Title: Identifying the proximate mechanisms that generate variation in nutritional plasticity for fecundity in *Drosophila melanogaster*

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Abstract: Nutrition is an important determinant of an animal's survival and fitness. Phenotypic plasticity allows a genotype to adjust life history traits to changes in its nutritional environment, and it varies among individuals. To understand how variation in plasticity is achieved, we made use of a *Drosophila melanogaster* isogenic panel to characterize nutritional plasticity for fecundity by feeding flies diets differing in their yeast content and counting the number of eggs produced. We then identified lines with the highest and lowest plastic responses to diet and dissected the potential proximate mechanisms responsible for these differences in plasticity, including morphology, behaviour, and physiology. Our results suggest that genetic variation in plasticity is not due to differences in ovariole number, but due to both increased food intake, and higher efficiency at converting food into eggs. Our results show that, in this population of *D. melanogaster*, variation in behaviour and physiology, but not morphology, underlies differences in plasticity for fecundity. Further, they set the stage for future studies aiming to understand how the proximate mechanisms that generate genetic variation in plasticity contribute to a population's persistence when faced with environmental changes.

Introduction

Diet impacts animal wellbeing by shaping a wide variety of life-history traits including growth and development, response to disease, metabolism, lifespan, and reproduction (Simpson & Raubenheimer, 2012; Bozinovic et al., 2011; Pavan et al., 2013; Sgrò et al., 2016). However, not all individuals respond to diet in the same way. The ability of a genotype to adjust its phenotypes in response to environmental conditions, known as phenotypic plasticity, underpins whether a population or species will persist in the face of nutritional stress (Lynch

& Walsh, 1998; Hoffmann & Merilä, 1999; Gause, 1947; Bradshaw, 1965; Ørsted et al., 2017). This is particularly important because both the availability and quality of food resources accessible to animals is predicted to vary with climate change (Long et al., 2014; Rosenblatt & Schmitz, 2016). For this reason, understanding how individuals differ in their response to diet, and the underlying causes of these differences, is key to understanding how animals will cope with changes in their food resources.

Genetic variation in plasticity is quantified as genotype-by-environment (G×E) interactions (Schlichting & Pigliucci, 1998; Lande, 2009). Populations residing along environmental gradients respond differently to nutritional stress, suggesting that genetic variation in nutritional plasticity can evolve (Chakraborty et al., 2020, Klepsatel et al., 2020). In addition, individuals within a population show genotype-specific responses to diet (Ng'oma et al., 2018; King et al., 2011, Bergland et al., 2008). Characterising this genetic variation in plasticity is the first step in understanding how populations might respond and eventually adapt to nutritional stress (Narum et al., 2013; Chevin & Hoffmann, 2016; Chakraborty et al., 2020).

While many studies of genetic variation in plasticity within populations have focused on developmental, morphological, behavioural, or stress resistance traits (Van Buskirk & Steiner, 2009; Ghalambor et al., 2007, LaFuente et al., 2018; Cunningham et al., 2020), few studies have directly examined genetic variation in fitness across environments. This is largely because fitness is difficult to measure, and so studies often focus on life-history traits that represent close proxies of fitness, such as lifespan and fecundity (Fisher, 1930). Fecundity is tightly correlated with fitness and is sensitive to changes in nutritional availability and quality (Wallin et al., 1992, Behmer et al., 2001, Lee et al., 2008; Piper et al., 2014), which means small changes in the environment have potential to critically impact fecundity, and therefore fitness. Genetic variation in nutritional plasticity within populations has been documented for fecundity in the fruit fly *Drosophila melanogaster*, measured as the number of eggs laid (Ng'oma et al., 2018; King et al., 2011; Camus et al., 2017). However, little is known about the proximate mechanisms that regulate nutritional plasticity for female fecundity.

Variation in the plastic response of egg laying to nutrition can be regulated by numerous underlying mechanisms, including morphology, behaviour, and physiology. In insects, the ovaries are composed of one or more ovarioles, which are functional units for egg production (David, 1970). For insects like *Drosophila,* the number of ovarioles in an ovary both varies with nutrition and dictates the number of eggs a female can produce at any time (David, 1970). Furthermore, genotypes differ in their plasticity for ovariole number in response to protein restriction (Bergland et al., 2008). Thus, genetic variation in the plastic responses of fecundity

69 across diets could be due to differences in ovary morphology, as determined by ovariole
70 number.

71 Differences in behaviour, such as rates of food intake, across genotypes can also buffer
72 the extent to which individuals will experience nutritional stress on poor diets. Food intake is
73 carefully regulated to ensure animals reach their nutritional requirements to maximise life-
74 history traits (Simpson & Raubenheimer, 2009; Simpson & Raubenheimer, 1995). Plasticity in
75 food intake and other feeding behaviours occurs when organisms are faced with choices
76 between food sources with different nutritional content or change their intake strategy when
77 forced to eat sub-optimal diets (Carvalho & Mirth, 2017; Rodrigues et al., 2015; Silva-Soares
78 et al., 2017; Diegelmann et al., 2017; Fuhita & Tanimura, 2011). As a result, variation in
79 animals' feeding behaviours alters whole body physiology, and has the potential to mediate
80 plastic responses in fecundity.

81 Finally, genetic differences in physiological processes, such as the efficiency with which
82 animals absorb and assimilate the nutrients ingested, can also dictate the degree of plastic
83 response. Traits such as body size have been shown to be regulated by nutrient assimilation
84 (Sibly, 1981; Clissold et al., 2010). Even when animals ingest large quantities of nutrients, they
85 can still develop into smaller adults if they are not able to absorb the nutrients efficiently or are
86 unable to allocate them to the correct organs (Urabe & Watanabe, 1991; Neat et al., 1995). For
87 example, when fed a fixed quantity of the same diet, populations of *D. melanogaster* that are
88 adapted to cold environments reach larger body sizes than those adapted to warm environments
89 (James & Partridge, 1995). Potentially, genetic variation in the ability to absorb and assimilate
90 nutrients could underlie differences in nutritional plasticity for fecundity as well.

91 One way to study genetic variance in plasticity within a population is to use panels of
92 highly inbred lines originated from natural populations that are exposed to a gradient of
93 environmental conditions. This approach has been used with panels of isogenic lines, such as
94 the *Drosophila melanogaster* Genetic Reference Panel (DGRP) and the *Drosophila* Synthetic
95 Population Resource (DSRP) (Mackay et al., 2012; King et al., 2012). The advantage of using
96 such isogenic lines is that any differences between lines in responses to environmental variation
97 can be attributed to genetic differences in plasticity. Using this approach, studies have revealed
98 genetic variation in plasticity in response to temperature or nutrition for a range of traits such
99 as body size, olfactory behaviour, and wing-body scaling relationships (Lafuente et al., 2018;
100 Sambandan et al., 2008; Frankino et al., 2019).

