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

André N. Alves[a,b], Avishikta Chakraborty[a,c], Mia Wansbrough[a], Greg M. Walter[a], Matthew D. W. Piper[a], Carla M. Sgrò[a] *, Christen K. Mirth[a]*[1]

[a] School of Biological Sciences, Monash University, Melbourne, VIC, Australia

[b] School of BioSciences, The University of Melbourne, Melbourne, VIC, Australia

[c] Division of BioSciences, University College London, London, United Kingdom

* Equal supervision of the research. Order decided by coin toss.

[1] Corresponding author: Christen K. Mirth, email: christen.mirth@monash.edu

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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. The origin of this variation comes from differences in proximate mechanisms regulating trait expression. 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 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 the ability of a population or species to 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 and Pigliucci, 1998; Lande, 2009). Populations residing along environmental gradients have been shown to 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). 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 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 is able to 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 across diets could be due to differences in ovary morphology, as determined by ovariole number.

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

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

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

In this study, we used a newly-derived set of isogenic lines of *D. melanogaster* to 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. We proceeded to count the number of ovarioles, measure food intake, and assess protein-to-egg conversion efficiency in these lines across diets varying in protein content. Our study provides insight into how within-population genetic variation in plasticity might derive from differences in proximate mechanisms.

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 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 ^{o}C, on a 12 h light/dark cycle.

Generating the isogenic lines

Inbreeding was applied to each of the 400 isofemale lines through single pair brother-sister mating, for a minimum of 20 generations, resulting in a panel of 81 fertile isogenic lines used for this study (Chakraborty et al., 2022) 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

For the isogenic lines, we focused on fecundity because we were interested in quantifying the plastic response of a trait that was closely linked to fitness. 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 described in Linford et al., (2013), for 24 h at 25 °C. We then distributed 50 eggs from each line into each of 20 vials containing standard yeast-dextrose-potato food at 25 °C. Adult flies that emerged from these vials were collected over a 48 h period, transferred to new bottles containing yeast-dextrose-potato medium, and females were left to mate with males from their corresponding lines for 48 h prior to the assessments of fecundity described below.

After 48 h of mating, females were separated from males, and five females from each isogenic line were transferred into vials that contained one of three diets, to assess nutritional plasticity in fecundity. To test for changes in fecundity due to diet, we compared a 100% standard diet (yeast-dextrose-potato) with two other diets that contained 5% or 50% of yeast (relative to the 100% diet), but the same amount of dextrose and potato. We chose these diets because yeast is the main source of protein for *D. melanogaster*, and protein concentration correlates tightly with egg production (Piper et al., 2014; Ortega et al., 2021; Alves et al., 2022). The low protein diets chosen for this study simulate not only variation in diet that animals already experience (Markow & O'Grady, 2008; Matavelli et al., 2015; Silva-Soares et al., 2016), but also the predicted reductions in nutrition expected under climate change (DaMatta et al., 2010; Sardans et al., 2017).

To quantify changes in fecundity for the three diets, we established ten replicate vials, each containing five females from each isogenic line for each diet/line combination. Females were transferred to fresh food every 24 h. Eggs laid on days 5, 6 and 7 of the assays (days post-mating) were counted since this interval is known to capture the peak period in fecundity for *D. melanogaster* and is often used as a measurement for fecundity (Novoseltsev et al., 2003).

Testing for significant interactions between diet and genotype for absolute fecundity

We first tested for significant effects of diet on fecundity quantified for all isogenic lines exposed to three diets. An ANOVA was applied to the model with the best fit, using absolute number of eggs as a Poisson-distributed response variable. Diet, genotype, and their interaction were included as fixed effects to assess the effect of diet and genotype on absolute fecundity of the isogenic lines. Experimental block and replicate vial were included as random effects.

Testing for overall genotype-by-environment interactions for relative fecundity

To test for significant genotype-by-environment interactions for relative fecundity, we applied a linear mixed-effects model using a normal distribution with relative fecundity (the sum of eggs laid from day 5 to 7 standardised to the average within each diet) as the response variable. We used relative fecundity (rather than absolute fecundity) to equalise differences in mean across environments. To do so, we compared the extent of differences among lines relative to the mean fecundity for each diet. To apply the linear model, we used the lme4 package in R. We fit sequential models (Table 1) with diet as a fixed effect. The first model included experimental block, and replicate vial as random effects (model 1.1, Table 1). Isogenic line was included as a random effect in model 1.2 and a likelihood ratio test used to test for improved model fit (model 1.2, Table 1). To test for genotype-by-environment interactions, the interaction between isogenic line and diet was then included as an additional random effect (model 1.3, Table 1) and a likelihood ratio test was used to test for improved model fit over the model that did not include this interaction (model 1.2 vs model 1.3, Table 1).

Model 1.1: Relative # Eggs ~ Diet + (1|Block) + (1|Replicate)

Model 1.3: Relative # Eggs ~ Diet + (1|Genotype) + (1+Diet|Genotype) + (1|Block) + (1|Replicate)

Quantifying genetic variance in fecundity

We then explored whether G×E across diets was the result of changes in genetic variation for fecundity across diets or due to genetic correlations across diets of less than 1 (Falconer 1952; Via & Lande 1985). We used Bayesian models to estimate genetic variance within environments, and genetic correlations in relative fecundity between diets using a generalized linear mixed model with the R package ‘MCMCglmm’ (Hadfield, 2010):

$$\text{Eq 1: } y_{ijkl} = D_i + g_{j(i)} + b_{g(kij)} + e_{m(ijkg)}$$

where the sum of eggs laid from day 5 to 7 was again standardised to the average in each diet (to reflect relative fecundity) and included as a univariate Poisson-distributed response variable (y_{ijkl}). The *i*th diet was included as the only fixed effect (D_i). The *j*th isogenic line ($g_{j(i)}$) and experimental block ($b_{l(kij)}$) were included as random effects to account for variation among lines within each diet, and among experimental blocks. Residual variance is represented by $e_{m(ijkg)}$. The model was implemented with 1.1 million iterations, a burn-in of 100,000 iterations, and a thinning interval of 500 iterations. We checked autocorrelation for model convergence by confirming that the effective sample size exceeded 85% of the number of samples that were saved. For each diet we estimated random intercepts and slopes, which calculated among-genotype genetic variance within each diet as well as the genetic covariance between diets, resulting in a 3×3 genetic covariance matrix.

