly,
104   *Bactrocera cucurbitae* (Miyatake and Shimizu 1999), and (2) evolved body size differences, and
105   correlated levels of sexual conflict, in *Drosophila melanogaster* (Ghosh and Joshi 2012).
106   Likewise, divergent selection on body size results in premating isolation, through mechanical
107   incompatibilities during attempted copulations between *Columbicola* feather mites (Villa *et al.*
108   2019).

109   Age-at-reproduction has been extensively manipulated using experimental evolution in
110   *Drosophila* to investigate correlated changes in longevity, development time, body size and a
111   slew of associated traits (Prasad and Joshi 2003). In this study, we use an exceptionally long term
112   evolution experiment (LTEE) with population pairs separated by over 1,500 generations and
113   three decades under divergent selection in allopatry. The two *D. melanogaster* life-history
114   selection regimes used in this study are selected to reproduce late in life (4 weeks from egg; CO)
115   and extremely early in life (9 days from egg; Accelerated CO / ACO). The ACO populations
116   have evolved small body size, rapid development time, and reduced lifespan (Chippindale *et al.*
117   1997a; Chippindale *et al.* 2004a). On the other hand, the CO flies are big, develop slowly and
118   likely have an increased ability to resist copulations as females and to inflict harm as males
119   (Chippindale *et al.* 2004b; Chippindale *et al.* 1997b; Mital *et al.* 2022, Verma *et al.* 2022).

120   In the speciation literature, body size is identified as a crucial trait that can drive the
121   evolution of reproductive isolation (Servedio et al. 2011). In *D. melanogaster*, body size is
122   strongly correlated with juvenile development time (Chippindale *et al.* 1997a; Nunney 1996;
123   Prasad and Joshi 2003; Prasad *et al.* 2000; Zwaan *et al.* 1995) and life-history selection on
124   development has been shown to result in the evolution of body size (Chippindale *et al.* 1997a;
125   Prasad *et al.* 2000). An important sexually selected trait for both sexes, large females tend to be
126   more fecund (Roff 2002; Stearns 1992) and attract higher courtship effort from males (Byrne and
127   Rice 2006); while large males have increased competitive abilities (Markow and Ricker 1992;
128   Partridge and Farquhar 1983), and secure more frequent copulations as a consequence of female
129   preference (Markow 1986; Partridge *et al.* 1987, but see Prasad et al. 2007). Given the evolved
130   size differences between the ACOs and COs, a simple prediction is that individuals of both
131   population-types display a mating preference for individuals from the larger CO populations,
132   producing conditions of partial, directional, reproductive isolation driven by sexual selection on
133   the basis of body size, along the lines observed by Ghosh and Joshi (2012).

134   In *D. melanogaster,* divergent reproductive strategies between the sexes have long been
135   recognized (Bateman 1948) potentially resulting in interlocus sexual conflict. Frequent matings
136   increase male fitness but can decrease female fitness as a result of male harassment and the
137   harmful effects of genital wounding and seminal fluid (Chapman et al. 1995; Bonduriansky *et al.*

138 2008; Travers *et al.* 2015). The harm experienced by females as a result of mating, measured by
139 lifespan and egg production rate, is correlated with male body size (Pitnick and García–González
140 2002; Friberg and Arnqvist 2003). In the context of our two selection treatments, we predict that
141 male harm and female resistance traits will be stronger in the CO populations compared to the
142 ACO populations, due to the difference in body size between the large COs and smaller ACOs.
143 In experimental hybridization between CO and ACO lines, this difference could drive premating
144 isolating barriers to evolve in one direction, with CO females resistant to mating with the smaller
145 ACO males, and ACO females vulnerable to unwanted mating with CO males. Indeed, working
146 with a similar system, Ghosh and Joshi (2012) found such one-directional isolation, driven by
147 large females resisting mating attempts by small males, and a preference for (or lack of
148 resistance to) large males in females of both treatments. On the other hand, especially with the
149 large number of generations in allopatry, mating signals and preferences may have diverged
150 between populations in our selection experiment creating the possibility for positive assortment
151 in mate choice experiments.

152   We investigated mate choice through individual female, male, and group mating choice
153 assays in the ACO/CO complex of selected lines. We further looked for potential gametic
154 incompatibilities (postcopulatory prezygotic isolation) by comparing egg hatchability in hybrid
155 matings to the parental populations. Finally we looked for postzygotic effects (hybrid breakdown
156 or vigour) in developmental traits, body size, and adult fitness.

METHODS

Experimental populations

Our experiments employed the accelerated development (ACO) (Chippindale *et al.* 1997a) and control-old (CO) (Rose et al. 1992) populations. All populations are maintained under standard laboratory conditions: moderate density (larval density of 80 - 120 larvae / vial), 25ºC, 12:12 light:dark cycle on banana/agar/killed-yeast medium, and with a census population size of approximately 2,000 individuals per generation.

The five CO populations (CO_1-CO_5) were derived from like-numbered O populations in 1989 (Rose *et al.* 1992). Each CO population is maintained on a 28-day discrete life cycle, with flies placed into cages at a density of ~1000 flies/cage. On day 26, yeast paste is added to the medium to stimulate egg-laying, and on day 28/day 0, eggs are collected at 80-120 eggs per vial. In 1991, one A population (ACO_1-ACO_5) was derived from each of the five CO populations and initially subjected to direct selection for accelerated development. After generation 175, these accelerated populations were maintained on a discrete life cycle of 9 days.

Experimental populations were derived from three of the five pairs of ACO and CO populations (ACO_1, ACO_3, ACO_5 and CO_1, CO_3, CO_5). To remove environmental effects and synchronise experiments, all populations were maintained over a control 14 day life cycle for two generations prior to any assay. Prior to experiment, life cycles were staggered in light of evolved development time differences (ACO << CO) with a 48h difference in egg collection time to synchronise eclosion time of adults. Each assay was repeated for three replicate population pairs: CO_1-ACO_1, CO_3-ACO_3, and CO_5-ACO_5.

Female mate choice assay

To test for the existence of premating reproductive isolation between populations as mediated by female preference, we evaluated the mate choice of ACO and CO females. Flies were sorted by sex and collected as virgins within 6 hours of eclosion under light CO_2 anaesthesia. A food dye technique was used to differentiate between males from each regime (developed from Verspoor *et al.* 2015). Dye techniques were calibrated based on tests of mortality, and the volume of dye used did not result in detectable harmful side-effects, measured through changes in lifespan. Vials were dyed three days prior to the assay using 6 drops of red or blue food dye. One day prior to the experiment, male flies were added to coloured vials at a density of 10 flies/vial and left to ingest the dye for 24 hours. Females were acclimated to individual vials for at least 24 hours prior to the assay. At the time of the assay one male of each population identity and colour was mouth aspirated into an empty vial, then flipped into a vial containing an acclimatised female. Mating latency (time to amplexus formation), colour of the mated male and mating duration were recorded. Under the conditions we used, copulations lasted on average between 15-20 minutes. A viable mating was considered one that lasted for more than

195 two minutes, and vials in which mating did not occur within 60 minutes were discarded. This
196 represents a commonly employed design to test mate choice (Dukas 2005, Ghosh & Joshi 2012,
197 Arbuthnott *et al.* 2017). Vials were randomised so that observers were unaware of female
198 identity. Trials were colour balanced so that in half of the trials the ACO male was blue and the
199 CO male red, and in the other half the alternate dye pattern was applied. Flies were 2-3 days old
200 at the time of the assay.

201

202   This assay was repeated over two days, with female choice tested in both a morning (9am-
203 12pm) and afternoon (1pm-4pm) trial each day. In each of the 4 trials, we tested the choice of 50
204 females from both selection regimes for each of the 3 replicate populations. The ratio of
205 homotypic to heterotypic matings was calculated across the 50 replicate vials for each
206 combination of replicate population and selection regime.

207

208 *Male mate choice assay*

209

210   It is difficult to distinguish the influence of male competition and courtship effort from
211 female preference and resistance. By testing individual male mate choice in addition to female
212 choice and contrasting the results, we hoped to uncover the source of any observed non-random
213 mating pattern. From the time of virgin collection, males were maintained at 10 males/vial. Two
214 females, one from each selection regime, were acclimatised to the same vial 24 hours prior to the
215 assay. Female flies from ACO and CO selection treatments could be identified reliably based
216 upon size alone. On the day of the experiment, one male was "pootered" (mouth aspirated) into a
217 vial containing two females. As with the female choice assay, observers recorded mating latency,
218 the size of the mated female (large or small) and mating duration and all other procedures were
219 the same.

220

221 *Group mate choice assay*

222

223   To test for the existence of premating reproductive isolation between ACO and CO
224 populations when mating occurred in a group environment, we evaluated female mate choice in
225 vials containing 10 females and 12 males from each regime. Flies were handled and marked
226 using the procedures outlined above for individual assays. On the day of the assay, male vials of
227 paired ACO and CO populations were combined and then flipped into a female vial. Vials were
228 observed until at least 8 (out of a possible 10) simultaneously copulating pairs were observed,
229 and were then frozen for 60 minutes without disruption to the copulating pairs. The frozen pairs
230 were observed, and the colour of each mated male was recorded. This methodology was repeated
231 over two trials. During each trial, 10 vials were tested for each regime within each replicate
232 population.

233

234 *Hatchability*

235

236   In order to determine the existence of postmating prezygotic isolation we compared the
237 hatch rate of eggs produced from F1 and F2 crosses $CO_i♀ × ACO_i♂$ and $ACO_i♀ × CO_i♂$ as well
238 as the parental crosses $CO_i♀ × CO_i♂$, and $ACO_i♀ × ACO_i♂$ ($i = 1,3,5$). Crosses were performed
239 in vials with 12 virgin females and 10 virgin males. A period of at least 4 hours was allowed for
240 mating and egg laying. From each cross, male and female progeny were collected as virgins and
241 maintained in sex specific vials. Hybrid and parental crosses were staggered in an attempt to
242 synchronise time of eclosion.

243

244   For tests of hatchability, 100 flies of each sex were deposited in a collector bottle with a
245 plate of yeasted food. After an egg laying period of 3 hours the plate was removed. Eggs were
246 transferred into vials using fine brushes and egg collection solution (Ashburner 2005). Each vial
247 contained 90 eggs arranged in a grid pattern to facilitate easy counting. 9 such vials were created
248 for each cross identity, for each of the three replicate populations. 24 hours after egg collection,
249 the proportion of hatched eggs was determined. Vials continued to be maintained under standard
250 conditions to be used in tests of viability, development time, and size.

251

252 *Larvae to adult viability*

253

254   To uncover any survival differences, we compared the larvae to adult viability of hybrids
255 compared to flies from parental crosses. Twelve days after egg collection, the number of eclosed
256 adults in each vial was recorded. The difference between the number of viable eggs and eclosed
257 adults was used as a measure of larvae to adult viability. 9 vials were tested for each cross
258 identity in each replicate population.

259

260 *Development time*

261

262   Development time of flies from hybrid and parental crosses was also analysed as an
263 indicator of hybrid viability. Each vial was observed for eclosion over a period of 4 days,
264 beginning 24 hours prior to the time of expected peak eclosion for each line. For the first 2 days,
265 vials were observed every 6 hours. For the following 2 days, observations were made every 12
266 hours. During each observation, the number of eclosed flies was recorded. An average eclosion
267 time was then calculated for the vial. 9 vials were tested for each cross identity across each
268 replicate population.