101 In this study, we used a newly-derived set of isogenic lines of *D. melanogaster* to
102 quantify within-population genetic variance for nutritional plasticity. We then identified

genotypes that showed either high or low plasticity in female fecundity in response to dietary protein. To uncover the source of variation in plasticity, we next explored whether morphology, feeding behaviour, or physiology could explain the differences between high and low plasticity lines. To do this, we counted the number of ovarioles, measured food intake, and assessed protein-to-egg conversion efficiency in these lines across diets varying in protein content. Our study provides insight into the proximate mechanisms that generate within-population genetic variation in plasticity, serving as a platform for understanding how populations can respond to changes in their nutritional environment.

Methods

Field collections and establishing experimental lines

We collected 200 field-inseminated *Drosophila melanogaster* females from a banana plantation and Tropical Fruit World in Duranbah, on the east coast of Australia in January 2018 (28.3° S, 153.5° E). We generated two independent isofemale lines from each of the wild-caught females resulting in 400 lines in total. All lines were treated with tetracycline, to attempt to remove *Wolbachia,* and reared for two generations prior to 20 generations of inbreeding (described below). Flies were maintained on standard yeast-potato-dextrose medium (potato flakes 20 g/L; dextrose 30 g/L; Brewer's yeast 40 g/L; agar 7 g/L, Nipagin 6 mL/L; and propionic acid 2.5 mL/L) at 25 ºC, on a 12 h light/dark cycle.

Generating the isogenic lines

Inbreeding was applied to each of the 400 isofemale lines through full pair-sib mating, for a minimum of 20 generations, resulting in a final panel of 81 fertile isogenic lines which were used for this study (Chakraborty et al., 2023), with an inbreeding coefficient of F=0.986 (Mackay et al., 2012; Reddiex et al., 2018). All lines were maintained at a constant temperature of 18 °C, under a 12 h light/dark cycle, and fed yeast-potato-dextrose medium.

Fecundity assays

To assess fecundity, flies were maintained for two generations in a common environment of standard yeast-dextrose-potato medium at 25 °C to reduce effects of maternal or grand-maternal environment. To control for larval rearing density and to synchronise adult emergence time, eggs from each isogenic line were collected by allowing adults to lay in embryo collection cages (Genesee Scientific) on 60 mm petri dishes half-filled with apple juice/agar medium, as

136 described in Linford et al., (2013), for 24 h at 25 °C. We then distributed 50 eggs from each
137 line into each of 20 vials containing standard yeast-dextrose-potato food at 25 °C. Adult flies
138 from both sexes that emerged from these vials were collected over a 48 h period, transferred to
139 new vials containing yeast-dextrose-potato medium, and left to interact for 48 h, during which
140 they could mate multiple times, prior to the assessments of fecundity described below.
141 After 48 h of mating, females were separated from males, and five females from each
142 isogenic line were transferred into vials that contained one of three diets, to assess nutritional
143 plasticity in fecundity. To test for changes in fecundity due to protein restriction, we compared
144 a 100% standard diet (yeast-dextrose-potato) with two other diets that contained 5% or 50% of
145 yeast (relative to the 100% diet), but the same amount of dextrose and potato. We chose these
146 diets because yeast is the main source of protein for *D. melanogaster*, and protein concentration
147 correlates tightly with egg production (Piper et al., 2014; Ortega et al., 2021; Alves et al., 2022).
148 The low protein diets chosen for this study simulates not only variation in diet that animals
149 already experience (Markow & O'Grady, 2008; Matavelli et al., 2015; Silva-Soares et al.,
150 2016), but also the predicted reductions in nutrition expected under climate change (DaMatta
151 et al., 2010; Sardans et al., 2017).
152 To quantify changes in fecundity for the three diets, we established ten replicate vials,
153 each containing five females from each isogenic line for each diet/line combination. Females
154 were transferred to fresh food every 24 h. Eggs laid on days 5, 6 and 7 of the assays (days post-
155 mating) were counted since this interval is known to capture the peak period in fecundity for
156 *D. melanogaster* and is often used as a measurement for fecundity (Novoseltsev et al., 2003).
157
158 **Testing for overall genotype-by-environment interactions for fecundity**
159 To test for significant genotype-by-environment interactions for fecundity, we applied a
160 generalised linear mixed-effects model using a Poisson distribution with fecundity (the sum of
161 eggs laid from day 5 to 7) as the response variable. To apply the linear model, we used the
162 glmmTMB package in R. We fit sequential models (Table 1) with diet as a fixed effect and
163 experimental block as a random effect. Experimental block was defined as a set of isogenic
164 lines tested over the same period of time, as it was physically impossible to test all 81 lines
165 concurrently. To test for genotype-by-environment interactions, we used random regression.
166 This tested for improved fit between models in which only block was including as random
167 effect, block and isogenic line were included as a random effects, and block and the interaction
168 between diet and isogenic lines were included as random effects (Table 1). We tested for

169 improved fit using a likelihood ratio test and by comparing AIC values across models (Table
170 1).
171
172 **Quantifying genetic variance in fecundity**
173 We then explored whether G×E across diets was the result of changes in genetic variation
174 for fecundity across diets or due to genetic correlations across diets of less than 1 (Falconer
175 1952; Via & Lande, 1985). We used Bayesian models to estimate genetic variance within
176 environments, and genetic correlations in fecundity between diets using a generalized linear
177 mixed model with the R package 'MCMCglmm' (Hadfield, 2010):
178
179 $$\text{Eq 1: } y_{ijkl} = D_i + g_{j(i)} + b_{g(kij)} + e_{m(ijkg)}$$
180
181 where the sum of eggs laid from day 5 to 7 was included as a univariate Gaussian-distributed
182 response variable (y_{ijkl}). The *i*th diet was included as the only fixed effect (D_i). The *j*th isogenic
183 line ($g_{j(i)}$) and experimental block ($b_{l(kij)}$) were included as random effects to account for
184 variation among lines within each diet, and among experimental blocks. Residual variance is
185 represented by $e_{m(ijkg)}$. The model was implemented with 1.1 million iterations, a burn-in of
186 100,000 iterations, and a thinning interval of 500 iterations. We checked autocorrelation for
187 model convergence by confirming that the effective sample size exceeded 85% of the number
188 of samples that were saved. For each diet we estimated random intercepts and slopes, which
189 calculated among-genotype genetic variance within each diet as well as the genetic covariance
190 between diets, resulting in a 3×3 genetic covariance matrix.
191 To quantify the genetic correlation between fecundity across environments, a correlation
192 matrix was calculated from the covariance matrix of fecundity. To consider the uncertainty in
193 our estimates of genetic variance and genetic correlations between treatments, we calculated
194 the 95% Highest Posterior Density (HPD) credible intervals. Density distributions of genetic
195 variance and genetic correlations were created by extracting 2,000 samples from the model.
196
197 **Identifying lines with high/low plasticity in fecundity**
198 To characterise differences in plasticity across genotypes, we first eliminated any
199 isogenic lines that had laid less than three eggs over the time period measured across all diets
200 as we could not accurately characterise plasticity in these lines (Figure 2A). To obtain
201 coefficients of plasticity across the remaining isogenic lines, we calculated the normalised
202 difference in egg numbers by subtracting the number eggs laid on the 5% from those laid on