To quantify the genetic correlation between relative fecundity across environments, a correlation matrix was calculated from the covariance matrix of relative fecundity. To take into account the uncertainty in our estimates of genetic variance and genetic correlations between treatments, we calculated the 95% Highest Posterior Density (HPD) credible intervals. Density distributions of genetic variance and genetic correlations were created by extracting 2,000 samples from the model.

Identifying lines with high/low plasticity in fecundity

To obtain coefficients of plasticity across the isogenic lines, we calculated the best linear unbiased prediction (BLUP), obtained from the MCMCglmm model, between the 5% and 100% yeast content diets for each genotype (Walter et al., 2022). These BLUPs estimate how genotypes rank relative to each other and the mean. After inspecting the distribution of reaction norms, we decided to eliminate any lines that had laid less than three eggs over the time period measured across all diets. This was done to eliminate isogenic lines that laid low numbers of eggs irrespective of the diet, presumably due to the presence of deleterious alleles. The five genotypes with the smallest BLUP post-filtering, which indicates the highest change in slope relative to the mean, were chosen as the high plastic lines, and the five lines with the largest BLUP, after filtering for lines that produced very few eggs overall, were chosen as the low plasticity lines.

Ovariole dissection and counting

The five lines with the highest and five lines with the lowest plasticity chosen from the analysis above were maintained for two generations in common garden settings of standard yeast-dextrose-potato medium at 25 °C prior to all assays described below. To assess if the differences in plasticity in fecundity between the high and low plasticity lines arose from differences in the number of ovarioles, flies were reared at 25 ^{o}C in standard potato-yeast-dextrose medium at controlled densities of 50 eggs per vial of food with 3 replicate vials per line. Once eclosed, adult flies were collected over a 48-hour period, transferred to new vials containing standard yeast-dextrose-potato medium, and left to mate for 48 h. At the end of this period, female flies were transferred from three vials per line into two microtubes, placed in dry ice and stored at -80 °C until ready for dissection.

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

Food intake and fecundity assays

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

The adult flies that emerged from these vials were collected over a 48-hour period, transferred to new vials containing standard yeast-dextrose-potato medium, and left to mate for 48 h. Once mated, five female flies per line were transferred into vials that contained 3 mL of apple juice/agar medium to a total of 10 vials per line and per diet.

We used the experimental setup for the capillary feeding (CAFE) assays from Diegelmann et al. (2017) to measure food intake and number of eggs laid. We pierced narrow cotton flugs (Genesee Scientific, product code 076-49-103) three times with a sharp metal rod of the same diameter as the calibrated glass micropipettes used for the assays (5 µL, Sigma, product number P0549). Three glass micropipettes per vial were filled with liquid media, marked with a black marker at the meniscus, and inserted in the cotton flugs. The media used

consisted of either 5N or 100N holidic food without agar (a synthetic diet containing the proportion of each amino acid according to an exome-matching study (Piper et al., 2014), with N representing the amount of nitrogen available, and by association, amount of protein available in the food, with 100N equal to 100 mM of nitrogen available and 5N, 5 mM of available nitrogen (Piper et al., 2014)). This medium was used over a standard yeast-dextrose-potato medium since the former is soluble, whereas the latter settles to the bottom of the capillary, impeding flies from feeding from it. We also added 0.1% blue food colouring to the media for contrast when measuring food intake (Queen food dye, blue, batch #118106). We established ten replicate vials per line and diet combination, each containing five female flies.

To measure number of eggs laid each day, females from the capillary feeding vials were transferred to fresh apple juice/agar vials every 24 h for seven days, after which the eggs laid over the previous 24 h period were counted manually. Capillaries were replaced, and amount of food left in the capillary from the previous 24 h measured with the same frequency to obtain feeding intake measurements. This was done for seven consecutive days. The CAFE setup was placed inside a plastic storage box (25 cm (H) x 48 cm (W) x 34.5 cm (D)) containing wet paper towel and closed with a lid to avoid evaporation. The CAFE setup was placed in an incubator under a 12 h light/dark cycle kept at 25 °C and 80% humidity. Apple juice/agar vials containing media-filled capillaries, but no flies, were placed in the same boxes to measure evaporation of the media. Evaporation losses were subtracted from experimental readings to obtain true ingestion amounts (Diegelmann et al., 2017).

Testing for statistical significance across proximate mechanisms

To assess differences in ovariole number across plasticity lines, we fit the data with a linear mixed effects model using the ‘lme4’ package. The average number of ovarioles/ovary was used as a response variable, and plasticity group was used as a fixed effect. Microtube and isogenic line were included as random effects.

Next, we tested for significant differences in egg laying behaviour between high and low plasticity lines. A generalised mixed effects linear model was created with the sum of eggs laid throughout the experiment as a Poisson-distributed variable with a log link function. Diet and plasticity group were included as fixed effects. Block, replicate, storage box, and isogenic line were included as random effects.

To test if the amount of ingested food differed between high and low plasticity lines, the amount of food intake over the length of this experiment was calculated for each food/line

pairing. Since the food intake data was not normally distributed, we square-root transformed the data, which improved the fit. The transformed food intake was used as a response variable and diet and plasticity group were included as fixed effects. Block, replicate, evaporation box, and isogenic line were included as random effects.

To test if protein ingested was a significant predictor of female fecundity, we used a generalised mixed effects linear model as described above. The sum of eggs laid throughout the experiment was the response variable, which was transformed using a log(10) scale, and assuming a Poisson distribution with a log link function. The same transformation was applied for protein ingested, which was calculated considering the amount of nitrogen available in the food. Plasticity group, and protein ingested were included as fixed effects. Block, replicate, storage box, and isogenic line were included as random effects.