269

270 *Body Size*

271

272   Body size was measured using dry weight as a proxy. During peak eclosion, 20 female flies
273 were collected from each cross identity. These flies were given 1 hour to physically mature and
274 then were frozen. Flies were divided into 4 groups of 5 females and placed into tinfoil dishes to

275   be dried in an oven at 70 degrees Celsius for 24 hours. Each dish was measured in a
276   microbalance four times: twice with the flies and twice without. These totals were averaged and
277   divided by 5 to provide an estimate of the dry weight of an individual fly.
278
279   *Fertility - Competitive reproductive fitness*
280
281   An adult fitness assay was completed to test for parental line and hybrid fertility
282   differences. In this assay, red-eyed focal flies competed with control flies marked with a
283   recessive brown-eye marker (bw^1) derived from the baseline. The IVb population was selected as
284   these flies are a common ancestor to both the focal populations, also having an intermediate life-
285   history selection protocol. The recessive nature of bw allowed the proportion of red eyed
286   progeny to be used as a metric of paternity share.
287
288   Focal F1 and F2 flies of the hybrid crosses $CO_i♀ × ACO_i♂$, $ACO_i♀ × CO_i♂$ as well as the
289   parental crosses $CO_i♀ × CO_i♂$, and $ACO_i♀ × ACO_i♂$ (i = 1,3,5) were tested. Crosses were
290   performed in vials with 12 virgin females and 10 virgin males. A period of at least 4 hours was
291   allowed for mating and egg laying. From each cross, male and female progeny (focal flies) were
292   collected as virgins and maintained in sex specific vials at a density of 10 flies/vial.
293
294   Focal flies were anaesthetized under CO_2 and combined with four IVb flies of the same sex
295   (competitors) and four IVb flies of the opposite sex (potential mates). Vials were supplemented
296   with a sparse amount of yeast. Female focal and IVb flies were acclimatised to vials 24 hours
297   prior to the assay. After 48 hours, flies were flipped into new vials where oviposition occurred.
298   Flies were 5-7 days old at the time of the assay. After 18 hours flies were discarded. At the time
299   of eclosion, the number of red eyed and brown eyed offspring produced from the competition
300   assay were counted. Thirty vials for each sex and cross identity were tested for each of the 3
301   replicate populations.
302
303   *Statistical Analysis*
304
305   Statistical analysis was conducted in R version 4.2.3 -- "Shortstop Beagle" (R Core Team
306   2023). Data was visually assessed for residual normality and heterogeneity. Where generalised
307   linear models were used, residual dispersion was tested using the DHARMa package (Hartig
308   2022).
309
310   Female, male and group mate choice data was analysed with a repeated G-test for
311   goodness of fit (Ghosh & Joshi 2012, McDonald 2014). In each case, the ratio of homotypic to
312   heterotypic matings was tested for deviation from a 1:1 null expectation separately for each
313   replicate population and overall for each selection regime. To study the effect of the male and
314   female identity on mating latency and duration, we used linear mixed effects models (LMMs) fit

315   with mated male identity and mated female identity as fixed effects and trial ID and replicate
316   population as crossed random effects. Data from female choice and male choice experiments
317   were analysed independently. In both experiments, mating latency models with raw data
318   produced skewed residual distributions. To correct for this, we used mixed linear models using
319   log(mating latency + 1) as the response variable.
320
321   To determine the effect of cross identity on fly development time and body size, LMMs
322   were fit with cross identity as a fixed effect and replicate population as a random effect. To
323   determine the effect of cross identity on hatchability, viability and the fertility of flies, binomial
324   generalised linear mixed effects models (GLMMs) were fit with cross identity included as fixed
325   effect and parental replicate included as a random effect. Fertility data was analysed separately
326   for males and females. Due to over-dispersion issues, we remodelled the viability data and the
327   fertility data for both sexes with beta-binomial generalised linear mixed effects models using the
328   'glmmTMB' package (Brooks *et al.* 2017).
329
330   Models were fit using the 'lme4' package (Bates et al. 2015). Fixed effects and random
331   effects were analysed using 'anova()', 'ranova()', 'Anova()' or by backwards model selection
332   (Kuznetsova *et al.* 2017, Fox & Weisberg 2019). Post-hoc comparisons between cross identities
333   were performed using the 'emmeans' package (Lenth 2022).

334   **RESULTS**
335
336   *Female Mate Choice Assay*
337
338   Overall, ACO females showed a significantly higher proportion of homotypic (0.57 +/-
339   0.04, mean +/- 1.96*se) compared to heterotypic matings (0.42 +/- 0.04, $P = 0.01$, Figure 1a),
340   with no significant heterogeneity among replicate population pairs ($P = 0.66$, Table S1). The
341   ACO_5 population showed a significant deviation from the null hypothesis, with more homotypic
342   matings compared to heterotypic matings ($P = 0.04$; Figure S1a). Females from the ACO_3
343   population showed a similar trend towards more homotypic matings with borderline significance
344   ($P = 0.05$). ACO_1 females showed no significant difference between mating types ($P = 0.26$).
345   When data was pooled across trials within each replicate population, both ACO_3 and ACO_5
346   populations, but not the ACO_1 population, showed a significant deviation from a random mating
347   ratio ($P_1 = 0.21$, $P_3 = 0.02$, $P_5 = 0.02$). with no significant heterogeneity between individual trials
348   for any of the 3 replicate populations ($P_1 = 0.30$, $P_3 = 0.28$, $P_5 = 0.21$).
349
350   Overall, CO females showed a significantly higher proportion of homotypic (0.60 +/- 0.05)
351   compared to heterotypic matings (0.40 +/- 0.05, $P = < 0.0001$, Figure 1a), with no significant
352   heterogeneity among replicates ($P = 0.65$, Table S1). Two CO populations, CO_1 and CO_3,
353   showed an overall significant deviation from the null hypothesis, with more homotypic
354   copulations ($P_1 = < 0.01$, $P_3 = 0.01$, Figure S1a). CO_5 females showed a trend towards more
355   homotypic matings, but this was not significant ($P = 0.08$). When data was pooled across trials,
356   all three CO populations showed a significant deviation from the null hypothesis of random
357   mating ($P_1 = < 0.001$, $P_3 = 0.01$, $P_5 = 0.04$), with no significant heterogeneity among trials for
358   any of the replicate populations ($P_1 = 0.13$, $P_3 = 0.15$, $P_5 = 0.28$).
359
360   Only mated male identity had a significant effect on mating latency ($P = <0.00001$, Table
361   S2). The crossed random effects of the replicate population and trial were significant ($P =$
362   <0.0001), but the cumulative proportional variance attributed to the random effects of replicate
363   population and trial ID was minimal (0.05). Both ACO and CO females displayed significantly
364   shorter latencies in mating with CO males (11.8 +/- 1.2 min, 11.6 +/- 1.0 min respectively),
365   compared to ACO males (15.3 +/-1.3 min, 15.5 +/- 1.5 min respectively, Figure 2a). There was
366   no significant effect of female identity or interaction between male and female identity.
367
368   Female identity and interaction between male and female identity had significant effects on
369   mating duration ($P = <0.00001$, Table S2). The crossed random effects of the replicate
370   population and trial were significant ($P = <0.0001$), but cumulative proportional variance
371   attributed to the random effects of replicate population and trial was minimal (0.04). In general,
372   ACO females (15.0 +/- 3.8 min) displayed shorter mating durations than CO females (16.7 +/-
373   4.2 min), and both females mated longer with homotypic partners (ACO♀-ACO♂ 15.4 +/-0.4

374   min, ACO♀-CO♂ 14.3 +/- 0.5 min, CO♀-ACO♂ 16.1 +/-0.6 min, CO♀-CO♂ 17.1 +/- 0.4 min).
375   All pairwise contrasts were significant (Figure 2c) with the exception of ACO females mated
376   with ACO males vs CO females mated with ACO males. There was no significant effect of
377   mated male identity.

378

379   *Male Mate Choice Assay*

380

381   Overall, ACO males showed a significantly higher proportion of matings with ACO
382   females (0.64 +/- 0.05) compared to CO females (0.36 +/- 0.05, $P = < 0.00001$, Figure 1b), with
383   no significant heterogeneity between populations ($P = 0.81$, Table S3). All three A populations
384   showed an overall significant deviation from the null hypothesis, displaying more homotypic
385   compared to heterotypic matings ($P_1 = < 0.0001$, $P_3 = 0.01$, $P_5 = < 0.001$). There was significant
386   heterogeneity observed among trials within the ACO_1 population ($P = 0.01$), but not the ACO_3
387   ($P = 0.70$) or ACO_5 ($P = 0.38$) populations.

388

389   Overall, CO males displayed a nearly equal proportion of matings with ACO (0.51 +/-
390   0.02) and CO females (0.49 +/- 0.02, $P = 0.95$, Figure 1b), with no heterogeneity among replicate
391   populations ($P = 0.93$, Table S3). All three CO populations showed no significant deviation from
392   the null hypothesis of random mating ($P_1 = 0.99$, $P_3 = 0.65$, $P_5 = 0.96$). There was also no
393   significant heterogeneity among trials within any of the replicate populations ($P_1 = 0.97$, $P_3 =$
394   0.54, $P_5 = 0.91$).

395

396   None of male identity, female identity or interaction between male & female identity
397   influenced mating latency (Table S2). The crossed random effects of the replicate population and
398   trial were significant ($P = 0.01$), but cumulative proportional variance attributed to the random
399   effects of replicate population and trial was minimal (0.02). Females from the CO populations
400   displayed a slightly higher mating latency with both ACO and CO males (18.0+/-1.7 min,
401   18.1+/-1.6 min respectively), compared to the ACO females (16.6+/-1.1 min, 16.9+/-1.4 min
402   respectively) but these differences were non-significant (Figure 2b).

403

404   Only mated female identity had a significant effect on mating duration ($P = <0.00001$,
405   Table S2). The crossed random effects of the replicate population and trial were significant ($P =$
406   <0.0001), but cumulative proportional variance attributed to the random effects of replicate
407   population and trial ID was minimal (0.04). Males from the both populations displayed a slightly
408   higher mating latency with CO females (16.3+/-0.7 min, 17.3+/-0.6 min respectively), compared
409   to the ACO females (14.8+/-0.5 min, 15.0+/-0.7 min respectively) (Figure 2d). There was no
410   significant effect of male identity or interaction between male and female identity.

411

412   *Group Mate Choice Assay*

413

414   Overall, ACO females showed a significantly higher proportion of matings with CO males
415   (0.62 +/- 0.05) compared to ACO males (0.38 +/- 0.05, P = <0.00001, Figure 1c), with
416   significant heterogeneity being observed between replicate populations (P = 0.01, Table S4).
417   ACO_3 and ACO_5 females showed an overall significant deviation from the null hypothesis,
418   displaying more heterotypic compared to homotypic matings (P_3 = 0.02, P_5 = <0.0001). ACO_1
419   females showed no deviation from the null expectation (P = 0.78). There was no significant
420   heterogeneity observed among trials within any of the three replicate ACO populations (P_1 =
421   0.49, P_3 = 0.45, P_5 = 0.50).
422
423   Overall, CO females showed significantly more homotypic (0.61 +/- 0.04) compared to
424   heterotypic (0.39 +/- 0.04) copulations (P = <0.0001, Figure 1c), with no heterogeneity between
425   replicate populations (P = 0.12, Table S4). CO_5 females showed an overall significant deviation
426   from the null hypothesis, with more homotypic compared to heterotypic copulations (P =
427   <0.001). CO_1 and CO_3 populations showed no overall deviation from a random mating ratio of
428   1:1 (P_1 = 0.10, P_5 = 0.07). When data was pooled across trials, both CO_3 and CO_5 populations
429   showed significant deviation from a random mating ratio (P_3 = 0.02, P_5 = <0.0001). There was
430   no significant heterogeneity among trials within any of the populations (P_1 = 0.10, P_3 = 0.58, P_5
431   = 0.96).
432
433   ***Post-mating prezygotic reproductive isolation***
434
435   <u>*Hatchability*</u>
436
437   Cross identity had a significant effect on egg hatchability (P = <0.01, Table S5). The
438   random effect of replicate population was not significant (P = 0.89). The only significant
439   differences in hatch rate was the decline of $CO_i♀ × A_i♂$ F2 hybrids compared to ACO and CO
440   parental lines (Figure 3a). Overall, hatchability was high with average values between 0.96 +/-
441   0.01 ($CO_i♀ × A_i♂$ F2 hybrids) and 0.98 +/- 0.01 (ACO parentals).
442
443   ***Postzygotic reproductive isolation***
444
445   <u>*Larvae to adult viability*</u>
446
447   There was also a significant effect of cross identity (P = <0.0001, Table S5), but no
448   significant random effect of replicate population (P = 0.31) on the viability of flies. On average,
449   larvae to adult viability was lowest for ACO parental (0.78 +/- 0.03) and highest for $CO_i♀ ×$
450    $ACO_i♂$ F2 flies (0.93 +/- 0.02). Both $CO_i♀ × ACO_i♂$ hybrids and $ACO_i♀ × CO_i♂$ F2 hybrids
451   demonstrated significantly higher viability than ACO, CO and $ACO_i♀ × CO_i♂$ F1 flies(Figure
452   3b). .
453

454 *Development time*

455

456   There was a significant effect of cross identity (P = <0.0001, Table S6), but no
457 significant random effect of replicate population (P = 0.51) on the development time of flies. All
458 four hybrid crosses demonstrated development times that were significantly longer than ACO
459 parental flies (175.76 +/- 0.5 hours) and significantly shorter than CO parental flies (218.32 +/-
460 1.2 hours). There were no significant differences in development times among the four hybrid
461 crosses (Figure 3d).

462

463 *Body size*

464

465   Cross identity also had a significant effect on the body size of flies (P = <0.0001, Table
466 S6). The random effect of the replicate population was not significant (P =0.23). All four hybrid
467 lines showed average individual weights that were significantly higher than parental ACO lines
468 (0.21 +/- 0.01 g), and significantly lower than parental CO lines (0.31 +/- 0.01 g). Hybrid
469 ACO$_i$♀ × CO$_i$♂ F1 flies were also significantly heavier than ACO$_i$♀ × CO$_i$♂ F2 flies (Figure
470 3c).