203 the 100% yeast content diets for each replicate/genotype and normalising this difference by the
204 mean egg number in the 100% diet for each genotype (Figure 2B). We then ran linear mixed
205 effect models on the normalised difference in egg numbers using the genotype as a fixed effect
206 and block as a random effect ('lme4' package). We calculated the estimated marginal means
207 for each genotype using the 'emmeans' package. Finally, we selected five of the ten genotypes
208 with the highest emmeans values as the high plasticity lines, and five out of the ten lines with
209 the smallest emmeans values, as the low plasticity lines (Figure 2C). The lines characterised
210 for their high or low plasticity chosen from the analysis were maintained for two generations
211 in common garden settings of standard yeast-dextrose-potato medium at 25 °C prior to all
212 assays described below.
213

214 **Ovariole dissection and counting**

215 To assess if the differences in plasticity in fecundity between the high and low plasticity
216 lines arose from differences in the number of ovarioles, flies were reared at 25ºC in standard
217 potato-yeast-dextrose medium at controlled densities of 50 eggs per vial of food with 3
218 replicate vials per line. Once eclosed, adult flies were collected over a 48-hour period,
219 transferred to new vials containing standard yeast-dextrose-potato medium, and left to mate for
220 48 h. At the end of this period, female flies were transferred from three vials per line into two
221 microtubes, placed in dry ice and stored at -80°C until ready for dissection.

222 Ovariole number was measured on 10 females per replicate microtube (20 females per
223 line). Flies were submerged in 1 × phosphate-buffer solution (PBS) and their ovaries removed
224 and teased apart to count the number of ovarioles.
225

226 **Food intake and fecundity assays**

227 We next examined the relationship between food intake and fecundity in the high and
228 low plasticity lines. To control for larval rearing density and to synchronise adult emergence
229 time, we collected eggs from the parental generation by leaving them to lay in embryo
230 collection cages (Genesee Scientific) on 60 mm petri dishes half-filled with apple juice/agar
231 medium, as described in Linford et al. (2013), for 24 h at 25°C. Eggs were then transferred into
232 10 food vials per line, at a density of 50 eggs per vial, each of which contained 6 mL of standard
233 yeast-dextrose-potato medium at 25°C.

234 The adult flies that emerged from these vials were collected over a 48-hour period,
235 transferred to new vials containing standard yeast-dextrose-potato medium, and left in vials for

236 48 h to allow the flies to mate multiple times. Following the 48 h mating period, five female
237 flies per line were transferred into vials that contained 3 mL of apple juice/agar medium to a
238 total of 10 vials per line and per diet, as described below.
239 We used the experimental setup for the capillary-assisted feeding (CAFE) assays from
240 Diegelmann et al. (2017) to measure food intake and number of eggs laid. We pierced narrow
241 cotton flugs (Genesee Scientific, product code 076-49-103) three times with a sharp, hot metal
242 rod of the same diameter as the calibrated glass micropipettes used for the assays (5 µL, Sigma,
243 product number P0549). Three glass micropipettes per vial were filled with liquid media,
244 marked with a black marker at the meniscus, and inserted in the cotton flugs. The media used
245 consisted of either 5N or 100N holidic food without agar (a synthetic diet containing the
246 proportion of each amino acid according to an exome-matching study (Piper et al., 2014)). In
247 these diets N represents the amount of nitrogen available, and by association, amount of protein
248 available in the food, with 100N equal to 100 mM of nitrogen available and 5N, 5 mM of
249 available nitrogen (Piper et al., 2014). This medium was used over a standard yeast-dextrose-
250 potato medium since the former is soluble, whereas the latter settles to the bottom of the
251 capillary, impeding flies from feeding from it. We also added 0.1% blue food colouring to the
252 media for contrast when measuring food intake (Queen food dye, blue, batch #118106). We
253 established ten replicate vials per line and diet combination, each containing five females.
254 Concurrently, to measure number of eggs laid each day, females from the capillary
255 feeding vials were transferred to fresh apple juice/agar vials every 24 h for seven days, after
256 which the eggs laid over the previous 24 h period were counted manually. Capillaries were
257 replaced, and amount of food left in the capillary from the previous 24 h was measured with
258 the same frequency to obtain feeding intake measurements. This was done for seven
259 consecutive days. The CAFE setup was placed inside a plastic storage box (25 cm (H) x 48 cm
260 (W) x 34.5 cm (D)) containing wet paper towel and closed with a lid to avoid evaporation. The
261 CAFE setup was placed in an incubator under a 12 h light/dark cycle kept at 25 °C and 80%
262 humidity. Apple juice/agar vials containing media-filled capillaries, but no flies, were placed
263 in the same boxes to measure evaporation of the media. Evaporation losses were subtracted
264 from experimental readings to obtain true ingestion amounts (Diegelmann et al., 2017).
265
266 **Assessing statistical significance across proximate mechanisms**
267 To assess differences in ovariole number across plasticity lines, we fit the data with a
268 linear mixed effects model using the 'lme4' package. The average number of ovarioles/ovary

269 was used as a response variable, and plasticity group was used as a fixed effect. Microtube and
270 isogenic line were included as random effects.
271 Next, we tested for significant differences in egg laying behaviour between high and low
272 plasticity lines. A generalised mixed effects linear model was created with the sum of eggs laid
273 throughout the experiment as a Poisson-distributed variable with a log link function. Diet and
274 plasticity group were included as fixed effects. Experimental block, replicate, storage box, and
275 isogenic line were included as random effects.
276 To test if the amount of ingested food differed between high and low plasticity lines, food
277 intake over the length of this experiment was calculated for each food/line pairing. Since the
278 food intake data was not normally distributed, we square-root transformed the data, which
279 improved the fit. The transformed food intake was used as a response variable and diet and
280 plasticity group were included as fixed effects. Block, replicate, evaporation box, and isogenic
281 line were included as random effects.
282 To test if protein ingested was a significant predictor of female fecundity, we used a
283 generalised mixed effects linear model as described above. The sum of eggs laid throughout
284 the experiment was the response variable, which was transformed using a log(10) scale. The
285 same transformation was applied for protein ingested, which was calculated considering the
286 amount of nitrogen available in the food. Plasticity group, and protein ingested were included
287 as fixed effects. Block, storage box, and isogenic line were included as random effects.
288 All statistical analyses were performed in R Studio (version 3.4.1). Plots were produced
289 using ggplot2 ('tidyverse' package).
290

291 # Results

292 ***Testing for within-population genotype-by-environment interactions for fecundity***

293 To test for overall genetic variance in the plastic response of fecundity to nutrition, we
294 placed isogenic lines onto diets differing in yeast content and counted the number of eggs laid
295 on days 5-7. We found that total fecundity significantly increased with yeast content in the diet,
296 and that genotypes differed in the total number of eggs laid across all diets (Figure 1A).
297 Furthermore, genotypes differed in their response to diet, resulting in a significant diet by
298 genotype interaction. Including a genotype by diet interaction in the random effects
299 significantly improved the model fit over models that included either block alone or block and

genotype, as indicated by a significant likelihood ratio test and a lower AIC score (Table 1). Thus, we observed significant a genotype-by-environment (GxE) interaction for egg numbers.