All statistical analyses were performed in R (version 3.4.1). Plots were produced using ggplot2 ('tidyverse' package). All data and scripts are available in Figshare (DOI: 10.26180/18515291).

Results

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

To test for overall genetic variance in the plastic response of fecundity to nutrition, each of the 86 isogenic lines was placed onto diets containing either 5%, 50%, or 100% yeast and the number of eggs laid on days 5-7 was counted. We found that total fecundity significantly increased with yeast content in the diet, and that genotypes differed in the total number of eggs laid across all diets (Figure 1A, Supplementary Table 1). Furthermore, genotypes differed in their response to diet, resulting in a significant diet by genotype interaction.

Relative fecundity is a better measurement than absolute fecundity as it allows us to normalise differences in mean across environments and compare the differences in diet response among lines relative to the mean fecundity in each diet. For this reason, we assessed genotype-by-environment interactions using relative fecundity. We calculated relative fecundity across diets, by calculating the sum of eggs laid between days 5 and 7, standardised to each diet. When analysing the response of relative fecundity to diet, the inclusion of genotype as a random effect significantly improved the model fit, as indicated by a significant likelihood ratio test and a lower AIC score (Table 1, model 1.1 and 1.2). As evidence of significant G×E, the model that included the genotype by diet interaction as a random effect

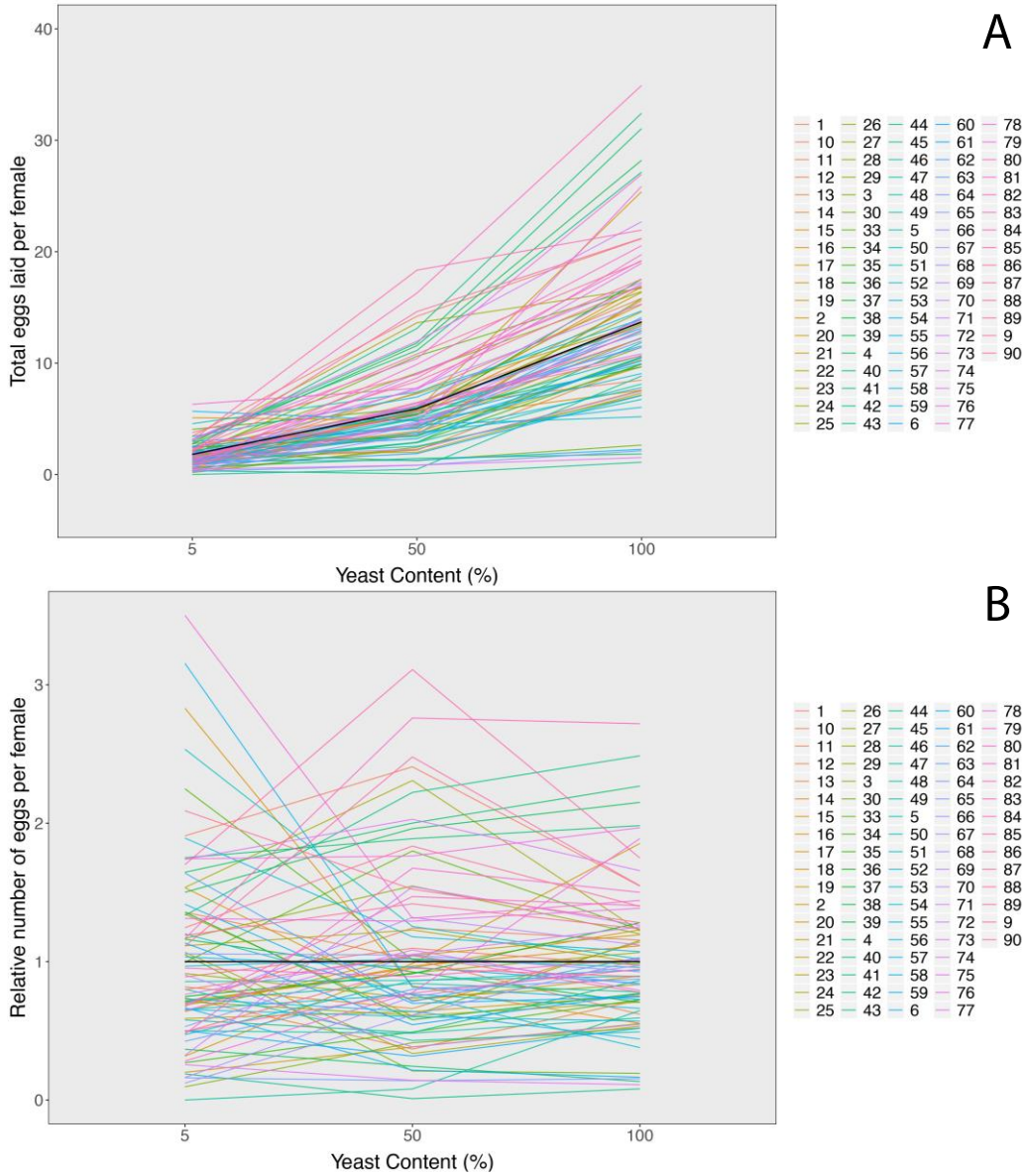

Figure 1: Phenotypic reaction norms of fecundity for each isogenic line across diets with 5, 50 and 100% yeast content. A) The sum of eggs laid between days 5 to 7 for each female were counted in each diet for each isogenic line. B) Relative fecundity, i.e., the number of eggs standardized to the mean of each diet, were counted for each isogenic line. Each colour represents a different isogenic line. The mean reaction norm of the population is represented by the black line.

further improved the model fit, with a significant likelihood ratio test and lower AIC value (Figure 1B, Table 1, model 1.2 and 1.3).

Table 1: Linear mixed model fit by maximum likelihood for isogenic lines and the effect of diet on egg production. lmer was performed to assess if diet had any effect on relative egg production taking into account the variation within each block, within replicates and within genotypes and its interaction with diet. The model was compared to a similar model lacking the

interaction term and tested with a likelihood ratio test for best fit. The summary of the model with the best fit is presented below.