471

472 *Reproductive fitness*

473

474   Cross identity had a significant effect on female reproductive fitness (P = <0.0001, Table
475 S7). Parental CO lines showed the highest average fitness (0.24 +/- 0.03), parental ACO lines the
476 lowest (0.12 +/- 0.02) and hybrids displayed intermediate fitness between parental lines (Figure
477 4). All cross identities produced significantly more offspring than parental ACO females and
478 flies from all cross identities, except for CO$_i$♀ × ACO$_i$♂ F1 females, produced significantly
479 fewer offspring than parental CO flies. In both cases, second generation (F2) females displayed
480 lower reproductive fitness than first generation (F1) hybrids, but this effect was only significant
481 for the CO$_i$♀ × ACO$_i$♂ crosses. There was no significant effect of population replicate on female
482 fertility (P = 1).

483

484   There was a significant fixed effect of cross identity (P = <0.01, Table S7) but not replicate
485 population (P = 1) on the fertility of male flies. CO$_i$♀ × ACO$_i$♂ F1 male hybrids showed the
486 highest average fitness (0.13 +/- 0.04) and ACO males the lowest (0.05 +/- 0.02). CO and CO$_i$♀
487 × ACO$_i$♂ F1 males demonstrated a significantly higher fitness than ACO males (Figure 4).
488 Similar to that observed in the female assay, male F2 hybrids displayed lower fitness than F1
489 hybrids, but these differences were not significant. While female flies displayed fitness values
490 around the expected value, males of both parental and hybrid cross identities produced a lower
491 proportion of offspring than expected when in competition with four competitors.

DISCUSSION

Our experimental assays show the striking differentiation between the ACO and CO populations after over three decades and 1500 generations of divergent selection on demography. The ACO population adults eclose nearly two days earlier than the CO population adults do. The clear tradeoff between speed and size is evident, as A adults are about 33% lighter than CO adults, and this is likely related to the strong differences in adult fitness we observed between the two selection treatments. Interestingly, the tradeoff between developmental speed and juvenile viability (Chippindale et al. 1997) is not apparent here or in other recent unpublished data on these populations, suggesting compensatory evolution over time. Given these differences in morphology and life history, we asked if we could detect the early stages of the speciation process for these populations in allopatry under different ecological circumstances.

To summarise our results, we found evidence for the evolution of premating barriers to gene flow between these diverged populations. Mate-choice assays generally reflected positive assortative mating patterns driven by female mate-choice. Females from both focal populations showed strong homotypic mate choice in individual tests, but this pattern was less clear in group tests, particularly as the smaller ACO females tended towards heterotypic mates. Under conditions of female choice, mating durations of homotypic pairs were consistently longer than heterotypic pairs. Despite this (and homotypic mate choice), the larger CO males showed lower latencies to mating with both females. In contrast, under conditions of male choice (no male-male competition), ACO and CO males showed no differences in mating latency. Further, under male choice conditions, both males mated for longer durations with the larger CO females, suggesting sperm catering based on body size rather than homotypic preference. We show that post-mating prezygotic barriers have not evolved to the point of detectability between these populations in the form of gamete incompatibility, measured through egg hatchability. We also find no evidence of postzygotic reproductive isolation in the form of survival to reproductive maturity, and measurements of development time and body size show that hybrids display intermediate trait values relative to the parental treatments. We observed only suggestive trends for postzygotic reproductive isolation in the form of hybrid breakdown in competitive reproductive fitness assays. F_1 hybrids display intermediate fitness to that of the two parentals, however, there is a tendency for F_2 hybrid offspring to display lower fitness than the F_1 hybrids; while potentially an indicator of evolving Dobzhansky-Muller incompatibilities, our data are merely suggestive at this juncture, meriting further investigation.

It has been suggested that prezygotic isolating barriers will typically evolve before postzygotic barriers, and *Drosophila* seems to adhere to this rule (Coyne & Orr 2004, Arthur & Dyer 2015). Within the category of prezygotic barriers, premating RI is expected to evolve before postmating-prezygotic RI (Turissini et al. 2018). Our data are generally in accord with

529 these predictions, as our diverged population pairs show marked premating reproductive
530 isolation, at least under individual choice tests, but clear evidence for later stage RI is lacking.
531
532   Strong divergence in body size exists between ACO and CO animals (Xiao et al. 2019; data
533 herein) and are presumed to correlate with differences in persistence/resistance capabilities
534 between the selection treatments. This size difference led us to expect the presence of some
535 premating reproductive isolation. However, we predicted premating isolation in one direction, on
536 the basis of greater resistance from larger (CO) females, greater persistence of CO males, and a
537 possible preference for large mates. That directional RI was noted by Ghosh & Joshi (2012) in a
538 study similar to ours: bigger mates were preferred by both large/slow and small/fast flies.
539 Contrary to that precedent, females of both regimes displayed strong homotypic mate choice and
540 longer mating durations with their homotypic partners in individual tests. This result is especially
541 intriguing for the ACO females, as they displayed a preference for smaller ACO males even
542 though *D. melanogaster* females are expected to prefer larger males (Pitnick 1991, Jagadeeshan
543 et al. 2015, but see Prasad et al. 2007).
544
545   Male mate choice tests may present trickier interpretations. ACO selected males largely
546 mated with homotypic females, but male Cos mated nearly randomly. While ACO males appear
547 to be showing positive assortative mating preferences, both types of males were observed
548 making repeated courtship attempts with both homotypic and heterotypic females (unquantified
549 observations) suggesting that the skewed mating pattern could be the result of the increased
550 capability of CO females to resist unwanted mating attempts, particularly from the smaller A
551 males. In alignment with this hypothesis, we observed a non-significant trend of longer mating
552 latencies with CO females. In populations of flies evolved under a similar pair of life history
553 selection regimes, Ghosh and Joshi (2012) found that small males attempted to court large
554 females, but were unsuccessful, and, as a result, mated more frequently with small females. We
555 therefore cannot easily ascribe mating assortment to the choice of the male, who may be
556 undiscriminating under the test conditions, particularly with no competition for mates.
557 Concurrent with this, we note no differences in the mating latencies of the two male types in the
558 absence of male-male competition. ACO-type females are also under tremendous pressure to
559 mate, as they are selected to lay eggs about 24h after becoming sexually mature. In the absence
560 of competition, many males may have simply taken the most receptive mate first; for the CO
561 males this may have counterbalanced a homotypic preference, creating the ambivalent outcome
562 observed. The results of the mate choice assays taken together suggest that premating isolation in
563 this system is most strongly driven by female choice.
564
565   Body size is a likely trait involved in the evolution of reproductive isolation between the
566 population pairs tested. However, it remains possible that the patterns of assortative mating stem
567 from traits that are yet to be characterised. Some possibilities include courtship behaviours
568 (mating call, wing movements) and cuticular hydrocarbon profiles that may have diverged under

569   the divergent selection regimes. Besides female preference, body size differences also play a
570   clear role in the differential capacities displayed by females to resist mating attempts by males,
571   which creates a further reproductive barrier between large females and small males. Despite
572   large body size differences between the selection treatments, it seems unlikely that the patterns
573   of positive assortative mating can be explained by mechanical incompatibilies in copulation
574   alone; some earlier anecdotal observations suggested they may exist, but we saw no clear
575   evidence in these trials.
576

577   In the group mate choice assays, female CO flies continued to show homotypic mating
578   preference, but A females displayed reversed preferences, mating more frequently with
579   heterotypic males than homotypic males. The existence of heterotypic mate choice in groups, in
580   spite of homotypic mate choice in individual assays suggests that the nature of the assay plays a
581   role in the mating patterns observed. In the group mating assays, vials were more crowded (34
582   individuals vs. 3) - potentially creating circumstances where resisting forcible mating-attempts is
583   more difficult for females. This is a likely explanation given that only the small A females
584   displayed reversed mate choice preferences, while the larger CO females largely reproduced the
585   same mate choice patterns as displayed in individual assays. It is worth testing if similar mate
586   choice assays conducted in larger arenas (such as 90mm petri dishes) would reverse this
587   preference to homotypic choice. At the time these experiments were conducted (since changed)
588   there was an important difference in the mating environment of the ACO and CO selection
589   treatments: whilst the early-reproducing ACO's primarily mated in vials, with only a brief phase
590   in cages for egg-laying, the CO's, with 14 days in cages probably do all mating relevant to
591   fitness in the cage environment. With these differences in the "natural" mating environment of
592   our LTEE, the mate choice patterns found in individual assays seem to show us that A-selected
593   females have a preference for homotypic mating but are unable to exercise it in a group mating
594   setting with larger males.
595

596   Asynchronous development time alone can contribute to reproductive isolation, as seen in
597   the experimental work of Miyatake and Shimuzu (1999) on the melon fly. Because the mating
598   times diverged in lines selected for development time, significant RI arose that was evidenced in
599   experimental mate choice trials. Similarly, the extreme early reproduction regime of the A
600   populations means that neither CO individuals nor A/CO hybrids could hybridize, as the latter
601   groups would not even be sexually mature before a generation is completed. This scenario is, of
602   course, artificial because natural populations generally do not grow on synchronised, discrete
603   generations. they are not directly responsible for the assortative mating patterns observed in the
604   controlled mate-choice assays.
605

606   Post-mating, we report no evidence of reproductive isolation deriving from gamete
607   incompatibility, as would be evident in reduced egg hatch rates. Similarly, our measurements of
608   larva to adult viability data show that there are no hybrid treatments with lower levels of viability

than the purebred parental populations. In fact, we observed trends of hybrid vigour, with some of the hybrid treatments displaying higher larva to adult viability than the parental treatments. Interestingly, the larva to adult viabilities in F2 treatments were significantly higher than that of the parental selection treatments and one F1 treatment. While the increased viability in hybrids could suggest the existence of homozygous deleterious variation in developmental loci in our selection regimes, this should be consistently seen in both F1 and F2 generations, instead of being more common in the F2 generation. Development time and body size displayed no suggestion of abnormalities indicative of developmental instability, with intermediate hybrid trait values relative to the parental treatments in both sexes.

The competitive reproductive fitness of hybrids was generally intermediate to the parental treatments, and we found no evidence of hybrid sterility. Given that both assays subjected a focal fly to 4 competitors of the same sex, we expect focal flies to sire/dam ⅕th of the offspring produced, if all else was equal. In female reproductive fitness assays, our populations did cluster around the predicted threshold, with hybrids scoring intermediate fitness levels relative to the low fitness ACOs and high fitness COs. In male reproductive fitness assays though, fitness fell well below the predicted threshold in all treatments. We rule out the possibility of this being a case of Haldane's rule, because male fitness falls well below predicted levels in parental males as well as hybrids. We believe that this is a consequence of the usage of IVb flies as competitors/mates in these assays. Selected as common ancestors to both selection regimes, the IVbs have evolved in allopatry with both the populations, with a slightly different maintenance regime. We believe that the reduced male fitness estimates may be a consequence of homotypic mate choice by IVb females in male fitness assays. While we do see different levels of competitive reproductive output for the different treatments in the female assay, it is not possible to disentangle male mate choice from innate fecundity and harm resistance differences in the females of different selection regimes. Overall, in spite of the depressed male fitness output relative to predictions, hybrids in both sexes still consistently showed intermediacy in fitness, suggesting no reproductive sterility and a link to an additive trait such as body size.

The comparison between F1 and F2 reproductive fitness revealed an interesting trend: F1 fitness was consistently higher than that of F2 offspring, however, this difference was statistically significant in only 1 out of 4 comparisons (2 reciprocal crosses *2 sexes). We interpret this with caution as a possible trend of hybrid breakdown as a consequence of incompatible gene complexes evolving in the two selection regimes.

We generally found high repeatability across our three independent replicates, which suggests that the characters measured and pre-mating RI observed stem from consistent treatment differences between the CO and ACO populations. Internal random processes of divergence in allopatry, such as the coevolution of arbitrary signaller-receiver relationships in allopatry may also drive the evolution of RI. A system like ours opens the door to a potential test

649   for such divergence if RI were investigated between replicate populations *within* selection

650   treatment, rather than *between* treatments. Although our three-decade experiment seems too

651   young to see more than hints of post-mating RI, more sensitive queries of characters like

652   developmental stability (e.g., as measured by fluctuating asymmetry) may be of interest. Further

653   investigation of the sources of premating RI and its environmental sensitivity are warranted.

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866

867   **FIGURE CAPTIONS**

868

869   **Figure 1.** The proportion of homotypic compared to heterotypic matings recorded during the
870   female (a), male (b) and group (c) mate choice assay. Female (a,c) and male population identity
871   is shown on the x-axis. An asterisk represents a significant deviation from a random mating ratio
872   as indicated by a repeated G-test for goodness of fit (p<0.05).

873

874   **Figure 2.** The latency (a,b) and duration (c,d) of matings in the choice assays. Female (a,c) and
875   male (b,d) population identity is shown on the x-axis. Letters indicate significant differences
876   between mating types.

877

878   **Figure 3.** The hatchability (a), egg to adult viability (b), body weights (c) and average
879   development time (d) of flies from parental and hybrid crosses. Letters indicate significant
880   differences between cross identities.