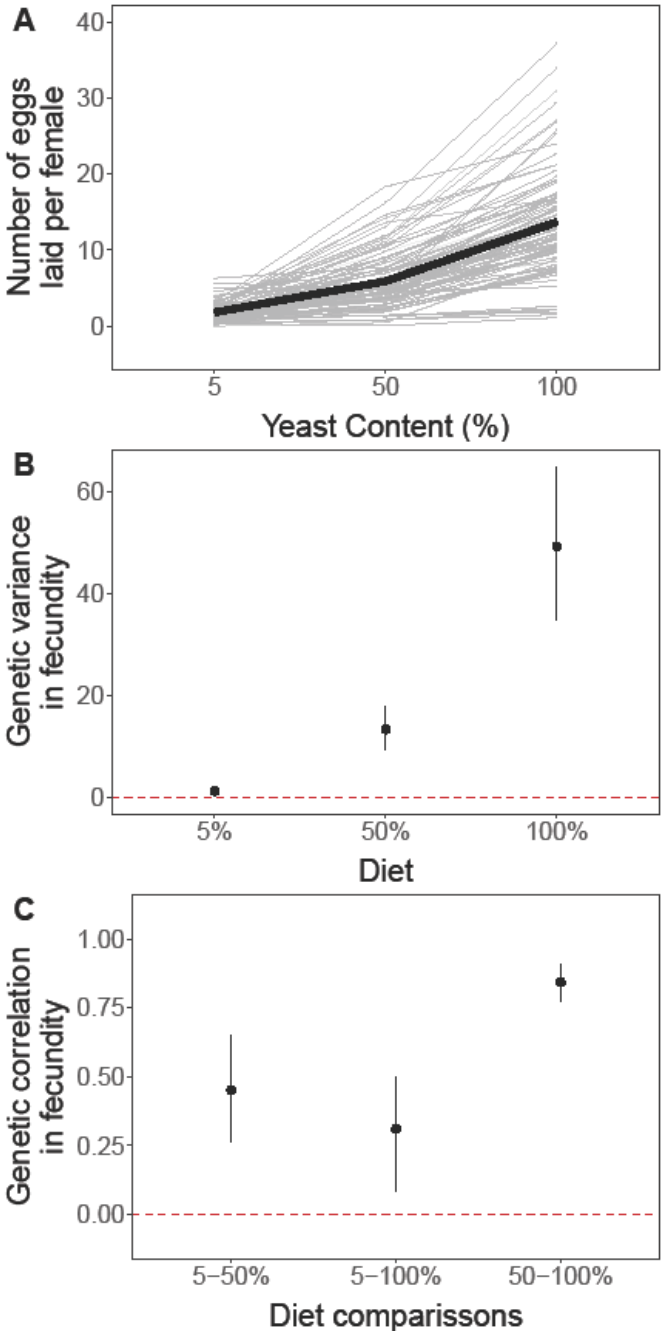

Figure 1: Nutritional plasticity for fecundity and its underlying variation. A) Reaction norms of fecundity for each isogenic line across diets with 5, 50 and 100% yeast content. The sum of eggs laid between days 5 to 7 for each female were counted in each diet for each isogenic line. The mean reaction norm of the population is represented by the black line and each isogenic line is represented in grey. B) Estimate of genetic variance in fitness and respective 95% credible interval for each diet. The dashed line represents 0 genetic variance, i.e. no variation within that diet. C) Estimates of genetic correlation and respective 95% credible intervals. The dashed line represents 0 genetic correlation, i.e. no genetic correlation between diets.

304 *Table 1: Random regression mixed effects models testing for genetic variation in plasticity for fecundity in response to the*
305 *yeast content of the diet. Data was fit using a Gaussian distribution.*

Model Number	Model	AIC	Likelihood ratio test (p-value)
1.1	# Eggs ~ Diet + (1|Block)	13595	-
1.2	# Eggs ~ Diet + (1|Block) + (1|Genotype)	11833	1.1 cf 1.2: p<0.001
1.3	# Eggs ~ Diet + (1|Block) + (1+Diet|Genotype)	11313	1.2 cf 1.3: p<0.001

306

307 ***Quantifying genetic variance in the plastic response of fecundity to diet***

308 Having detected significant GxE for fecundity, we next determined the origin of this
309 variation. G×E can be due to increased variation in fecundity on specific diets across genotypes
310 (Sheth et al., 2018). Alternatively, G×E can result from differences in the response of genotypes
311 to diets, resulting in genetic correlation of less than 1 across diets.

312 We found significant genetic variance for fecundity on all three diets, and the expression
313 of genetic variance increased with yeast concentration, as indicated by non-overlapping
314 credible intervals and density distributions (Figure 1B, Supplementary Figure 1). Thus,
315 changes in genetic variance across diets explains at least some of the significant G×E
316 interaction for fecundity.

317 Genetic correlations in fecundity among diets were all strong and positive (Figure 1C,
318 Supplementary Figure 1). Genetic correlations were highest between the 50% and 100% yeast
319 diets, and significantly lower between the 5% and 50% and 5% and 100% diets. The fact that
320 genetic correlations were less than one for all three diet comparisons indicates that the
321 significant genotype-by-environment interaction for fecundity also results from the fact that
322 the isogenic lines differ in some of the alleles that underpin nutritional plasticity for this trait.

323

324 ***Characterising plasticity across isogenic lines***

325 We next characterised differences in plasticity for female fecundity across low (5%)
326 and high (100%) dietary protein concentrations. We first excluded any isogenic lines that laid
327 less than 3 eggs across the time period observed for all diets as we could not accurately
328 characterise plasticity in these lines (Figure 2A). With the remaining lines, we calculated the
329 estimated marginal means (emmeans), using the normalised difference between the 5% and
330 100% yeast content diets for each genotype (Figure 2B). These means estimate how genotypes
331 rank relative to each other and the mean. We then selected five of the ten genotypes with the