Model Number	Model	AIC	Likelihood ratio test (p-value)
1.1	Relative # Eggs ~ Diet + (1|Block) + (1|Replicate)	5790.585	-
1.2	Relative # Eggs ~ Diet + (1|Block) + (1|Replicate) + (1|Genotype)	5276.211	1.2 cf 1.1: <2.2e-16 ***
1.3	Relative # Eggs ~ Diet + (1|Block) + (1|Replicate) + (1|Genotype) + (1+Diet|Genotype)	4837.132	1.3 cf 1.2: <2.2e-16 ***

Quantifying genetic variance in the plastic response of fecundity to diet

Having detected significant genotype-by-environment interactions for relative fecundity, we next determined the origin of this variation. GxE can be due to increased variation in relative fecundity on specific diets across genotypes (Walter et al., 2022; Sheth et al., 2018).

Alternatively, GxE can result from differences in the response of genotypes to diets, resulting in genetic correlation of less than 1 across diets.

While we found significant genetic variance for relative fecundity on all three diets, we did not detect a significant difference in the expression of genetic variance across diets, as indicated by the overlapping credible intervals and density distributions (Figure 2A,

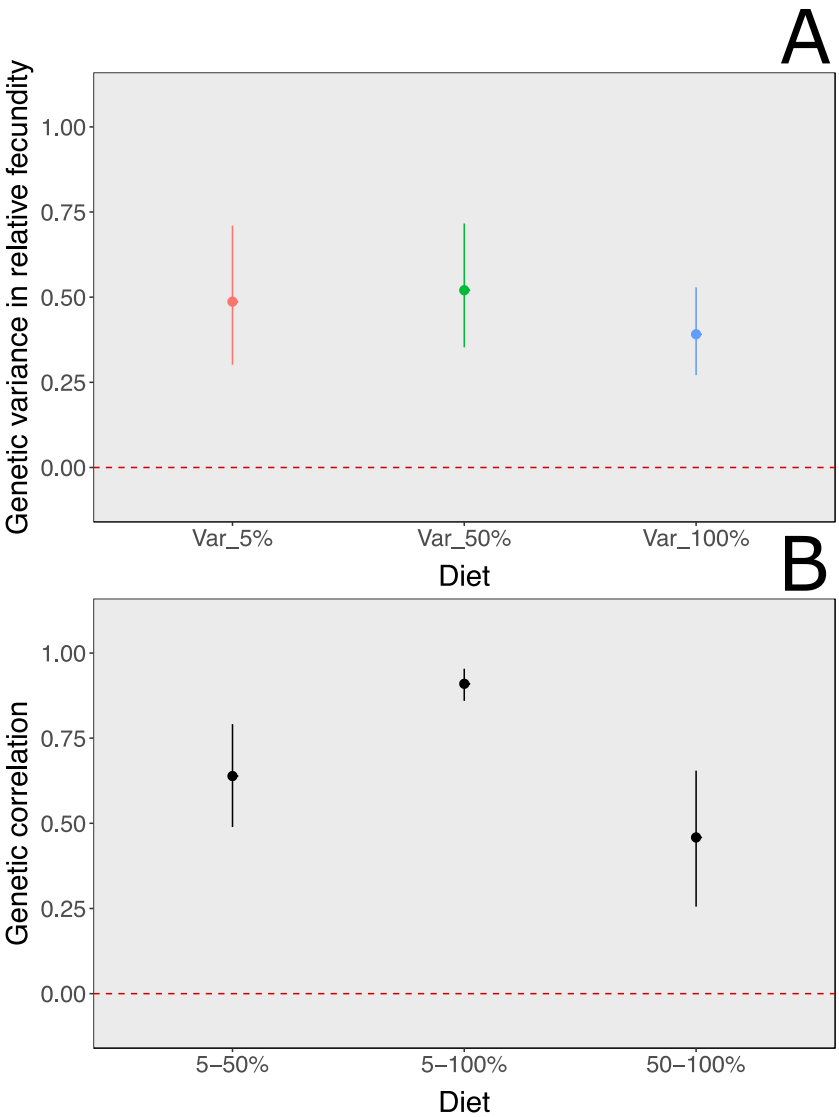

Figure 2: Estimates of genetic variance in relative fitness and genetic correlation across diets. A) Estimate of genetic variance in relative fitness and respective 95% credible interval for each diet. The dashed line represents 0 genetic variance, i.e. no variation within that diet. B) Estimates of genetic correlation and respective 95% credible intervals. The dashed line represents 0 genetic correlation, i.e. no genetic correlation between diets.

Supplementary Figure 1). Thus, the significant G×E interaction for relative fecundity is not explained by changes in genetic variance across diets.