881

882   **Figure 4.** The average proportion of red eyed offspring produced by flies from parental and
883   hybrid crosses, in competition with brown eyed competitors. Average proportions were
884   determined for each replicate population and plotted. The dashed line indicates the expected
885   proportion of ⅓ of the offspring. Letters indicate significant differences between cross identities.
886

1   **Evolution of reproductive isolation in a long-term evolution experiment with *Drosophila***
2   ***melanogaster*: 30 years of divergent life history selection.**
3

4   **ABSTRACT**
5

6   We ask if three decades and over 1 500 generations of divergent life history selection on
7   age at reproduction has resulted in the evolution of reproductive isolation (RI) between
8   laboratory populations of *Drosophila melanogaster*. We tested for premating, postmating-
9   prezygotic and postzygotic reproductive isolation between 3 replicate population pairs. Large
10 evolved differences in body size between selection treatments suggested the potential for
11 prezygotic barriers driven by sexual selection or physical incompatibilities between the sexes.
12 Although a simple prediction would be preference for larger size, creating directional isolation,
13 our results from individual mate choice trials indicate that populations from both selection
14 treatments show a marked bias towards homotypic mate choice; indicative of prezygotic RI
15 driven by sexual selection or sexual conflict. Hybridization between the focal populations
16 resulted in the production of viable adult flies with intermediate size and developmental traits.
17 We observed a suggestive but statistically non-significant trend of fitness decline in the F2
18 generation of hybrids, but no significant evidence suggesting the evolution of postmating-
19 prezygotic or postzygotic RI. Our findings are in accord with extant literature that posits that
20 premating RI evolves before postmating forms of RI.
21
22

INTRODUCTION

Understanding the mechanisms underlying the evolution of reproductive isolation and the timing of their origination is central to the study of speciation. With the fossil record yielding limited information about this process on microevolutionary timescales, there has been a focus on extant incipient species, either where populations naturally intermingle in contact zones or through experimental crosses. Such populations, part way through the process of RI, can help to illuminate the nature of divergence, as indicated by premating isolation or hybrid inferiority due, for example, to antagonistic epistasis between incompatible alleles (Dobzhansky 1937; Muller 1942) and hence offer a snapshot of speciation in action. But what selective agents or historical contingencies have driven the divergence may or may not be apparent, particularly as replication and controls may be absent or limited, and partial isolation and changes in the environment can lead to reversals in the development of RI (Lackey & Boughman 2017), complicating the inference of sequence in the evolution of isolating mechanisms.

Here we focus on early stages of ecological speciation. ~~In this work, we focus on the role of divergent life-history selection in ecological speciation.~~ Ecological speciation is the process through which reproductive isolation evolves as a consequence of local adaptation with limited gene flow. Divergent selection pressures can contribute to this process by acting directly, or through pleiotropy or tight linkage with genes that are directly responsible for reproductive isolation (Rice and Hostert 1993; Rundle and Nosil 2005). Divergent evolved responses between ecotypes may result in (1) changes in morphology, mating behaviours or physiological characters that hinder mating attempts mechanically or via mate choice (premating reproductive isolation); (2) changes in gametes/reproductive machinery that inhibit fertilization (postmating-prezygotic reproductive isolation); and/or (3) genetic incompatibilities that reduce survival or reproductive success of hybrid individuals (postzygotic reproductive isolation).

In addition to divergent natural selection in allopatric populations, divergent intersexual coevolution via sexual selection has long been recognized to have a potentially significant role in the evolution of reproductive isolation (Lande 1981; Maan and Seehausen 2011; Panhuis *et al*. 2001; Ritchie 2007; Singh and Singh 2014). Likewise, interlocus sexual conflict – where traits that increase the fitness of one sex directly cost the other – can result in rapid coevolution (an "evolutionary chase" or "arms race") between male and female reproductive traits (Rice & Holland 1997; Parker & Partridge 1998; Rice 1998) and potentially accelerate the process of speciation (Ritchie 2007; Gavrilets 2014; Syed *et al.* 2017); the evolution of female resistance to reduce the direct costs of mating is predicted to contribute to assortative mate choice (Gavrilets *et al.* 2001).

While natural systems such as hybrid zones have advanced our understanding of the evolution of RI immensely, researchers are frequently limited to indirect inferences, such as using estimates of gene flow and clines to infer reproductive isolation. Beyond knowledge of the

61 evolutionary history, these systems limit the number RI barriers that can be assessed, and
62 generally lack replication at the population-pair level. Notable exceptions include stickleback
63 fishes, where repeated invasion of freshwater from marine ancestors, and subsequent
64 differentiation into limnetic-benthic and lake-stream ecotypes provide extensive replication
65 (Lackey & Boughman 2017); and storm-petrels, where repeated separation of populations by
66 breeding time (allochrony) provides replicated evidence of reproductive isolation in sympatry
67 (Wallace *et al.* 2017). Experimental evolution can be a valuable tool for more direct
68 investigation, allowing us to probe potential drivers of speciation between populations with well
69 understood evolutionary histories. Experimental studies in speciation have attempted to test if (1)
70 non-ecological drivers like drift, (2) negative selection against hybridization between ecotypes,
71 (3) divergent selection on populations or (4) sexual conflict produced reproductive isolation
72 (reviewed in Rice and Hostert 1993; Coyne and Orr 2004; Fry 2009; White *et al.* 2020). An
73 obvious limitation to experimental evolutionary studies of speciation is time: research projects
74 rarely exceed granting cycles and few organisms lend themselves to experimental evolution. It is
75 little surprise then that *Drosophila* is far and away the genus most investigated in the laboratory.

76   Early divergent selection studies produced seemingly equivocal results with respect to
77 creating reproductive isolation. A closer examination suggested that divergent selection was
78 highly effective, given a pre-existent tendency to mate assortatively on the basis of the trait
79 selected upon (Rice and Hostert 1993; Fry 2009). Divergent selection upon behavioural traits
80 resulted in the correlated evolution of reproductive isolation (del Solar 1966; Soans *et al.* 1974;
81 Hurd and Eisenberg 1975; Lofdahl *et al.* 1992) while divergent selection on other traits like
82 bristle number (Barker and Cummins 1969; Santibañez and Waddington 1958) produced
83 negative results. More recently, experimental evolution studies have focused on selection
84 regimes that do not directly involve characters that are known to influence intra-population
85 assortment. For example, a number of studies have investigated the importance of local
86 adaptation to distinct nutrition sources for mating assortment. Divergent selection regimes with
87 different nutritional environments result in premating isolation through (1) the evolution of
88 signalling traits and mating preferences in *Drosophila serrata* (Rundle et al. 2005), (2) the
89 evolution of symbiotic microbiota in inbred strains of *D. melanogaster* (Najarro *et al.* 2015;
90 Sharon *et al.* 2010), (3) competitive ability differences between controls and selected individuals
91 in *D. melanogaster* (Belkina *et al.* 2018), (4) positive assortative mating preferences (albeit
92 unstable) in *D. melanogaster* (albeit unstable) (Nash *et al.* 2019) and in *Saccharomyces*
93 *cerevisiae* (Mahilkar *et al.* 2023); and (5) postzygotic reproductive isolation through genetic
94 incompatibilities in *Saccharomyces cerevisiae* (Dettman *et al.* 2007) and *Neurospora* (Dettman
95 *et al.* 2008).

96   Life history traits are less frequently studied in the context of reproductive isolation, but
97 have also received some attention – predominantly in studies of allochronic speciation. Powell *et*
98 *al.* (2020) note how adaptation to differences in seasonal timing has been shown to contribute to
99 reproductive isolation in a wide variety of organisms including plants (Savolainen *et al.* 2006;

100   Lowry *et al.* 2008), insects (Tauber *et al.* 1977; Wood & Guttman 1982; Horner *et al.* 1999;
101   Eubanks *et al.* 2003; Abbot & Withgott 2004; Forbes *et al.* 2009; Ording *et al.* 2009; Wadsworth
102   *et al.* 2013; Hood *et al.* 2015) and vertebrates (Friesen *et al.* 2007). Speciation through other life-
103   history traits and correlated changes in intersexual coevolution has received less attention, due to
104   the myriad challenges associated with studying these systems in nature. For example, dDivergent
105   selection on pre-adult development time has been shown to produce pre-mating isolation through
106   (1) disturbances in circadian rhythm resulting in changes in phases of mating in the melon fly,
107   *Bactrocera cucurbitae* (Miyatake and Shimizu 1999), and (2) evolved body size differences, and
108   correlated levels of sexual conflict, in *Drosophila melanogaster* (Ghosh and Joshi 2012).
109   Likewise, divergent selection on body size results in premating isolation, through mechanical
110   incompatibilities during attempted copulations between *Columbicola* feather mites (Villa *et al.*
111   2019).

112   Age-at-reproduction has been extensively manipulated using experimental evolution in
113   *Drosophila* to investigate correlated changes in longevity, development time, body size and a
114   slew of associated traits (Prasad and Joshi 2003). In this study, we use an exceptionally long term
115   evolution experiment (LTEE) with population pairs separated by over 1,500 generations and
116   three decades under divergent selection in allopatry. The two *D. melanogaster* life-history
117   selection regimes used in this study are selected to reproduce late in life (4 weeks from egg; CO)
118   and extremely early in life (9 days from egg; Accelerated CO / ACO). The ACO populations
119   have evolved small body size, rapid development time, and reduced lifespan (Chippindale *et al.*
120   1997a; Chippindale *et al.* 2004a). On the other hand, the CO flies are big, develop slowly and
121   likely have an increased ability to resist copulations as females and to inflict harm as males
122   (Chippindale *et al.* 2004b; Chippindale *et al.* 1997b; Mital *et al.* 20221, Verma *et al.* 2022).

123   In the speciation literature, body size is identified as a crucial trait that can drive the
124   evolution of reproductive isolation (Servedio et al. 2011). In *D. melanogaster*, body size is
125   strongly correlated with juvenile development time (Chippindale *et al.* 1997a; Nunney 1996;
126   Prasad and Joshi 2003; Prasad *et al.* 2000; Zwaan *et al.* 1995) and life-history selection on
127   development has been shown to result in the evolution of body size (Chippindale *et al.* 1997a;
128   Prasad *et al.* 2000). An important sexually selected trait for both sexes, large females tend to be
129   more fecund (Roff 2002; Stearns 1992) and attract higher courtship effort from males (Byrne and
130   Rice 2006); while large males have increased competitive abilities (Markow and Ricker 1992;
131   Partridge and Farquhar 1983), and secure more frequent copulations as a consequence of female
132   preference (Markow 1986; Partridge *et al.* 1987, but see Prasad et al. 2007). Given the evolved
133   size differences between the ACOs and COs, a simple prediction is that individuals of both
134   population-types display a mating preference for individuals from the larger CO populations,
135   producing conditions of partial, directional, reproductive isolation driven by sexual selection on
136   the basis of body size, along the lines observed by Ghosh and Joshi (2012).

137   In *D. melanogaster,* divergent reproductive strategies between the sexes have long been
138   recognized (Bateman 1948) potentially resulting in interlocus sexual conflict. Frequent matings

139 increase male fitness but can decrease female fitness as a result of male harassment and the
140 harmful effects of genital wounding and seminal fluid (Chapman et al. 1995; Bonduriansky *et al.*
141 2008; Travers *et al.* 2015). The harm experienced by females as a result of mating, measured by
142 lifespan and egg production rate, is correlated with male body size (Pitnick and García–González
143 2002; Friberg and Arnqvist 2003). In the context of our two selection treatments, we predict that
144 male harm and female resistance traits will be stronger in the CO populations compared to the
145 ACO populations, due to the difference in body size between the large COs and smaller ACOs.
146 In experimental hybridization between CO and ACO lines, this difference could drive premating
147 isolating barriers to evolve in one direction, with CO females resistant to mating with the smaller
148 ACO males, and ACO females vulnerable to unwanted mating with CO males. Indeed, working
149 with a similar system, Ghosh and Joshi (2012) found such one-directional isolation, driven by
150 large females resisting mating attempts by small males, and a preference for (or lack of
151 resistance to) large males in females of both treatments. On the other hand, especially with the
152 large number of generations in allopatry, mating signals and preferences may have diverged
153 between populations in our selection experiment creating the possibility for positive assortment
154 in mate choice experiments.

155   We investigated mate choice through individual female, male, and group mating choice
156 assays in the ACO/CO complex of selected lines. We further looked for potential gametic
157 incompatibilities (postcopulatory prezygotic isolation) by comparing egg hatchability in hybrid
158 matings to the parental populations. Finally we looked for postzygotic effects (hybrid breakdown
159 or vigour) in developmental traits, body size, and adult fitness.

METHODS

Experimental populations

162   Our experiments employed the accelerated development (ACO) (Chippindale *et al.* 1997a)
163   and control-old (CO) (Rose et al. 1992) populations. All populations are maintained under
164   standard laboratory conditions: moderate density (larval density of 80 - 120 larvae / vial), 25ºC,
165   12:12 light:dark cycle on banana/agar/killed-yeast medium, and with a census population size of
166   approximately 2,000 individuals per generation.