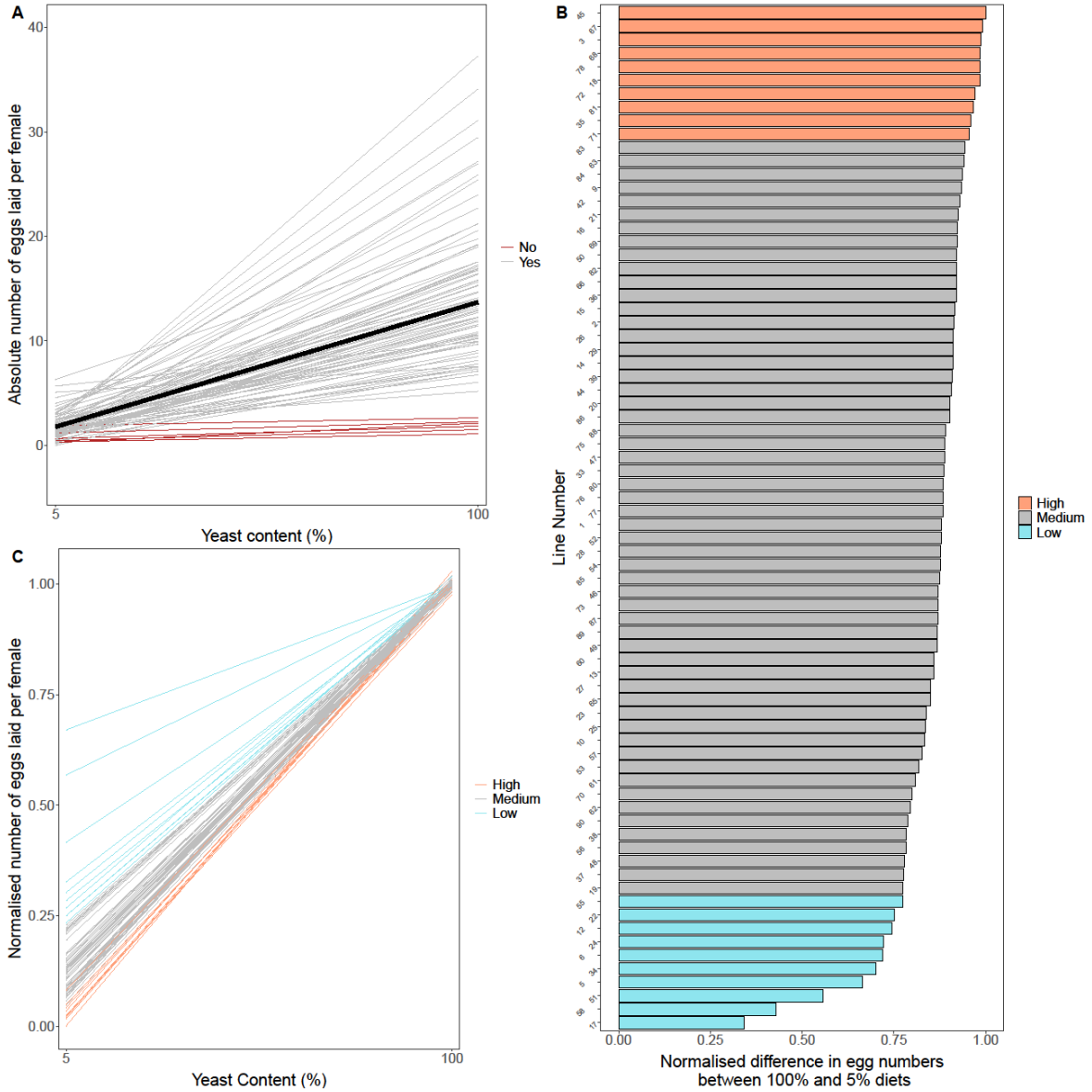

Figure 2 – Characterizing plasticity across isogenic lines. A) Isogenic lines excluded from further analysis. Lines in red represent the isogenic lines that laid less than 3 eggs across all diets and were excluded from further analysis. B) Ranking isogenic lines by Best Linear Unbiased Predictors (BLUP) value on the normalized difference between the number of eggs laid on 100% and 5% diets. Blue lines show low plasticity lines with low normalized difference between egg numbers between 100% and 5% diets. Orange lines represent high plasticity lines with a high normalized difference between egg numbers between 100% and 5% diets. C) Representation of reaction norms for each isogenic line. Normalised number of eggs per female was plotted for both diets selected, with blue lines representing the low plasticity lines, which have shallower slopes, and orange lines representing high plasticity lines, which have steeper slopes.

332 smallest emmean values as the high plasticity lines, and five out of the ten lines with the largest
333 emmean values, as the low plasticity lines (Figure 2B,C). We then used these chosen lines to
334 explore three proximate sources of variation in plasticity for fecundity: 1) ovary morphology,
335 measured by ovariole number, 2) feeding behaviour, and 3) physiology, determined by the
336 efficiency at which genotypes convert ingested protein into eggs.
337
338 ***Measuring ovariole number across plasticity lines***
339 Ovariole number is known to limit the number of eggs produced (David, 1970), but it
340 is unclear whether it plays a role in plastic responses of egg laying to diet. We hypothesised
341 that isogenic lines that have capacity to lay more eggs in the most protein rich diets might have
342 higher ovariole number, and vice-versa. To test whether plastic responses were determined by
343 ovariole number, we reared our high and low plasticity isogenic lines on the same media and
344 dissected groups of females, teasing apart their ovaries to count ovarioles. The number of
345 ovarioles did not differ between plasticity groups (Figure 3A, Supplementary Table 1). These
346 data indicate that differences seen in nutritional plasticity for fecundity are not due to
347 differences in ovariole number between isogenic lines.