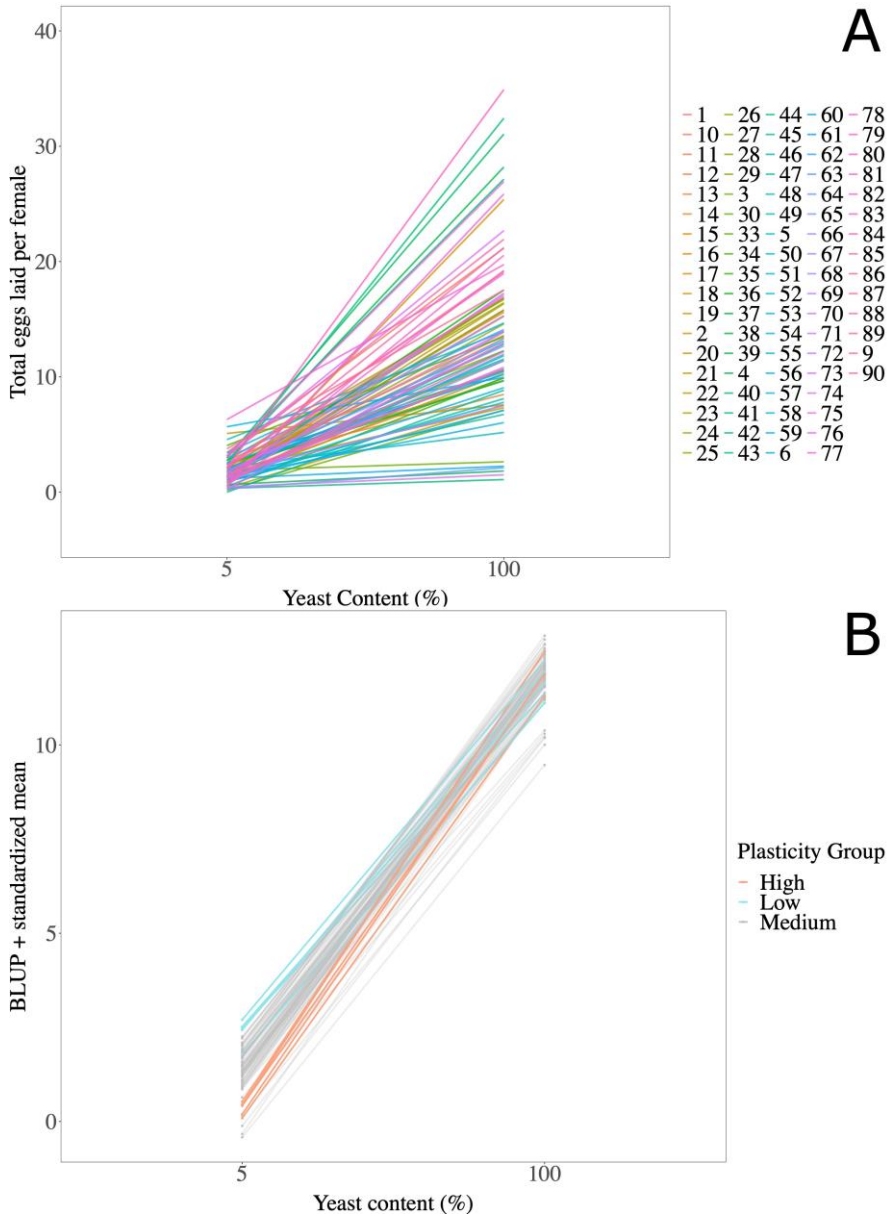

Figure 3 - Isogenic lines with either high or low plasticity for egg production between the standard and the 5% yeast content diets were chosen. A) Isogenic lines were subjected to diets with different yeast content and the number of eggs laid during days 5 to 7 were counted. Each colour represents a different isogenic line. B) Isogenic lines were subjected to two diets differing in yeast content and their BLUPs calculated to further categorise their plasticity in fecundity for yeast deprivation. The light salmon lines are the five isogenic lines with high plasticity, whereas the cadet blue lines are the five lines with low plasticity for this trait. All other isogenic lines are in grey.

Genetic correlations in fecundity among diets were all strong and positive (Figure 2B, Supplementary Figure 2). Interestingly, the genetic correlation between the 5% and 100% yeast diets was significantly greater than the remaining genetic correlations, as indicated by greater and non-overlapping credible intervals and density distributions (Figure 2B, Supplementary Figure 2). The fact that all three genetic correlation values were less than one indicates that the

significant genotype-by-environment interaction for relative fecundity results from the fact that the isogenic lines differ in some of the alleles that underpin nutritional plasticity for this trait.

Characterising plasticity across isogenic lines

To characterise differences in plasticity for female fecundity across low (5%) and high (100%) dietary protein concentrations, we subset the data to these two diets. These diets were chosen as they are characterised by the highest genetic correlation between them. Then, we standardised the number of eggs laid in the 5% diet to the mean of the 100% diet, and used the standardised values in our linear mixed effects models (Supplementary Table 2). We extracted the BLUPs from our models, which estimate how genotypes rank relative to each other and the mean. The five genotypes with the smallest BLUP, which indicates the highest change in slope relative to the mean, were chosen as the high plasticity lines. The five lines with the largest BLUP, after filtering for lines that produced less than three eggs overall, were chosen as the low plasticity lines (Figure 3B). We then used these chosen lines to identify the proximate mechanism(s) underlying differences in plasticity. We explored three proximate sources of variation in plasticity for fecundity: 1) ovary morphology, measured by ovariole number, 2)