167   The five CO populations (CO_1-CO_5) were derived from like-numbered O populations in
168   1989 (Rose *et al.* 1992). Each CO population is maintained on a 28-day discrete life cycle, with
169   flies placed into cages at a density of ~1000 flies/cage. On day 26, yeast paste is added to the
170   medium to stimulate egg-laying, and on day 28/day 0, eggs are collected at 80-120 eggs per vial.
171   In 1991, one A population (ACO_1-ACO_5) was derived from each of the five CO populations and
172   initially subjected to direct selection for accelerated development. After generation 175, these
173   accelerated populations were maintained on a discrete life cycle of 9 days.

174   Experimental populations were derived from three of the five pairs of ACO and CO
175   populations (ACO_1, ACO_3, ACO_5 and CO_1, CO_3, CO_5). To remove environmental effects and
176   synchronise experiments, all populations were maintained over a control 14 day life cycle for
177   two generations prior to any assay. Prior to experiment, life cycles were staggered in light of
178   evolved development time differences (ACO << CO) with a 48h difference in egg collection
179   time to synchronise eclosion time of adults. Each assay was repeated for three replicate
180   population pairs: CO_1-ACO_1, CO_3-ACO_3, and CO_5-ACO_5.
181
182   *Female mate choice assay*
183
184   To test for the existence of premating reproductive isolation between populations as
185   mediated by female preference, we evaluated the mate choice of ACO and CO females. Flies
186   were sorted by sex and collected as virgins within 6 hours of eclosion under light CO_2
187   anaesthesia. A food dye technique was used to differentiate between males from each regime
188   (developed from Verspoor *et al.* 2015). Dye techniques were calibrated based on tests of
189   mortality, and the volume of dye used did not result in detectable harmful side-effects, measured
190   through changes in lifespan. Vials were dyed three days prior to the assay using 6 drops of red or
191   blue food dye. One day prior to the experiment, male flies were added to coloured vials at a
192   density of 10 flies/vial and left to ingest the dye for 24 hours. Females were acclimated to
193   individual vials for at least 24 hours prior to the assay. At the time of the assay one male of each
194   population identity and colour was mouth aspirated into an empty vial, then flipped into a vial
195   containing an acclimatised female. Mating latency (time to amplexus formation), colour of the
196   mated male and mating duration were recorded. Under the conditions we used, copulations lasted
197   on average between 15-20 minutes. A viable mating was considered one that lasted for more than

198   two minutes, and vials in which mating did not occur within 60 minutes were discarded. This
199   represents a commonly employed design to test mate choice (Dukas 2005, Ghosh & Joshi 2012,
200   Arbuthnott *et al.* 2017). Vials were randomised so that observers were unaware of female
201   identity. Trials were colour balanced so that in half of the trials the ACO male was blue and the
202   CO male red, and in the other half the alternate dye pattern was applied. Flies were 2-3 days old
203   at the time of the assay.
204
205   This assay was repeated over two days, with female choice tested in both a morning (9am-
206   12pm) and afternoon (1pm-4pm) trial each day. In each of the 4 trials, we tested the choice of 50
207   females from both selection regimes for each of the 3 replicate populations. The ratio of
208   homotypic to heterotypic matings was calculated across the 50 replicate vials for each
209   combination of replicate population and selection regime.
210
211   *Male mate choice assay*
212
213   It is difficult to distinguish the influence of male competition and courtship effort from
214   female preference and resistance. By testing individual male mate choice in addition to female
215   choice and contrasting the results, we hoped to uncover the source of any observed non-random
216   mating pattern. From the time of virgin collection, males were maintained at 10 males/vial. Two
217   females, one from each selection regime, were acclimatised to the same vial 24 hours prior to the
218   assay. Female flies from ACO and CO selection treatments could be identified reliably based
219   upon size alone. On the day of the experiment, one male was "pootered" (mouth aspirated) into a
220   vial containing two females. As with the female choice assay, observers recorded mating latency,
221   the size of the mated female (large or small) and mating duration and all other procedures were
222   the same.
223
224   *Group mate choice assay*
225
226   To test for the existence of premating reproductive isolation between ACO and CO
227   populations when mating occurred in a group environment, we evaluated female mate choice in
228   vials containing 10 females and 12 males from each regime. Flies were handled and marked
229   using the procedures outlined above for individual assays. On the day of the assay, male vials of
230   paired ACO and CO populations were combined and then flipped into a female vial. Vials were
231   observed until at least 8 (out of a possible 10) simultaneously copulating pairs were observed,
232   and were then frozen for 60 minutes without disruption to the copulating pairs. The frozen pairs
233   were observed, and the colour of each mated male was recorded. This methodology was repeated
234   over two trials. During each trial, 10 vials were tested for each regime within each replicate
235   population.
236
237   *Hatchability*

238

239   In order to determine the existence of postmating prezygotic isolation we compared the
240   hatch rate of eggs produced from F1 and F2 crosses $CO_i♀ × ACO_i♂$ and $ACO_i♀ × CO_i♂$ as well
241   as the parental crosses $CO_i♀ × CO_i♂$, and $ACO_i♀ × ACO_i♂$ ($i = 1,3,5$). Crosses were performed
242   in vials with 12 virgin females and 10 virgin males. A period of at least 4 hours was allowed for
243   mating and egg laying. From each cross, male and female progeny were collected as virgins and
244   maintained in sex specific vials. Hybrid and parental crosses were staggered in an attempt to
245   synchronise time of eclosion.

246

247   For tests of hatchability, 100 flies of each sex were deposited in a collector bottle with a
248   plate of yeasted food. After an egg laying period of 3 hours the plate was removed. Eggs were
249   transferred into vials using fine brushes and egg collection solution (Ashburner 2005). Each vial
250   contained 90 eggs arranged in a grid pattern to facilitate easy counting. 9 such vials were created
251   for each cross identity, for each of the three replicate populations. 24 hours after egg collection,
252   the proportion of hatched eggs was determined. Vials continued to be maintained under standard
253   conditions to be used in tests of viability, development time, and size.

254

255   *Larvae to adult viability*

256

257   To uncover any survival differences, we compared the larvae to adult viability of hybrids
258   compared to flies from parental crosses. Twelve days after egg collection, the number of eclosed
259   adults in each vial was recorded. The difference between the number of viable eggs and eclosed
260   adults was used as a measure of larvae to adult viability. 9 vials were tested for each cross
261   identity in each replicate population.

262

263   *Development time*

264

265   Development time of flies from hybrid and parental crosses was also analysed as an
266   indicator of hybrid viability. Each vial was observed for eclosion over a period of 4 days,
267   beginning 24 hours prior to the time of expected peak eclosion for each line. For the first 2 days,
268   vials were observed every 6 hours. For the following 2 days, observations were made every 12
269   hours. During each observation, the number of eclosed flies was recorded. An average eclosion
270   time was then calculated for the vial. 9 vials were tested for each cross identity across each
271   replicate population.

272

273   *Body Size*

274

275   Body size was measured using dry weight as a proxy. During peak eclosion, 20 female flies
276   were collected from each cross identity. These flies were given 1 hour to physically mature and
277   then were frozen. Flies were divided into 4 groups of 5 females and placed into tinfoil dishes to

278   be dried in an oven at 70 degrees Celsius for 24 hours. Each dish was measured in a
279   microbalance four times: twice with the flies and twice without. These totals were averaged and
280   divided by 5 to provide an estimate of the dry weight of an individual fly.
281
282   *Fertility - Competitive reproductive fitness*
283
284   An adult fitness assay was completed to test for parental line and hybrid fertility
285   differences. In this assay, red-eyed focal flies competed with control flies marked with a
286   recessive brown-eye marker (bw^1) derived from the baseline. The IVb population was selected as
287   these flies are a common ancestor to both the focal populations, also having an intermediate life-
288   history selection protocol. The recessive nature of *bw* allowed the proportion of red eyed
289   progeny to be used as a metric of paternity share.
290
291   Focal F1 and F2 flies of the hybrid crosses $CO_i♀ × ACO_i♂$, $ACO_i♀ × CO_i♂$ as well as the
292   parental crosses $CO_i♀ × CO_i♂$, and $ACO_i♀ × ACO_i♂$ ($i = 1,3,5$) were tested. Crosses were
293   performed in vials with 12 virgin females and 10 virgin males. A period of at least 4 hours was
294   allowed for mating and egg laying. From each cross, male and female progeny (focal flies) were
295   collected as virgins and maintained in sex specific vials at a density of 10 flies/vial.
296
297   Focal flies were anaesthetized under CO_2 and combined with four IVb flies of the same sex
298   (competitors) and four IVb flies of the opposite sex (potential mates). Vials were supplemented
299   with a sparse amount of yeast. Female focal and IVb flies were acclimatised to vials 24 hours
300   prior to the assay. After 48 hours, flies were flipped into new vials where oviposition occurred.
301   Flies were 5-7 days old at the time of the assay. After 18 hours flies were discarded. At the time
302   of eclosion, the number of red eyed and brown eyed offspring produced from the competition
303   assay were counted. Thirty vials for each sex and cross identity were tested for each of the 3
304   replicate populations.
305
306   *Statistical Analysis*
307
308   Statistical analysis was conducted in R version 4.2.3 -- "Shortstop Beagle" (R Core Team
309   2023). Data was visually assessed for residual normality and heterogeneity. Where generalised
310   linear models were used, residual dispersion was tested using the DHARMa package (Hartig
311   2022).
312
313   Female, male and group mate choice data was analysed with a repeated G-test for
314   goodness of fit (Ghosh & Joshi 2012, McDonald 2014). In each case, the ratio of homotypic to
315   heterotypic matings was tested for deviation from a 1:1 null expectation separately for each
316   replicate population and overall for each selection regime. To study the effect of the male and
317   female identity on mating latency and duration, we used linear mixed effects models (LMMs) fit

318   with mated male identity and mated female identity as fixed effects and trial ID and replicate
319   population as crossed random effects. Data from female choice and male choice experiments
320   were analysed independently. In both experiments, mating latency models with raw data
321   produced skewed residual distributions. To correct for this, we used mixed linear models using
322   log(mating latency + 1) as the response variable.
323
324   To determine the effect of cross identity on fly development time and body size, LMMs
325   were fit with cross identity as a fixed effect and replicate population as a random effect. To
326   determine the effect of cross identity on hatchability, viability and the fertility of flies, binomial
327   generalised linear mixed effects models (GLMMs) were fit with cross identity included as fixed
328   effect and parental replicate included as a random effect. Fertility data was analysed separately
329   for males and females. Due to over-dispersion issues, we remodelled the viability data and the
330   fertility data for both sexes with beta-binomial generalised linear mixed effects models using the
331   'glmmTMB' package (Brooks *et al.* 2017).
332
333   Models were fit using the 'lme4' package (Bates et al. 2015). Fixed effects and random
334   effects were analysed using the 'anova()' and 'ranova()', ~~and~~ 'Anova()' or ~~and~~ by backwards
335   model selection ~~for LMMs and GLMMs, respectively~~ (Kuznetsova *et al.* 2017, Fox & Weisberg
336   2019). Post-hoc comparisons between cross identities were performed using the 'emmeans'
337   package (Lenth 2022).~~Statistical analysis was conducted in RStudio version 1.3.1073 (RStudio~~
338   ~~Team 2020) and R version 1.3.1073 (R Core Team 2020). Data was visually assessed for~~
339   ~~residual normality and heterogeneity. Female, male and group mate choice data was analysed~~
340   ~~with a repeated G-test for goodness of fit (Ghosh & Joshi 2012, McDonald 2014). In each case,~~
341   ~~the ratio of homotypic to heterotypic matings was tested for deviation from a 1:1 null expectation~~
342   ~~separately for each replicate population and overall for each selection regime. To determine the~~
343   ~~effect of cross identity on fly development time and body size, linear mixed effects models~~
344   ~~(LMMs) were fit with cross identity as a fixed effect and replicate population as a random effect.~~
345   ~~To determine the effect of cross identity on hatchability, viability and the fertility of flies,~~
346   ~~binomial generalised linear mixed effects models (GLMMs) were fit with cross identity included~~
347   ~~as fixed effect and parental replicate included as a random effect. Fertility data was analysed~~
348   ~~separately for males and females. Models were fit using the 'lme4' package (Bates et al. 2015).~~
349   ~~Fixed effects and random effects were analysed using the 'anova()' and 'ranova()', and~~
350   ~~'Anova()' and by backwards model selection for LMMs and GLMMs, respectively (Kuznetsova~~
351   ~~et al. 2017, Fox & Weisberg 2019). Post-hoc comparisons between cross identities were~~
352   ~~performed using the 'emmeans' package (Lenth 2022).~~
353
354

RESULTS

Female Mate Choice Assay

Overall, ACO females showed a significantly higher proportion of homotypic (0.57 +/- 0.0$\underline{48}$, mean +/- $\underline{1.96*}$se$\underline{d}$) compared to heterotypic matings (0.42 +/- 0.0$\underline{48}$, $P = 0.01$, Figure 1a), with no significant heterogeneity among replicate population pairs ($P = 0.66$, Table S1). The ACO$_5$ population showed a significant deviation from the null hypothesis, with more homotypic matings compared to heterotypic matings ($P = 0.04$; Figure S1a). Females from the ACO$_3$ population showed a similar trend towards more homotypic matings with borderline significance ($P = 0.05$). ACO$_1$ females showed no significant difference between mating types ($P = 0.26$). When data was pooled across trials within each replicate population, both ACO$_3$ and ACO$_5$ populations, but not the ACO$_1$ population, showed a significant deviation from a random mating ratio ($P_1 = 0.21$, $P_3 = 0.02$, $P_5 = 0.02$). with no significant heterogeneity between individual trials for any of the 3 replicate populations ($P_1 = 0.30$, $P_3 = 0.28$, $P_5 = 0.21$).