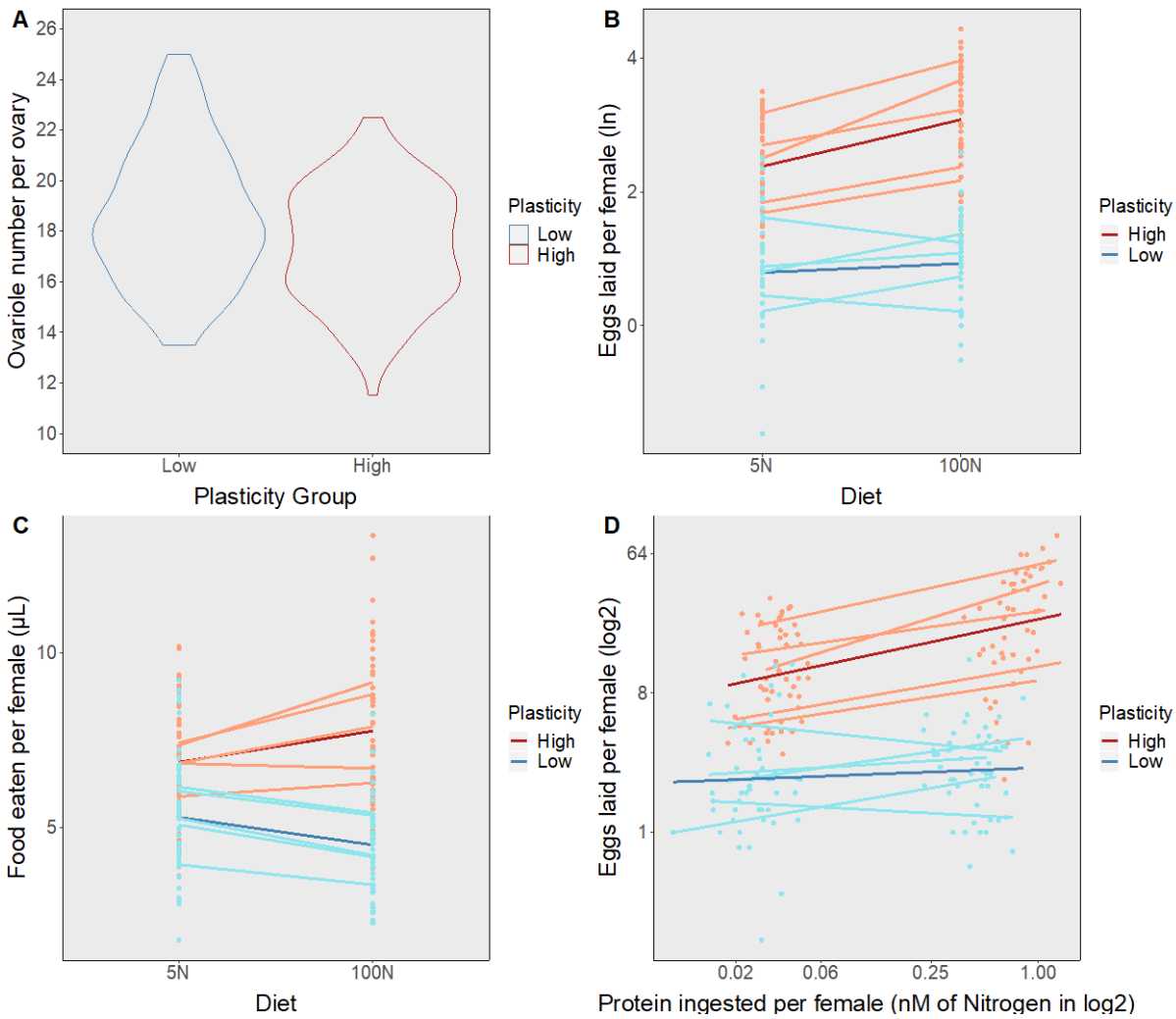

Figure 3: The relationship between differences in plasticity across isogenic lines, ovary size, food intake, and the capacity to convert food into eggs. A) Ovariole number in isogenic lines with different levels of plasticity. Number of ovarioles per ovary in flies from low (cadet blue) or high (light salmon) plasticity groups. Points represent data from each isogenic line/microtube. B) Eggs laid per female in diets containing 5% (5N) or 100% (100N) protein across isogenic lines with different levels of plasticity. C) Food intake per female in diets containing 5% (5N) or 100% (100N) protein across isogenic lines with either high or low levels of plasticity. D) The relationship between number of eggs laid and amount of protein ingested in high and low plasticity lines subjected to diets differing in protein content and allowed to lay eggs for 7 days. Points represent data from each replicate vial/diet/line. Salmon coloured points and lines are high plasticity lines and cadet blue lines are low plasticity lines. Darker, bold lines with standard error represent the average for each plasticity group.

Characterising feeding behaviour between high and low plasticity lines

Differences in plasticity might also arise due to differences in food intake between high and low plasticity lines in response to differing diets. Female flies from each of the high and low plasticity lines were placed in vials with access to three glass microcapillaries filled with holidic medium containing either 5% or 100% of the total protein of the standard holidic diet (Piper et al., 2014), allowing us to quantify the total amount of protein consumed.

As expected, the number of eggs laid was influenced by the protein concentration in the capillaries (Figure 3B, Table 3, Supplementary Table 2), but this depended on the plasticity

366 grouping. On average, high plasticity lines laid five to nine more eggs than the low plasticity
367 lines across both diets (Supplementary Table 2). In addition, high plasticity lines showed a
368 steeper response in the number of eggs laid in response to the protein concentration in the diet
369 than the low plasticity lines, resulting in a significant interaction term between diet and
370 plasticity group (Figure 3B, Table 3). Furthermore, the fact that these lines retained either high
371 or low plasticity on the holidic medium (this experiment) in addition to that on yeast-based
372 food (Figure 1A) suggests that this grouping method is accurate to ascertain plasticity across
373 diet types and lines.

374 The concentration of protein in the diet also had a significant impact on food intake
375 (Table 3). The high plasticity lines ate significantly more food than the low plasticity lines, and
376 this difference was more apparent on the high protein diet (Figure 3C, Table 3, Supplementary
377 Table 2), averaging 3.2 µL more food on a high protein diet. Furthermore, the high plasticity
378 lines showed a mild increase in food intake with increasing protein content of the diet, while
379 the low plasticity lines reduced their food intake slightly with increasing dietary protein
380 concentration, resulting in a significant interaction between diet type and plasticity group for
381 food consumption (Figure 3C, Table 3). This suggests that high and low plasticity lines differ
382 in the way they regulate their food intake in response to the protein content of the diet.
383

384 *Table 3 – Manipulating both diet and plasticity groups (high or low plasticity) alters egg laying or food ingestion behaviours.*
385 *Egg laying data was fit with a generalized mixed effects model, whereas ingestion was fit with a linear mixed effects model.*
386 *A generalized mixed effects model was fit to test the effect of diet, food ingestion and plasticity on egg laying.*

Number of eggs laid			
Term	**Chi-Square**	**Df**	**P-value**
Diet	188.825	1	<2.2e-16 ***
Plasticity Group	25.501	1	4.422e-07***
Diet*Plasticity Group	100.051	1	<2.2e-16 ***
Model			
ln(# of Eggs) ~ Diet*Plasticity Group + (1|Block) + (1|Box) + (1|Replicate) + (1|Line)			
Food Intake			
Term	**Chi-Square**	**Df**	**P-value**
Diet	4.8563	1	0.02755 *
Plasticity Group	17.4369	1	2.971e-05 ***

Diet*Plasticity Group	133.0921	1	<2.2e-16 ***
Model			
Sqrt(Food Intake) ~ Diet*Plasticity Group + (1|Block) + (1|Box) + (1|Replicate) + (1|Line)			
Food-to-egg conversion rate			
Term	**Chi-square**	**Df**	**P-value**
log(Protein ingested)	253.621	1	<2.2e-16 ***
Plasticity Group	24.393	1	7.85e-07 ***
log(Protein ingested) * Plasticity Group	96.198	1	<2.2e-16 ***
Model			
log(# Eggs) ~ log(Protein ingested) * Plasticity Group+ (1|Block) + (1|Box) + (1|Replicate) + (1|Line)			

Measuring differences in food-to-egg conversion rates between low and high plasticity lines

To understand if variation in egg laying plasticity was due to the amount of food eaten or ability to utilise nutrients more efficiently, we next examined the relationship between amount of protein ingested and the number of eggs laid across plasticity groups. In general, the number of eggs laid increased with protein ingested, suggesting that amount of protein ingested is a reasonable predictor of fecundity (Figure 3D, Table 3). The interaction between protein ingested and plasticity group was also significant, showing that high and low plasticity lines vary in the relationship between the number of eggs laid and the amount of protein ingested (Figure 3D, Table 3). The number of eggs laid increased at a faster rate with each nM of nitrogen ingested in high plasticity lines when compared to low plasticity lines for the same amount of protein ingested. This difference in conversion rate is seen as a higher slope for the high plasticity lines (Figure 3D, Table 3, Supplementary Table 2). These results suggest that food intake alone cannot explain the difference in plasticity between high and low plasticity lines, and that other mechanisms such as food absorption and/or assimilation also contribute to the response.