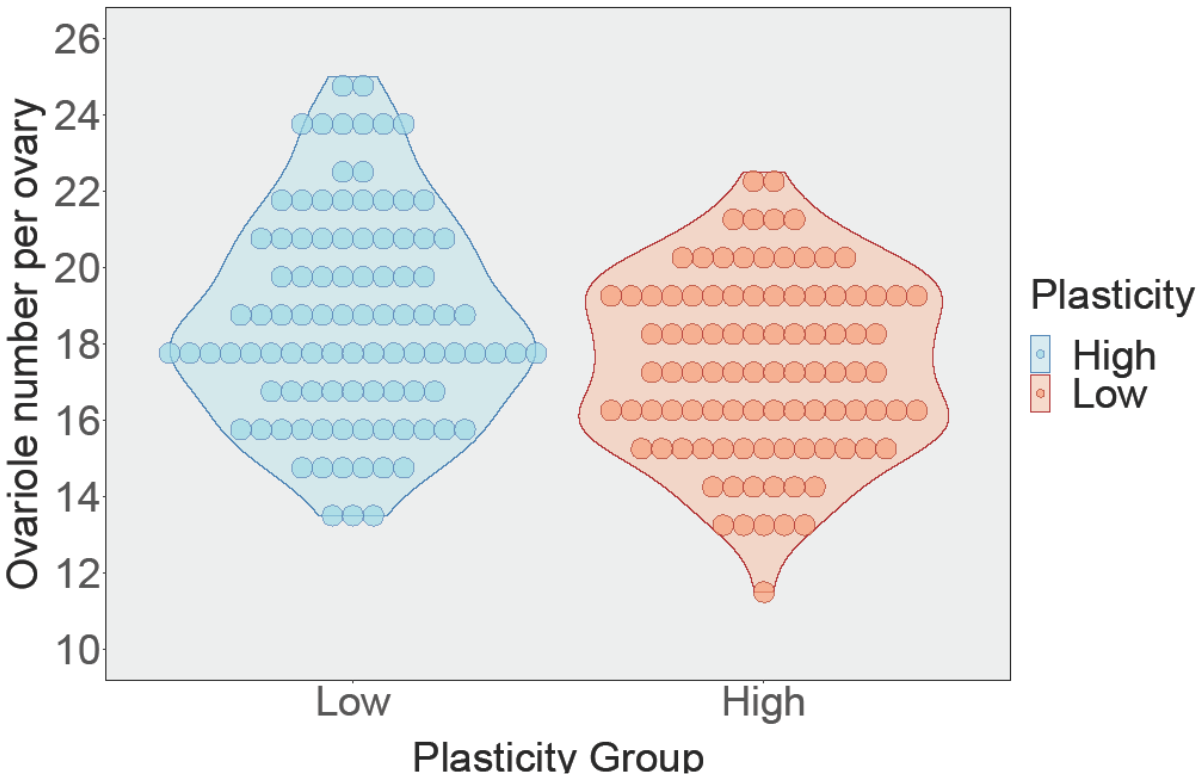

Figure 4: Ovariole number in isogenic flies with different levels of plasticity. Number of ovarioles per ovary in flies from low (cadet blue) or high (light salmon) plasticity groups. Points represent data from each isogenic line/microtube.

feeding behaviour, and 3) physiology, determined by the efficiency at which genotypes convert ingested protein into eggs.

Ovariole number does not explain differences in plasticity

Ovariole number is known to limit the number of eggs produced (David, 1970), but it is unclear whether it plays a role in plastic responses of egg laying to diet. To test whether plastic responses were determined by ovariole number, we reared our high and low plasticity isogenic lines on the same yeast-dextrose-potato media. Groups of 20 female flies from each of the high and low isogenic lines were dissected and their ovaries teased apart for ovariole counting. The number of ovarioles did not differ between plasticity groups (Figure 4, Supplementary Table 3). These data indicate that differences seen in plasticity for fecundity are not due to differences in ovariole number between isogenic lines.

Feeding behaviour differs between high and low plasticity lines

Differences in plasticity might also arise as a result of differences in food intake between high and low plasticity lines in response to differing diets. Groups of five female flies from each of the high and low plasticity lines were placed in vials filled with apple juice/agar medium. Into the cotton flug of each vial, we inserted 3 glass microcapillaries filled with holidic medium containing either 5% or 100% of the total protein of the standard holidic diet (Piper et al., 2014). Thus, the only source of protein for the flies was delivered via liquid medium in the capillaries, which could therefore be quantified. We allowed females to feed and to lay eggs for seven days.

As expected, the number of eggs laid was influenced by the protein concentration in the capillaries (Figure 5A, Table 4, Supplementary Table 4), but this depended on the plasticity grouping. On average, high plasticity lines laid more eggs than the low plasticity lines across both diets. In addition, high plasticity lines showed a steeper response in the number of eggs laid in response to the protein concentration in the diet than the low plasticity lines, resulting in a significant interaction term between diet and plasticity group (Figure 5A, Table 3). Furthermore, the fact that these lines retained either high or low plasticity on the holidic medium (this experiment) in addition to that on yeast based food (Figure 1A) suggests that this grouping method is accurate to ascertain plasticity across diet types and lines.

The concentration of protein in the diet also had a significant impact on food intake (Table 3). The high plasticity lines ate significantly more food than the low plasticity lines, and this difference was more apparent on the high protein diet (Figure 5B, Table 3, Supplementary Table 4). Furthermore, the high plasticity lines showed a mild increase in food intake with increasing protein content of the diet, while the low plasticity lines reduced their food intake slightly with increasing dietary protein concentration, resulting in a significant interaction between diet type and plasticity group for food consumption (Figure 5B, Table 3). This suggests that high and low plasticity lines differ in the way they regulate their food intake in response to the protein content of the diet.