Overall, CO females showed a significantly higher proportion of homotypic (0.60 +/- 0.0$\underline{58}$) compared to heterotypic matings (0.40 +/- 0.0$\underline{58}$, $P = < 0.0001$, Figure 1a), with no significant heterogeneity among replicates ($P = 0.65$, Table S1). Two CO populations, CO$_1$ and CO$_3$, showed an overall significant deviation from the null hypothesis, with more homotypic copulations ($P_1 = < 0.01$, $P_3 = 0.01$, Figure S1a). CO$_5$ females showed a trend towards more homotypic matings, but this was not significant ($P = 0.08$). When data was pooled across trials, all three CO populations showed a significant deviation from the null hypothesis of random mating ($P_1 = < 0.001$, $P_3 = 0.01$, $P_5 = 0.04$), with no significant heterogeneity among trials for any of the replicate populations ($P_1 = 0.13$, $P_3 = 0.15$, $P_5 = 0.28$).

Only mated male identity had a significant effect on mating latency ($P = <0.00001$, Table S2$\underline{?}$). The crossed random effects of the replicate population and trial ~~ID~~ were significant ($P = <0.0001$), but the cumulative proportional variance attributed to the random effects of replicate population and trial ID was minimal (0.05). Both ACO and CO females displayed significantly shorter latencies in mating with CO males (11.8 +/- 1.2 min, 11.6 +/- 1.0 min respectively), compared to ACO males (15.3 +/-1.3 min, 15.5 +/- 1.5 min respectively, Figure 2a). There was no significant effect of female identity or interaction between male and female identity.

Female identity and interaction between male and female identity had significant effects on mating duration ($P = <0.00001$, Table S2$\underline{?}$). The crossed random effects of the replicate population and trial ID were significant ($P = <0.0001$), but cumulative proportional variance attributed to the random effects of replicate population and trial ~~ID~~ was minimal (0.04). In general, ACO females (15.0 +/- 3.8 min) displayed shorter mating durations than CO females (16.7 +/- 4.2 min), and both females mated longer with homotypic partners (ACO♀-ACO♂ 15.4

395   +/-0.4 min, ACO♀-CO♂ 14.3 +/- 0.5 min, CO♀-ACO♂ 16.1 +/-0.6 min, CO♀-CO♂ 17.1 +/-
396   0.4 min). All pairwise contrasts were significant (Figure 2c~~p<0.05, Table ?~~) with the exception of
397   ACO females mated with ACO males vs CO females mated with ACO males. There was no
398   significant effect of mated male identity.

399

400   *Male Mate Choice Assay*

401

402   Overall, ACO males showed a significantly higher proportion of matings with ACO
403   females (0.64 +/- 0.058) compared to CO females (0.36 +/- 0.058, $P = < 0.00001$, Figure 1b),
404   with no significant heterogeneity between populations ($P = 0.81$, Table S32). All three A
405   populations showed an overall significant deviation from the null hypothesis, displaying more
406   homotypic compared to heterotypic matings ($P_1 = < 0.0001$, $P_3 = 0.01$, $P_5 = < 0.001$). There was
407   significant heterogeneity observed among trials within the ACO_1 population ($P = 0.01$), but not
408   the ACO_3 ($P = 0.70$) or ACO_5 ($P = 0.38$) populations.

409

410   Overall, CO males displayed a nearly equal proportion of matings with ACO (0.51 +/-
411   0.024) and CO females (0.49 +/- 0.024, $P = 0.95$, Figure 1b), with no heterogeneity among
412   replicate populations ($P = 0.93$, Table S32). All three CO populations showed no significant
413   deviation from the null hypothesis of random mating ($P_1 = 0.99$, $P_3 = 0.65$, $P_5 = 0.96$). There was
414   also no significant heterogeneity among trials within any of the replicate populations ($P_1 = 0.97$,
415    $P_3 = 0.54$, $P_5 = 0.91$).

416

417   None of male identity, female identity or interaction between male & female identity
418   influenced mating latency (Table S2). The crossed random effects of the replicate population and
419   trial were significant ($P = 0.01$), but cumulative proportional variance attributed to the random
420   effects of replicate population and trial ~~ID~~ was minimal (0.02). Females from the CO populations
421   displayed a slightly higher mating latency with both ACO and CO males (18.0+/-1.7 min,
422   18.1+/-1.6 min respectively), compared to the ACO females (16.6+/-1.1 min, 16.9+/-1.4 min
423   respectively) but these differences were non-significant (Figure 2b).

424

425   Only mated female identity had a significant effect on mating duration ($P = <0.00001$,
426   Table S2 ~~?~~). The crossed random effects of the replicate population and trial ~~ID~~ were significant
427   ($P = <0.0001$), but cumulative proportional variance attributed to the random effects of replicate
428   population and trial ID was minimal (0.04). Males from the both populations displayed a slightly
429   higher mating latency with CO females (16.3+/-0.7 min, 17.3+/-0.6 min respectively), compared
430   to the ACO females (14.8+/-0.5 min, 15.0+/-0.7 min respectively) (Figure 2d). There was no
431   significant effect of male identity or interaction between male and female identity.

432

433   *Group Mate Choice Assay*

434

435   Overall, ACO females showed a significantly higher proportion of matings with CO males
436   (0.62 +/- 0.05~~20~~) compared to ACO males (0.38 +/- 0.05~~20~~, P = <0.00001, Figure 1c), with
437   significant heterogeneity being observed between replicate populations (P = 0.01, Table S4~~3~~).
438   ACO_3 and ACO_5 females showed an overall significant deviation from the null hypothesis,
439   displaying more heterotypic compared to homotypic matings (P_3 = 0.02, P_5 = <0.0001). ACO_1
440   females showed no deviation from the null expectation (P = 0.78). There was no significant
441   heterogeneity observed among trials within any of the three replicate ACO populations (P_1 =
442   0.49, P_3 = 0.45, P_5 = 0.50).
443
444   Overall, CO females showed significantly more homotypic (0.61 +/- 0.04~~17~~) compared to
445   heterotypic (0.39 +/- 0.04~~17~~) copulations (P = <0.0001, Figure 1c), with no heterogeneity
446   between replicate populations (P = 0.12, Table S4~~3~~). CO_5 females showed an overall significant
447   deviation from the null hypothesis, with more homotypic compared to heterotypic copulations (P
448   = <0.001). CO_1 and CO_3 populations showed no overall deviation from a random mating ratio of
449   1:1 (P_1 = 0.10, P_5 = 0.07). When data was pooled across trials, both CO_3 and CO_5 populations
450   showed significant deviation from a random mating ratio (P_3 = 0.02, P_5 = <0.0001). There was
451   no significant heterogeneity among trials within any of the populations (P_1 = 0.10, P_3 = 0.58, P_5
452   = 0.96).
453

454   ***Post-mating prezygotic reproductive isolation***

455

456   *Hatchability*

457

458   Cross identity had a significant effect on egg hatchability (P = <0.01, Table S5~~4~~). The
459   random effect of replicate population was not significant (P = 0.89). The only significant
460   differences in hatch rate was the decline of $CO_i♀ × A_i♂$ F2 hybrids compared to ACO and CO
461   parental lines (Figure 3~~2~~a). Overall, hatchability was high with average values between 0.96 +/-
462   0.01~~2~~ ($CO_i♀ × A_i♂$ F2 hybrids) and 0.98 +/- 0.01 (ACO parentals).
463

464   ***Postzygotic reproductive isolation***

465

466   *Larvae to adult viability*

467

468   There was also a significant effect of cross identity (P = <0.0001, Table S5~~4~~)<ins>, but no</ins>
469   <ins>random effect of</ins>t ~~and~~ replicate population (P = ~~<0.01~~0.31) on the viability of flies. On average,
470   larvae to adult viability was lowest for ACO parental (0.78 +/- 0.03~~8~~) and highest for $CO_i♀ ×$
471      $ACO_i♂$ F2 flies (0.93 +/- 0.02~~6~~). Both $CO_i♀ × ACO_i♂$ hybrids and $ACO_i♀ × CO_i♂$ F2 hybrids
472   demonstrated significantly higher viability than ACO, CO and $ACO_i♀ × CO_i♂$ F1 flies ~~and CO~~
473   ~~flies showed significantly higher viability than ACO flies~~ (Figure 3~~2~~b). .
474

475   *Development time*

476

477   There was a significant effect of cross identity (P = <0.0001, Table S6~~5~~), but no random
478   effect of~~t~~ replicate population (P = 0.51) on the development time of flies. All four hybrid
479   crosses demonstrated development times that were significantly longer than ACO parental flies
480   (175.76 +/- 0.5~~1.33~~ hours) and significantly shorter than CO parental flies (218.32 +/- 1.23~~.22~~
481   hours). There were no significant differences in development times among the four hybrid
482   crosses (Figure 3~~2~~d).

483

484   *Body size*

485

486   Cross identity also had a significant effect on the body size of flies (P = <0.0001, Table
487   S6~~5~~). The random effect of the replicate population was not significant (P =0.23). All four hybrid
488   lines showed average individual weights that were significantly higher than parental ACO lines
489   (0.21 +/- 0.01 g), and significantly lower than parental CO lines (0.31 +/- 0.01~~2~~ g). Hybrid
490   ACO$_i$♀ × CO$_i$♂ F1 flies were also significantly heavier than ACO$_i$♀ × CO$_i$♂ F2 flies (Figure
491   3~~2~~c).

492

493   *Reproductive fitness*

494

495   Cross identity had a significant effect on female reproductive fitness (P = <0.0001, Table
496   S7~~6~~). Parental CO lines showed the highest average fitness (0.24 +/- 0.~~12~~03), parental ACO lines
497   the lowest (0.12 +/- 0.~~10~~02) and hybrids displayed intermediate fitness between parental lines
498   (Figure 4~~3~~). All cross identities produced significantly more offspring than parental ACO
499   females and flies from all cross identities, except for CO$_i$♀ × ACO$_i$♂ F1 females, produced
500   significantly fewer offspring than parental CO flies. In both cases, second generation (F2)
501   females displayed lower reproductive fitness than first generation (F1) hybrids, but this effect
502   was only significant for the CO$_i$♀ × ACO$_i$♂ crosses. There was no significant effect of
503   population replicate on female fertility (P = 1).

504

505   There was a significant fixed effect of cross identity (P = <0.00~~0~~1, Table S7~~6~~) ~~and random~~
506   ~~effect of~~but not replicate population (P = ~~0.02~~1) on the fertility of male flies. CO$_i$♀ × ACO$_i$♂ F1
507   male hybrids showed the highest average fitness (0.13 +/- 0.~~04~~20) and ACO males the lowest
508   (0.05 +/- 0.~~11~~02). ~~F1 hybrid and~~ CO and CO$_i$♀ × ACO$_i$♂ F1 males demonstrated a significantly
509   higher fitness than ~~F2 and~~ ACO males (Figure 4~~3~~). ~~F2 hybrid males sired significantly more~~
510   ~~offspring than A males.~~ Similar to that observed in the female assay, male F2 hybrids displayed
511   ~~significantly~~ lower fitness than F1 hybrids, but these differences were not significant. While
512   female flies displayed fitness values around the expected value, males of both parental and
513   hybrid cross identities produced a lower proportion of offspring than expected when in
514   competition with four competitors ~~(Figure 3)~~.

515
516

DISCUSSION

Our experimental assays show the striking differentiation between the ACO and CO populations after over three decades and 1-500 generations of divergent selection on demography. The ACO population adults eclose nearly two days earlier than the CO population adults do. The clear tradeoff between speed and size is evident, as A adults are about 33% lighter than CO adults, and this is likely related to the strong differences in adult fitness we observed between the two selection treatments. Interestingly, the tradeoff between developmental speed and juvenile viability (Chippindale et al. 1997) is not apparent here or in other recent unpublished data on these populations, suggesting compensatory evolution over time. Given these differences in morphology and life history, we asked if we could detect the early stages of the speciation process for these populations in allopatry under different ecological circumstances.