Discussion

Nutritional stress is one of the most common stressors faced by animals in nature and is likely to become increasingly so with climate change (Scheffers et al., 2016; Houghton et al.,

1990; Dawson et al., 2011; Thomas et al., 2004; Foden et al., 2013). Phenotypic plasticity will play a key role in shaping how animals will respond to climate change, at least in the short to mid-term (Lynch & Walsh, 1998; Hoffmann & Merilä, 1999; Franks et al., 2007; Geerts et al., 2015). Genetic variation in plasticity exists both across (Chakraborty et al., 2020, Klepsatel et al., 2020) and within (Schlichting and Pigliucci 1998; Lande 2009; LaFuente et al., 2018; Chakraborty et al., 2022; Dick et al., 2011) populations. Moreover, modulating the plastic response of life-history traits to nutrition is vital for an organism to persist through changes in their environment. Despite its importance, the relative contribution of variation in the proximate mechanisms that regulate plastic responses to diet is poorly understood. Here, we used isogenic lines of *D. melanogaster* to analyse the source of genetic variation for egg laying plasticity, and to understand how ovariole number, food intake, and protein-to-egg conversion efficiency impacted fecundity, a key trait correlated with fitness.

The source of genotype-by-environment interactions in isogenic lines

Numerous studies have revealed within-population genetic variation for plasticity in a range of life history traits (Van Buskirk & Steiner, 2009; Ghalambor et al., 2007, LaFuente et al., 2018; Cunningham et al., 2020, Dick et al., 2011), but only a few have assessed plasticity for fecundity, an important proxy for fitness (Ng'oma et al., 2018; King et al., 2011; Camus et al., 2017). Such studies emphasise that the distribution of reaction norms for individual genotypes can vary markedly from the population-level reaction norm. Our current study adds to the existing body of work by revealing that individual genotypes within a population can vary not only in the distribution of reaction norms for individual genotypes, but also in the extent and direction of plasticity for fecundity.

We then went on to reveal the cause of this within-population G×E. Significant G×E can arise as the result of differences in the expression of genetic variation across environments and/or cross-environment genetic correlations that are less than one (Falconer 1952; Via & Lande 1985; Sheth et al., 2018). While some studies have found that exposure to environmental stress may increase the expression of genetic variance for life-history traits, including fecundity (Service & Rose, 1985; Van Noordwijk et al., 1988; Etges, 1993; Holloway et al., 1990), other studies suggest that this might depend on the trait and the environmental condition studied (Larsson et al., 1997; Jenkins et al., 1997; Sgrò & Hoffmann, 1998). We found significant levels of genetic variance in fecundity in all three nutritional environments, however the expression of genetic variance increased with increasing diet quality. In this case, stressful diets reduced genetic variance instead of increasing it.

441 Although several theoretical and experimental studies have shown that genetic
442 correlations can change in sign and magnitude when individuals are exposed to new or stressful
443 environments (Krebs & Loeschcke, 1994; Norry & Loeschcke, 2002; Sgrò & Hoffmann, 2004),
444 we found that all three cross-diet genetic correlations were large and positive, which is
445 consistent with other studies that show positive genetic correlations across environments (Ebert
446 et al., 1993, Etges, 1993, Windig, 1994). The significant G×E we found for fecundity can be
447 explained by both changes in genetic variance across diets and the fact that genetic correlations
448 were less than one, indicating that the alleles that contribute to fecundity are in part independent
449 across environments. Alleles that contribute to fecundity independently across environments
450 might, therefore, explain differences in plasticity across different genotypes.
451
452 **Proximate mechanisms that regulate plasticity**
453 Ovariole number is a significant predictor of fecundity in *Drosophila*, as it caps the
454 maximum number of eggs that can be produced in optimal conditions (David, 1970).
455 Differences in ovariole number have been studied extensively between *Drosophila* species,
456 and it is known that environmental factors such as nutrition and temperature regulate this
457 number (Markow & Grady, 2008; Hodin & Riddiford, 2000; Bergland et al., 2008). Genetic
458 differences in ovariole number result from processes occurring during larval development,
459 when the ovarian structures first develop (Sarikaya et al., 2012). We hypothesized that genetic
460 differences in these developmental processes might lead to differences in ovariole number,
461 thereby limiting plasticity in fecundity. However, our data show that variation in plasticity for
462 fecundity in this population is unlikely to arise due to differences in ovariole number.
463 Previous studies have shown that flies regulate their food intake by choosing to eat
464 different diets to maximise life-history traits. For instance, flies eat salt-rich diets to increase
465 fecundity (Walker et al., 2015), diets with intermediate protein to carbohydrate ratios to
466 minimise development time (Rodrigues et al., 2015), and diets with lower protein to
467 carbohydrate ratios to modulate survival to infection (Ponton et al., 2019). Our study highlights
468 those differences in food intake correlate with differences in nutritional plasticity in fecundity
469 as well.
470 However, variation in food intake does not account for all the differences in nutritional
471 plasticity in fecundity observed across the high and low plasticity lines in the current study.
472 Especially with the low protein diet, we found that high and low plasticity lines showed little
473 difference in food intake, despite significant differences in the number of eggs laid. This
474 suggests that other post-ingestive, physiological mechanisms, such as amino acid absorption

475 or nitrogen retention, underlie genetic variation in this trait. Previous studies using cabbage
476 white, *Pieris rapae,* larvae that fed on different plants showed that larvae differ in their amino
477 acid absorption depending on the diet they are fed (Slansky & Feeny, 1977). Variation in the
478 efficiency of nutrient absorption and/or assimilation can also impact growth, development, and
479 longevity (Patt et al., 2003; Min et al., 2007). Thus, variation in the efficiency with which
480 animals can convert nutrients into phenotypic outputs is common.

481 A complementary, powerful approach that could provide further insight into the
482 proximate mechanisms that generate differences in plasticity in fecundity would be using
483 Genotype Wide Association Studies (GWAS) in isogenic panels such as the *Drosophila*
484 Genetic Reference Panel (DGRP) to identify genes that contribute to differences in food intake
485 and nutrient assimilation. This approach has been used successfully to identify loci contributing
486 to variation in thermal plasticity in body size (LaFuente et al., 2018). Other studies have found
487 genes associated with traits such as starvation resistance, olfactory behaviour, and body mass
488 composition that increase variation in plasticity when genotypes are exposed to different diets
489 (Nelson et al., 2016; Sambandan et al., 2008). A SNP affecting mean food intake has already
490 been discovered through this approach (Garlapow et al., 2015). An approach like this could be
491 applied to the isogenic panel used for this study to uncover new genes associated with the
492 differences in intake or nutrient utilization observed.