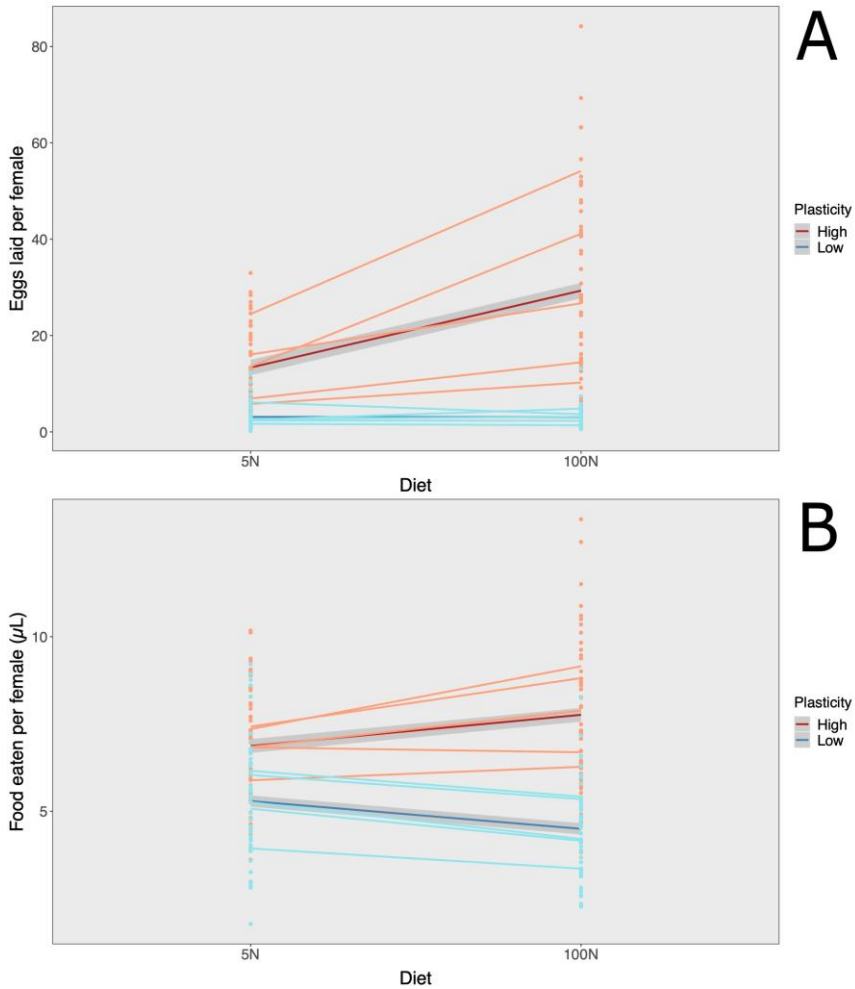

Figure 5 – Egg production and food intake in isogenic lines with different levels of plasticity. A) Eggs laid per female in diets containing 5% (5N) or 100% (100N) protein. B) Food intake per female in diets containing 5% (5N) or 100% (100N) protein. Points represent data from each replicate vial/diet/line. 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.

*Table 3 – Manipulating both diet and plasticity groups (high or low plasticity) alters egg laying or food ingestion behaviours. Egg laying data was fit with a generalized mixed effects model, whereas ingestion was fit with a linear mixed effects model. * 0.05 < P < 0.01, ** 0.01< P < 0.001, *** P < 0.001*

Number of eggs laid			
Term	**Chi-Square**	**Df**	**P-value**
Diet	1411.086	1	<2.2e-16 ***
Plasticity Group	31.255	1	2.263e-08 ***
Diet*Plasticity Group	317.383	1	<2.2e-16 ***
Model			
# 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	17.4365	1	2.971e-05 ***
Diet*Plasticity Group	133.0922	1	<2.2e-16 ***
Model			
Food Intake ~ Diet*Plasticity Group + (1|Block) + (1|Box) + (1|Replicate) + (1|Line)			

Differences in food assimilation underlie variation in plasticity

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 6, Table 4). 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 6, Table 4). 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 resulted in higher slopes for the high plasticity lines (Figure 6, Table 4, Supplementary Table 4). 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 assimilation also contribute to the response.

*Table 4 – Effect of amount of food eaten on egg laying behavior when manipulating diet and plasticity (high or low plastic lines). A generalized mixed effects model was fit to test the effect of diet, food ingestion and plasticity on egg laying. * 0.05 < P < 0.01, ** 0.01< P < 0.001, *** P < 0.001*

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)			

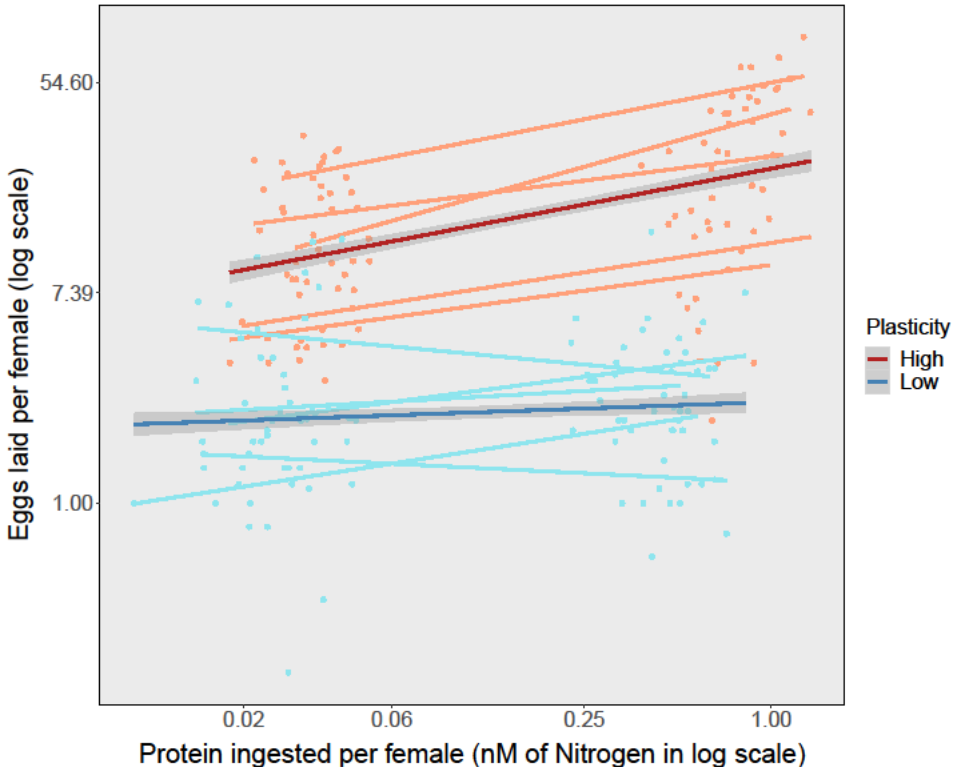

Figure 6 – Relationship between number of eggs laid and amount of protein ingested. High and low plasticity lines subjected to diets differing in protein content were allowed to lay eggs for 7 days. Cadet blue lines represent low plastic lines, salmon lines represent high plastic lines. Darker lines represent the average across lines within the same plasticity group. Both axes are represented in log scale.

Discussion

Nutritional stress is one of the most common stressors faced by animals in nature and will 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) 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), but 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; Walter et al., 2022; 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; Larsson et al., 1997; Jenkins et al., 1997; Sgrò & Hoffmann, 1998), other studies suggest that this might depend on the trait and the environmental condition studied. We found significant levels of genetic variance in relative fitness in all three nutritional environments, however the expression of genetic variance did not differ across environments. Changes in genetic variance in relative fecundity across environments, therefore, do not contribute to the significant G×E for relative fecundity across the range of diets we tested.