To summarise our results, we found evidence for the evolution of premating barriers to gene flow between these diverged populations. Mate-choice assays generally reflected positive assortative mating patterns driven by female mate-choice. Females from both focal populations showed strong homotypic mate choice in individual tests, but this pattern was less clear in group tests, particularly as the smaller ACO females tended towards heterotypic mates. Under conditions of female choice, mating durations of homotypic pairs were consistently longer than heterotypic pairs. Despite this (and homotypic mate choice), the larger CO males showed lower latencies to mating with both females. In contrast, under conditions of male choice (no male-male competition), ACO and CO males showed no differences in mating latency. Further, under male choice conditions, both males mated for longer durations with the larger CO females, suggesting sperm catering based on body size rather than homotypic preference. We show that post-mating prezygotic barriers have not evolved to the point of detectability between these populations in the form of gamete incompatibility, measured through egg hatchability. We also find no evidence of postzygotic reproductive isolation in the form of survival to reproductive maturity, and measurements of development time and body size show that hybrids display intermediate trait values relative to the parental treatments. We observed only suggestive trends for postzygotic reproductive isolation in the form of hybrid breakdown in competitive reproductive fitness assays. F_1 hybrids display intermediate fitness to that of the two parentals, however, there is a tendency for F_2 hybrid offspring to display lower fitness than the F_1 hybrids; while potentially an indicator of evolving Dobzhansky-Muller incompatibilities, our data are merely suggestive at this juncture, meriting further investigation.

It has been suggested that prezygotic isolating barriers will typically evolve before postzygotic barriers, and *Drosophila* seems to adhere to this rule (Coyne & Orr 2004, Arthur & Dyer 2015). Within the category of prezygotic barriers, premating RI is expected to evolve before postmating-prezygotic RI (Turissini et al. 2018). Our data are generally in accord with

556   these predictions, as our diverged population pairs show marked premating reproductive
557   isolation, at least under individual choice tests, but clear evidence for later stage RI is lacking.
558
559   Strong divergence in body size exists between ACO and CO animals (Xiao et al. 2019; data
560   herein) and are presumed to correlate with differences in persistence/resistance capabilities
561   between the selection treatments. This size difference led us to expect the presence of some
562   premating reproductive isolation. However, we predicted premating isolation in one direction, on
563   the basis of greater resistance from larger (CO) females, greater persistence of CO males, and a
564   possible preference for large mates. That directional RI was noted by Ghosh & Joshi (2012) in a
565   study similar to ours: bigger mates were preferred by both large/slow and small/fast flies.
566   Contrary to that precedent, females of both regimes displayed strong homotypic mate choice and
567   longer mating durations with their homotypic partners in individual tests. This result is especially
568   intriguing for the ACO females, as they displayed a preference for smaller ACO males even
569   though *D. melanogaster* females are expected to prefer larger males (Pitnick 1991, Jagadeeshan
570   et al. 2015, but see Prasad et al. 2007).
571
572   Male mate choice tests may present trickier interpretations. ACO selected males largely
573   mated with homotypic females, but male Cos mated nearly randomly. While ACO males appear
574   to be showing positive assortative mating preferences, both types of males were observed
575   making repeated courtship attempts with both homotypic and heterotypic females (unquantified
576   observations) suggesting that the skewed mating pattern could be the result of the increased
577   capability of CO females to resist unwanted mating attempts, particularly from the smaller A
578   males. In alignment with this hypothesis, we observed a non-significant trend of longer mating
579   latencies with CO females. -In populations of flies evolved under a similar pair of life history
580   selection regimes, Ghosh and Joshi (2012) found that small males attempted to court large
581   females, but were unsuccessful, and, as a result, mated more frequently with small females. We
582   therefore cannot easily ascribe mating assortment to the choice of the male, who may be
583   undiscriminating under the test conditions, particularly with no competition for mates.
584   Concurrent with this, we note no differences in the mating latencies of the two male types in the
585   absence of male-male competition. ACO-type females are also under tremendous pressure to
586   mate, as they are selected to lay eggs about 24h after becoming sexually mature. In the absence
587   of competition, many males may have simply taken the most receptive mate first; for the CO
588   males this may have counterbalanced a homotypic preference, creating the ambivalent outcome
589   observed. The results of the mate choice assays taken together suggest that premating isolation in
590   this system is most strongly driven by female choice.
591
592   Body size is a likely trait involved in the evolution of reproductive isolation between the
593   population pairs tested. However, it remains possible that the patterns of assortative mating stem
594   from traits that are yet to be characterised. Some possibilities include courtship behaviours
595   (mating call, wing movements) and cuticular hydrocarbon profiles that may have diverged under

596   the divergent selection regimes. Besides female preference, body size differences also play a
597   clear role in the differential capacities displayed by females to resist mating attempts by males,
598   which creates a further reproductive barrier between large females and small males. Despite
599   large body size differences between the selection treatments, it seems unlikely that the patterns
600   of positive assortative mating can be explained by mechanical incompatibilities in copulation
601   alone; some earlier anecdotal observations suggested they may exist, but we saw no clear
602   evidence in these trials.
603
604   In the group mate choice assays, female CO flies continued to show homotypic mating
605   preference, but A females displayed reversed preferences, mating more frequently with
606   heterotypic males than homotypic males. The existence of heterotypic mate choice in groups, in
607   spite of homotypic mate choice in individual assays suggests that the nature of the assay plays a
608   role in the mating patterns observed. In the group mating assays, vials were more crowded (32 34
609   individuals vs. 3) - potentially creating circumstances where resisting forcible mating-attempts is
610   more difficult for females. This is a likely explanation given that only the small A females
611   displayed reversed mate choice preferences, while the larger CO females largely reproduced the
612   same mate choice patterns as displayed in individual assays. It is worth testing if similar mate
613   choice assays conducted in larger arenas (such as 90mm petri dishes) would reverse this
614   preference to homotypic choice. At the time these experiments were conducted (since changed)
615   there was an important difference in the mating environment of the ACO and CO selection
616   treatments: whilst the early-reproducing ACO's primarily mated in vials, with only a brief phase
617   in cages for egg-laying, the CO's, with 14 days in cages probably do all mating relevant to
618   fitness in the cage environment. With these differences in the "natural" mating environment of
619   our LTEE, the mate choice patterns found in individual assays seem to show us that A-selected
620   females have a preference for homotypic mating but are unable to exercise it in a group mating
621   setting with larger males.
622
623   Asynchronous development time alone can contribute to reproductive isolation, as seen in
624   the experimental work of Miyatake and Shimuzu (1999) on the melon fly. Because the mating
625   times diverged in lines selected for development time, significant RI arose that was evidenced in
626   experimental mate choice trials. Similarly, the extreme early reproduction regime of the A
627   populations means that neither CO individuals nor A/CO hybrids could hybridize, as the latter
628   groups would not even be sexually mature before a generation is completed. This scenario is, of
629   course, artificial because natural populations generally do not grow on synchronised, discrete
630   generations. they are not directly responsible for the assortative mating patterns observed in the
631   controlled mate-choice assays.
632
633   Post-mating, we report no evidence of reproductive isolation deriving from gamete
634   incompatibility, as would be evident in reduced egg hatch rates. Similarly, our measurements of
635   larva to adult viability data show that there are no hybrid treatments with lower levels of viability

than the purebred parental populations. In fact, we observed trends of hybrid vigour, with some of the hybrid treatments displaying higher larva to adult viability than the parental treatments. Interestingly, the larva to adult viabilities in F2 treatments were significantly higher than that of the parental selection treatments and one F1 treatment. While the increased viability in hybrids could suggest the existence of homozygous deleterious variation in developmental loci in our selection regimes, this should be consistently seen in both F1 and F2 generations, instead of being more common in the F2 generation. Development time and body size displayed no suggestion of abnormalities indicative of developmental instability, with intermediate hybrid trait values relative to the parental treatments in both sexes.

The competitive reproductive fitness of hybrids was generally intermediate to the parental treatments, and we found no evidence of hybrid sterility. Given that both assays subjected a focal fly to 4 competitors of the same sex, we expect focal flies to sire/dam ⅕th of the offspring produced, if all else was equal. In female reproductive fitness assays, our populations did cluster around the predicted threshold, with hybrids scoring intermediate fitness levels relative to the low fitness ACOs and high fitness COs. In male reproductive fitness assays though, fitness fell well below the predicted threshold in all treatments. We rule out the possibility of this being a case of Haldane's rule, because male fitness falls well below predicted levels in parental males as well as hybrids. We believe that this is a consequence of the usage of IVb flies as competitors/mates in these assays. Selected as common ancestors to both selection regimes, the IVbs have evolved in allopatry with both the populations, with a slightly different maintenance regime. We believe that the reduced male fitness estimates may be a consequence of homotypic mate choice by IVb females in male fitness assays. While we do see different levels of competitive reproductive output for the different treatments in the female assay, it is not possible to disentangle male mate choice from innate fecundity and harm resistance differences in the females of different selection regimes. Overall, in spite of the depressed male fitness output relative to predictions, hybrids in both sexes still consistently showed intermediacy in fitness, suggesting no reproductive sterility and a link to an additive trait such as body size.

The comparison between F1 and F2 reproductive fitness revealed an interesting trend: F1 fitness was consistently higher than that of F2 offspring, however, this difference was statistically significant in only 1 out of 4 comparisons 2 out of 12 comparisons (2 reciprocal crosses* 3 replicate treatment pairs*2 sexes). We interpret this with caution as a possible trend of hybrid breakdown as a consequence of incompatible gene complexes evolving in the two selection regimes.

We generally found high repeatability across our three independent replicates, which suggests that the characters measured and pre-mating RI observed stem from consistent treatment differences between the CO and ACO populations. Internal random processes of divergence in allopatry, such as the coevolution of arbitrary signaller-receiver relationships in

676   allopatry may also drive the evolution of RI. A system like ours opens the door to a potential test
677   for such divergence if RI were investigated between replicate populations *within* selection
678   treatment, rather than *between* treatments. Although our three--decade experiment seems too
679   young to see more than hints of post-mating RI, more sensitive queries of characters like
680   developmental stability (e.g., as measured by fluctuating asymmetry) may be of interest. Further
681   investigation of the sources of premating RI and its environmental sensitivity are warranted.
682
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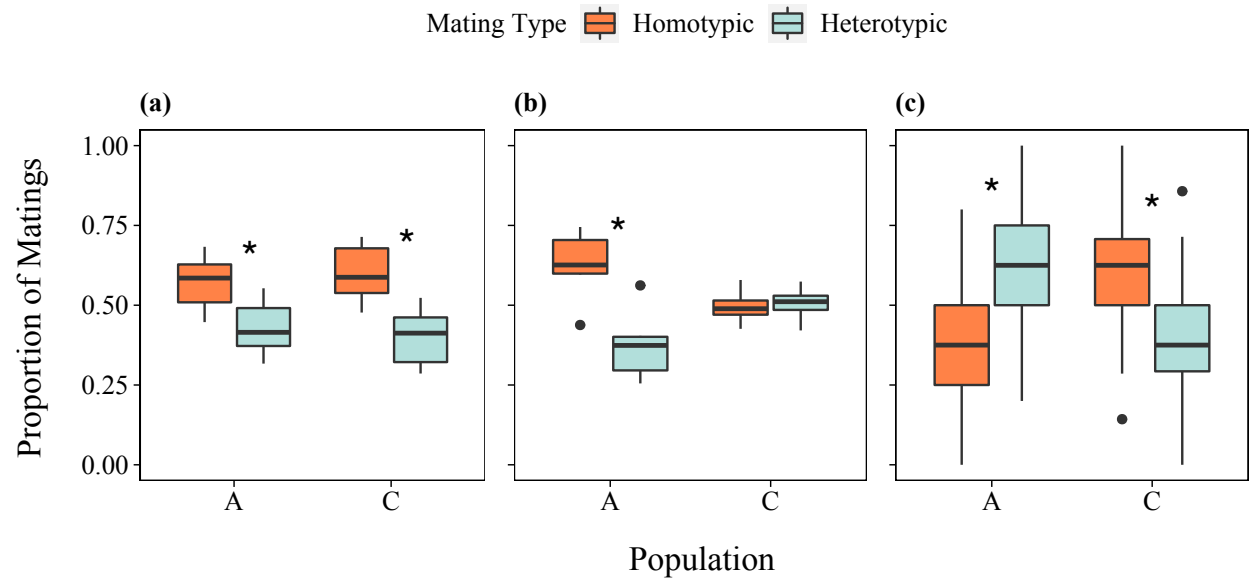898   *melanogaster*" *Evolution* 49(4):649-659.

899

900   **FIGURE CAPTIONS**

901

902   **Figure 1.** The proportion of homotypic compared to heterotypic matings recorded during the
903   female (a), male (b) and group (c) mate choice assay. Female (a,c) and male population identity
904   is shown on the x-axis. An asterisk represents a significant deviation from a random mating ratio
905   as indicated by a repeated G-test for goodness of fit (p<0.05).