493 To further understand plasticity, it would be of great value to examine plasticity in male
494 fitness in these isogenic lines. Differences in this trait could be a by-product of sexually
495 dimorphic gene expression. Previous studies have uncovered genetic variation in male and
496 female dietary requirements, which leads to differences in feeding behaviour between the two
497 sexes (Camus et al., 2017). In isogenic lines, gene expression might be severely skewed
498 towards one sex across diets, which can lead to a detrimental gene expression for the other sex
499 (Connallon & Clark, 2011). This could potentially lead to differences in plasticity, and using
500 GWAS studies, we could uncover loci associated with sexually dimorphic gene expression and
501 associated with variation in plasticity.

502 Variation in plasticity can be achieved in several ways. Here we show that variation in
503 nutritional plasticity for fecundity is not explained by morphological differences in ovariole
504 number, but due to differences in food intake and the ability to convert ingested nutrients into
505 eggs. Additional research into the potential loci responsible for creating these differences will
506 provide insight into the genetic mechanisms underlying variation in plasticity. Furthermore,
507 this study enhances the importance of understanding the proximate mechanisms that generate

variation in plasticity, since these will regulate the fitness-related traits that will ultimately contribute to species' persistence and adaptation.

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Author contributions

ANA contributed with experimental design, execution, data analysis and interpretation. AC contributed with experimental design and execution and data analysis. MW contributed with experimental design and execution. GMW contributed with data analysis and interpretation. MDW, CMS and CKM contributed with experimental design, and data analysis and interpretation. ANA, AC, GMW, MDW, CMS, and CKM contributed to writing and revising the manuscript.

Data availability

All data and scripts are available in Figshare (DOI: 10.26188/28079516).

Conflict of interest

The authors declare no potential competing interests.

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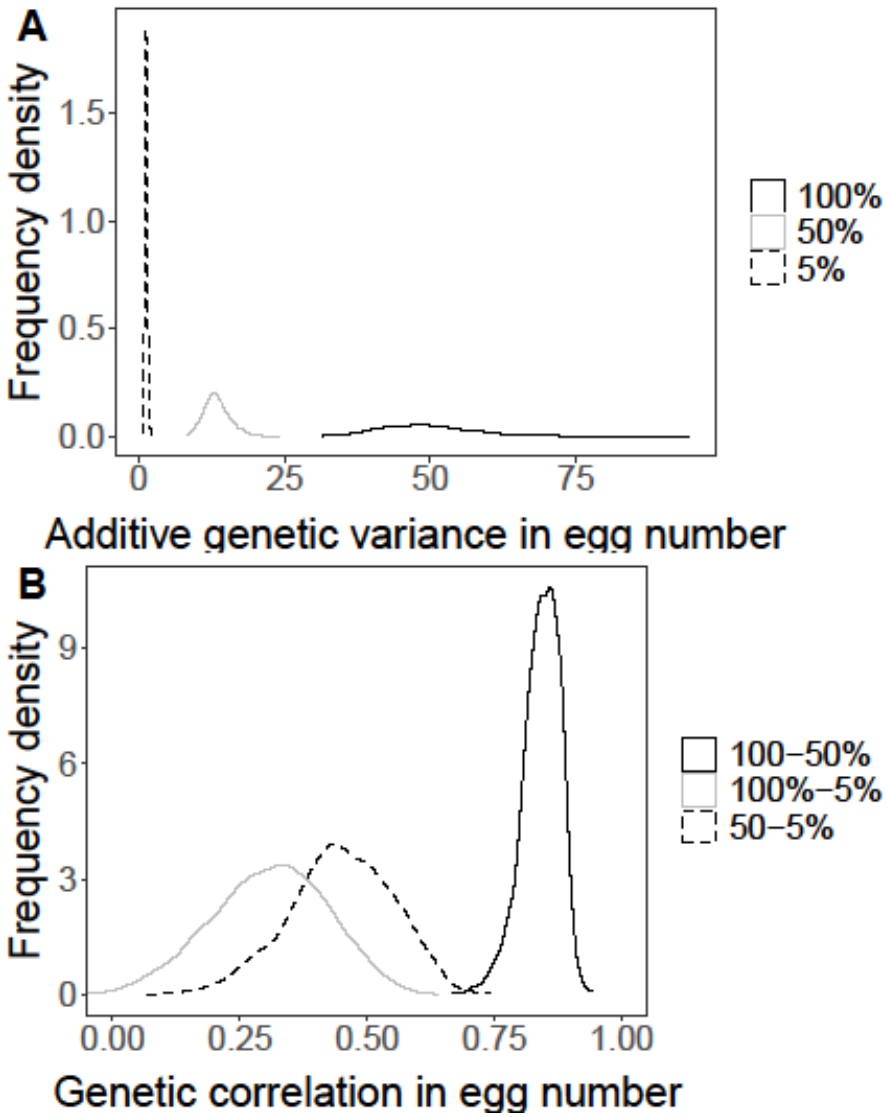
Supplementary Figures and Tables

Supplementary Table 1 – Ovariole number does not differ among plasticity groups (high or low plasticity). Ovariole number was fit with a linear mixed effects model.

Ovariole number			
Term	**Chi-square**	**Df**	**P-value**
Plasticity	1.3248	1	0.2497
Model			
Number of ovarioles ~ Plasticity Group + (1|Microtube) + (1| Line)			

Supplementary Table 2: Means for number of eggs produced, and amount of food ingested and trends for amount of eggs produced by function of protein ingested. The group column represents statistically significant groups that differ from each other with p-value <0.05.

Egglay				
Diet	**Plasticity Group**	**emmean**	**SE**	**group**
100N	Low	1.16	0.228	1
5N	Low	1.20	0.228	1
5N	High	2.46	0.226	2
100N	High	3.21	0.226	3
Food Intake				
Diet	**Plasticity Group**	**emmean**	**SE**	**group**
100N	Low	2.09	0.132	1
5N	Low	2.29	0.132	2
5N	High	2.62	0.132	2
100N	High	2.75	0.132	3
# Eggs ~ Protein ingested				
	Plasticity Group	**emtrend**	**SE**	**group**
	Low	0.0515	0.0138	1
	High	0.2320	0.0126	2



Supplementary Figure 1 – Density distributions of additive genetic variance and genetic correlations in egg number. A) Each line represents the density distribution of additive genetic variance in egg number for a particular diet. Solid black corresponds to the control diet, 100% yeast content, solid grey corresponds to 50% yeast content and dashed black line corresponds to the 5% yeast content diet. B) Each line represents the density distribution of genetic correlation in egg number for a particular pair of diets. Solid black corresponds to the comparison between control diet, 100% yeast content and 5% yeast content, solid grey corresponds to the comparison between 100% and 50% yeast content and dashed black line corresponds to the comparison between 50% and 5% yeast content diet.

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