Although several theoretical and experimental studies have shown that genetic correlations can change in sign and magnitude when individuals are exposed to new or stressful environments (Krebs & Loeschcke, 1994; Norry & Loeschcke, 2002; Sgrò & Hoffmann, 2004), we found that all three cross-diet genetic correlations were large and positive, which is consistent with other studies that show positive genetic correlations across environments (Ebert et al., 1993, Etges, 1993, Windig, 1994). The significant G×E we found for fecundity can be explained by the fact that these correlations were less than one, indicating that the alleles that contribute to relative fecundity are in part independent across environments.

Theory suggests that genetic correlations should have higher positive values between similar environments (Sgrò & Hoffmann, 2004). Thus, we expected that the genetic correlation between the standard 100% and the 50% yeast diet would be significantly larger than the correlation between the 100% and 5% yeast diets. However, we found the opposite to be true; the genetic correlation between the standard 100% and the 5% yeast diets was significantly higher than the correlations between the 100%-50% and 50%-5% yeast diets.

Usually, deleterious mutations expressed in common environments are quickly removed by selection. However, mutations that are only deleterious in rare environments may persist and disrupt existing genetic correlations, which results in reduced genetic correlations between the rare and the common environment (Schmalhausen, 1949; Kawecki et al., 1997; Holt & Gaines, 1992; Hoffmann & Merilä, 1999; Szafraniec et al., 2001). The yeast dilutions used in this study are within the range of conditions *D. melanogaster* would face in nature, throughout the rotting process of the fruits they inhabit (Matavelli et al., 2015; Silva-Soares et al., 2016). One possible explanation as to why genetic correlations were lowest when the 100% and 5% diets were compared to the 50% diet, is that the 50% yeast diet used in this study could represent a rare dietary environment. This could be because yeast grows exponentially as the fruit rots (Matavelli et al., 2015; Silva-Soares et al., 2016), thus this intermediate yeast concentration might represent a highly transient state, and therefore, unlikely to shape selection. To understand more about genetic correlations between diets of different protein contents, comprehensive field studies analysing the dietary composition of *D. melanogaster*'s food sources are required.

Proximate mechanisms that regulate plasticity

Ovariole number is a significant predictor of fecundity in *Drosophila*, as it caps the maximum number of eggs that can be produced in optimal conditions (David, 1970). Differences in ovariole number have been studied extensively between *Drosophila* species,

and it is known that environmental factors such as nutrition and temperature regulate this number (Markow & Grady, 2008; Hodin & Riddiford, 2000; Bergland et al., 2008). Genetic differences in ovariole number result from processes occurring during larval development, when the ovarian structures first develop (Sarikaya et al., 2012). We hypothesized that genetic differences in these developmental processes might lead to differences in ovariole number, thereby limiting plasticity in fecundity. However, our data show that variation in plasticity for fecundity is unlikely to arise due to differences in ovariole number.

Previous studies have shown that flies regulate their food intake by choosing to eat different diets to maximise life-history traits. For instance, flies eat salt-rich diets to increase fecundity (Walker et al., 2015), diets with intermediate protein to carbohydrate ratios to minimise development time (Rodrigues et al., 2015), and diets with lower protein to carbohydrate ratios to modulate survival to infection (Ponton et al., 2019). Our study highlights those differences in food intake correlate with differences in nutritional plasticity in fecundity as well.

However, variation in food intake does not account for all the differences in nutritional plasticity in fecundity observed across the high and low plasticity lines in the current study. Especially with the low protein diet, we found that high and low plasticity lines showed little difference in food intake, but significantly larger differences in the number of eggs laid. This suggests that other post-ingestive, physiological mechanisms, such as amino acid absorption or nitrogen retention, underlie genetic variation in this trait. Previous studies using cabbage white, *Pieris rapae*, larvae that fed on different plants showed that larvae differ in their amino acid absorption depending on the diet they are fed (Slansky & Feeny, 1977). Variation in the efficiency of nutrient absorption and/or assimilation can also impact growth, development, and longevity (Patt et al., 2003; Min et al., 2007). Thus, it appears as though variation in the efficiency with which animals can convert nutrients into phenotypic outputs is common.

A complementary, powerful approach that could provide further insight into the proximate mechanisms that generate differences in plasticity in fecundity would be using Genotype Wide Association Studies (GWAS) in isogenic panels such as the *Drosophila* Genetic Reference Panel (DGRP) to identify genes that contribute to differences in food intake and nutrient assimilation. This approach has been used successfully to identify loci contributing to variation in thermal plasticity in body size (LaFuente et al., 2018). Other studies have found genes associated with traits such as starvation resistance, olfactory behaviour, and body mass composition that increase variation in plasticity when genotypes are exposed to different diets (Nelson et al., 2016; Sambandan et al., 2008). A SNP affecting mean food intake has already

been discovered through this approach (Garlapow et al., 2015). An approach like this could be applied to the isogenic panel used for this study to uncover new genes associated with the differences in intake or nutrient utilization observed.

To further understand plasticity, it would be of great value to examine plasticity in male fecundity in these isogenic lines. Differences in this trait could be a by-product of sexually dimorphic gene expression. Previous studies have uncovered genetic variation in male and female dietary requirements, which leads to differences in feeding behaviour between the two sexes (Camus et al., 2017). In isogenic lines, where there is a high inbreeding coefficient and allele fixation rate, gene expression might be severely skewed towards one sex across diets, which can lead to a detrimental gene expression for the other sex (Connallon & Clark, 2011). This could potentially lead to differences in plasticity, and using GWAS studies, one can uncover loci associated with sexually dimorphic gene expression and associated with variation in plasticity.

Variation in plasticity can be achieved in several ways. Here we show that variation in nutritional plasticity for fecundity is not explained by morphological differences in ovariole number, but due to differences in food intake and the ability to convert ingested nutrients into eggs. Additional research into the potential loci responsible for creating these differences will provide insight into how variation in plasticity can be achieved. Furthermore, 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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