906

907   **Figure 2.** The latency (a,b) and duration (c,d) of matings in the choice assays. Female (a,c) and
908   male (b,d) population identity is shown on the x-axis. Letters indicate significant differences
909   between mating types.
910
911   **Figure 3**2**.** The hatchability (a), egg to adult viability (b), body weights (c) and average
912   development time (d) of flies from parental and hybrid crosses. Letters indicate significant
913   differences between cross identities.
914
915   **Figure 4**3**.** The average proportion of red eyed offspring produced by flies from parental and
916   hybrid crosses, in competition with brown eyed competitors. Average proportions were
917   determined for each replicate population and plotted. The dashed line indicates the expected
918   proportion of ⅕ of the offspring. Letters indicate significant differences between cross identities.
919

Figure 1. The proportion of homotypic compared to heterotypic matings recorded during the female (a), male (b) and group (c) mate choice assay. Female (a,c) and male population identity is shown on the x-axis. An asterisk represents a significant deviation from a random mating ratio as indicated by a repeated G-test for goodness of fit (p<0.05).

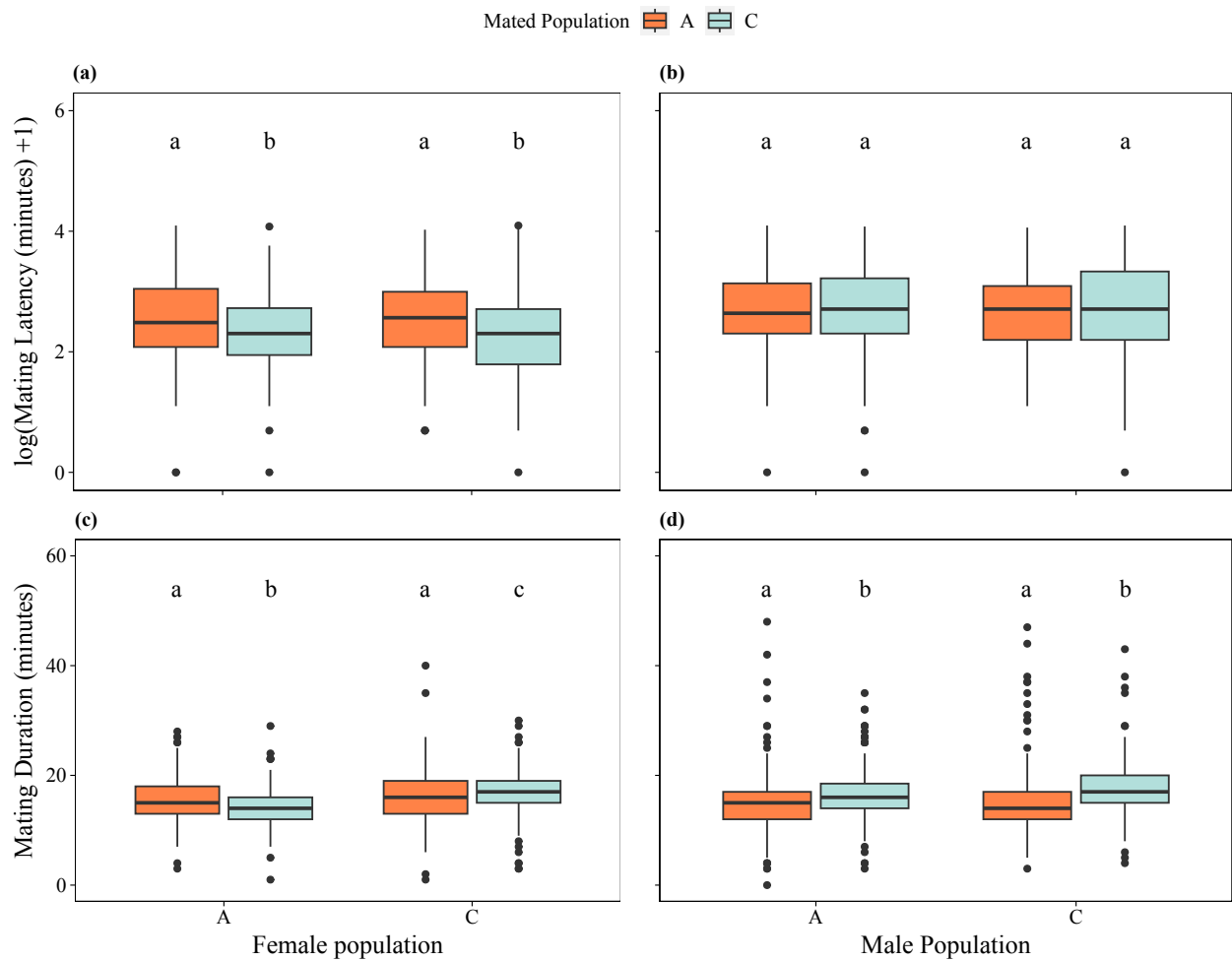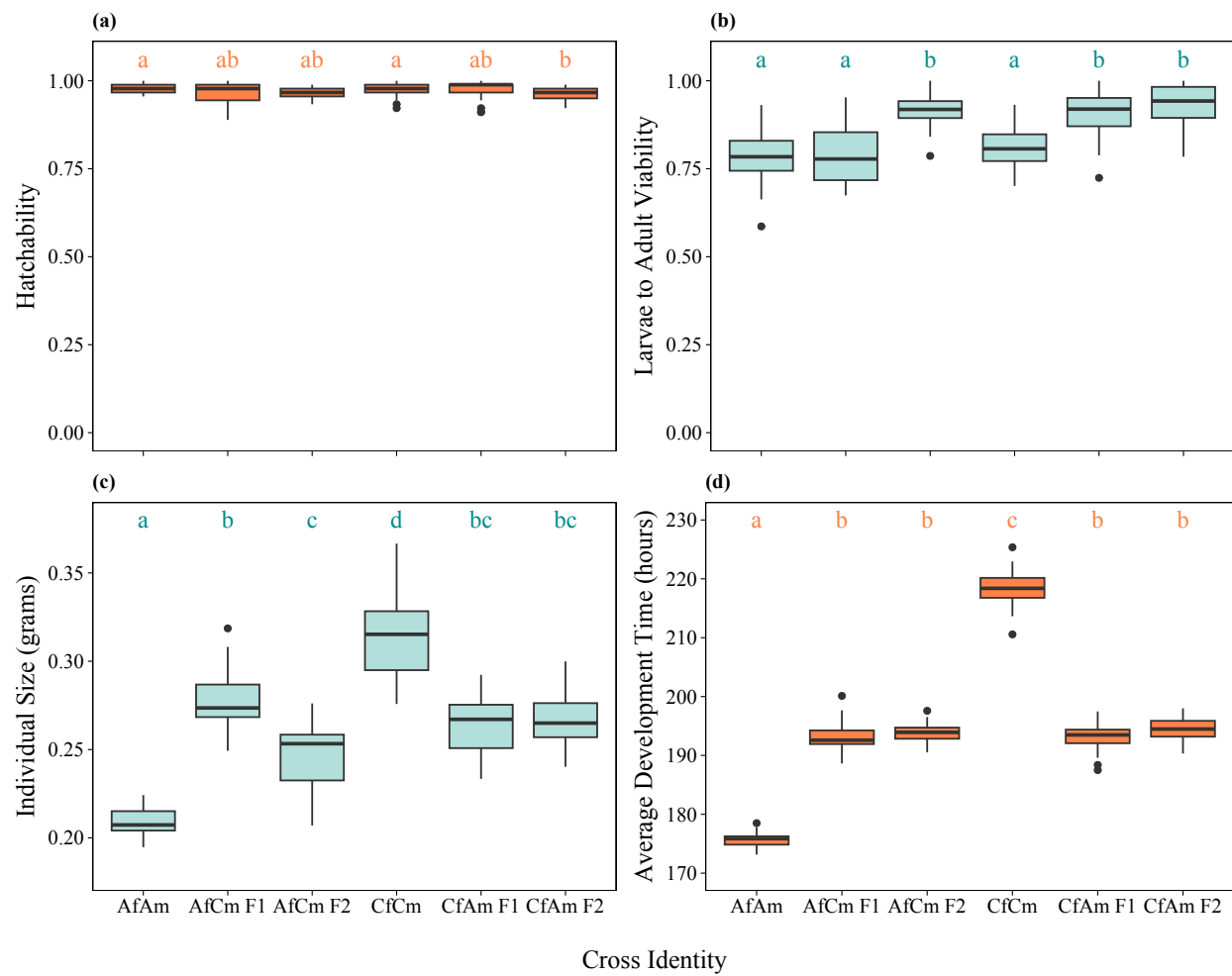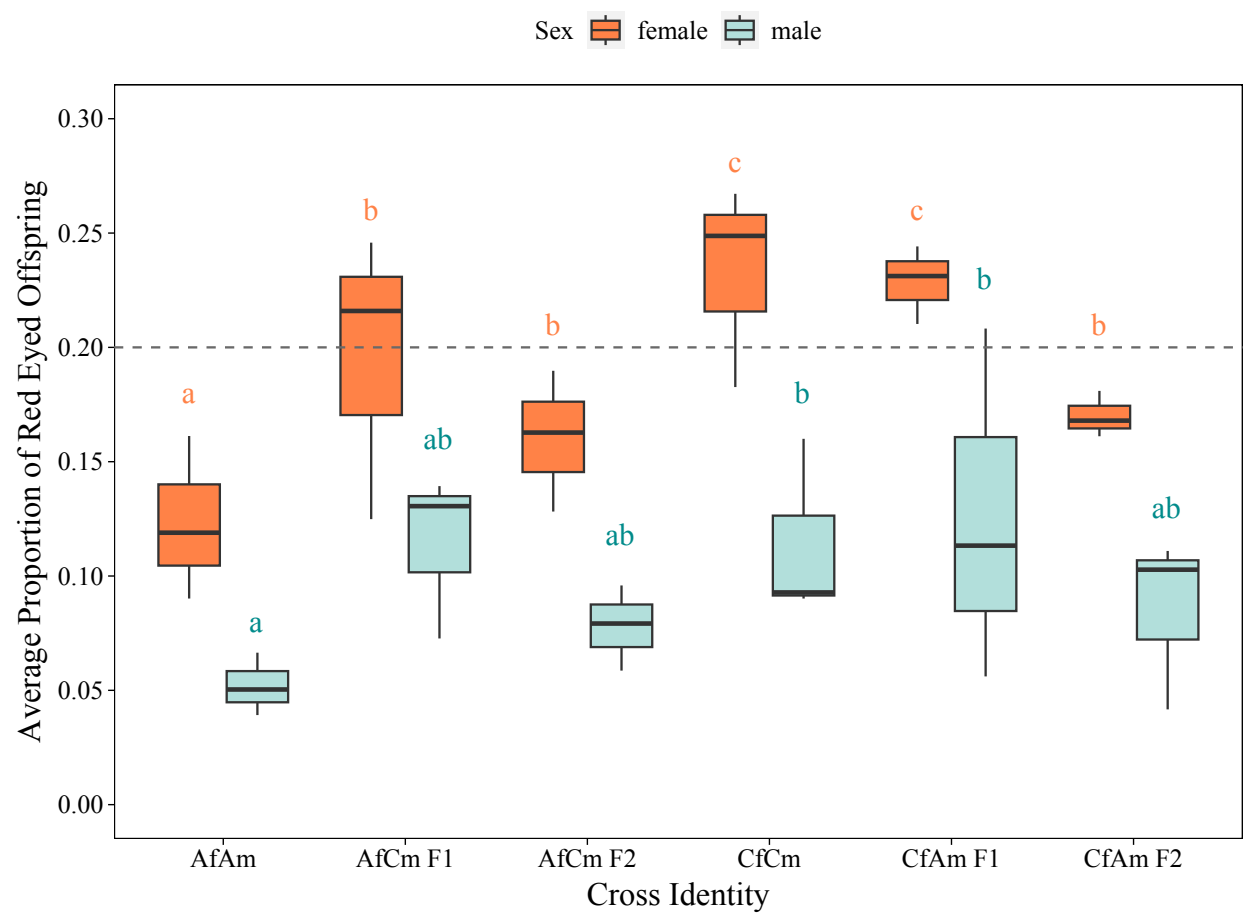**Figure 2.** The latency (a,b) and duration (c,d) of matings in the choice assays. Female (a,c) and male (b,d) population identity is shown on the x-axis. Letters indicate significant differences between mating types.

Figure 3. The hatchability (a), egg to adult viability (b), body weights (c) and average development time (d) of flies from parental and hybrid crosses. Letters indicate significant differences between cross identities.

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Figure 4. The average proportion of red eyed offspring produced by flies from parental and

hybrid crosses, in competition with brown eyed competitors. Average proportions were

determined for each replicate population and plotted. The dashed line indicates the expected

proportion of ⅕ of the offspring. Letters indicate significant differences between cross identities.