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RESEARCH ARTICLE

Within-population variation in body size plasticity in response to combined nutritional and thermal stress is partially independent from variation in development time

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Abstract

Ongoing climate change has forced animals to face changing thermal and nutritional environments. Animals can adjust to such combinations of stressors via plasticity. Body size is a key trait influencing organismal fitness, and plasticity in this trait in response to nutritional and thermal conditions varies among genetically diverse, locally adapted populations. The standing genetic variation within a population can also influence the extent of body size plasticity. We generated near-isogenic lines from a newly collected population of *Drosophila melanogaster* at the mid-point of east coast Australia and assayed body size for all lines in combinations of thermal and nutritional stress. We found that isogenic lines showed distinct underlying patterns of body size plasticity in response to temperature and nutrition that were often different from the overall population response. We then tested whether plasticity in development time could explain, and therefore regulate, variation in body size to these combinations of environmental conditions. We selected five genotypes that showed the greatest variation in response to combined thermal and nutritional stress and assessed the correlation between response of developmental time and body size. While we found significant genetic variation in development time plasticity, it was a poor predictor of body size among genotypes. Our results therefore suggest that multiple developmental pathways could generate genetic variation in body size plasticity. Our study emphasizes the need to better understand genetic variation in plasticity within a population, which will help determine the potential for populations to adapt to ongoing environmental change.

KEYWORDS
adult size, development time, isogenic lines, nutrition, plasticity, temperature

Christen K. Mirth and Carla M. Sgrò: Equal supervision of the research. Order decided by coin toss.

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1 | INTRODUCTION

Rapid climate change is making the world warmer and more variable (Blunden & Arndt, 2016; Chevin et al., 2010; Foden et al., 2013). By altering CO_2 levels, water availability, and temperature, which are essential for plant growth and productivity, climate change also impacts the nutritional quality and quantity of food resources available to animals (Long et al., 2014; Rosenblatt & Schmitz, 2016). This has the potential to increase nutritional stress for many animal populations. One mechanism through which animals can immediately buffer the deleterious effects of ongoing environmental change is phenotypic plasticity, where a single genotype produces multiple phenotypes depending on its environment (Bonamour et al., 2019; Bradshaw, 1965; Sgro et al., 2016). Phenotypic plasticity can enable an animal to adjust immediately within a generation (Bonamour et al., 2019; Sgro et al., 2016), and in the long run to enable persistence and adaptation to environmental change across generations (Arnold et al., 2019; Noble et al., 2019; West-Eberhard, 2003), although plasticity may not always be adaptive (Ghalambor et al., 2007). Sufficient genetic variation in plasticity can allow plastic responses to evolve, which is expected to increase the potential for adaptation that promotes the long-term persistence of the population (Chevin et al., 2010; Lande, 2009). However, few studies examine how genetic variance in plasticity emerges in response to combinations of environmental stressors; as a result, we have a poor understanding of how populations may adapt to environmental change.

Plastic responses to the environment have been found in a variety of fitness-related traits that respond to a range of environmental stressors (Acasuso-Rivero et al., 2019). Thermal and nutritional stress in particular are predicted to have wide-ranging impacts on animal populations (Bonamour et al., 2019; Sgro et al., 2016). Studies have documented the individual effects of the nutritional and thermal environment on plasticity in development, body size, stress resistance, fecundity, and lifespan (Angilletta, 2009; Gray et al., 2018; Guzzo et al., 2019; Kellermann et al., 2018; MacLean et al., 2017; Mirth & Shingleton, 2012; Roeder & Behmer, 2014; Sgro et al., 2016; Simpson et al., 2004; Sisodia & Singh, 2012). Such studies highlight that phenotypic plasticity is an important mechanism that allows animals to cope with thermal and nutritional stress (Bonamour et al., 2019; Sgro et al., 2016).

Under climate change, organisms will be subjected to simultaneous changes in multiple environmental stressors. As a consequence of rising temperatures and CO_2 levels, and lower water availability, climate change is shifting the timing and abundance of food resources. (Cross et al., 2015; Raubenheimer et al., 2012). Herbivorous and frugivorous animals are therefore increasingly faced with combined thermal and nutritional stress (Rosenblatt & Schmitz, 2016). Combinations of thermal and nutritional stress have the potential to interact to generate unpredictable phenotypic responses in numerous life-history traits (Alton et al., 2020; Chakraborty et al., 2020; Kim et al., 2020; Kutz et al., 2019). For example, the caterpillars of either the beet armyworm, *Spodoptera exigua*, or the cotton leafworm, *Spodoptera litura*, change how their development (e.g. growth rate, pupal mass, and viability) responds to diet depending on the rearing temperature (Lee et al., 2015; Lee & Roh, 2010). Growth rates in *S. exigua* caterpillars increased with a greater protein to carbohydrate (P:C) ratio and with increasing temperature. Maximum growth, however, was observed at a combination of high temperature and moderate P:C ratio (Lee & Roh, 2010). Thermal stress can therefore influence how traits respond to the nutritional environment, which means that predictions of climate change responses based on single environmental factors are likely to be unreliable.

Plastic responses to thermal and nutritional stress are also known to vary across populations which reflects genetic variation in plasticity (genotype-by-environment interactions, G × E; Schlichting & Pigliucci, 1998; Lande, 2009). Some of the best evidence for genetic variation in plasticity of life-history traits comes from studies in insects sampled along environmental gradients. Numerous studies have identified significant differences in plasticity in response to single stressors like temperature (Kivelä et al., 2012; Liefting et al., 2009; Rohr et al., 2018; Sgro et al., 2010; Sørensen et al., 2016) as well as combinations of diet and temperature (Chakraborty et al., 2020) across insect populations collected from different latitudes and thus adapted to different thermal conditions. These differences in sensitivity among populations matter, as theoretical studies have shown they confer differences in susceptibility to environmental change (Dreyer et al., 2016).

Even within a population, not all individuals are equally sensitive to environmental stress because of genetic variation in plasticity (G × E interactions) between individual genotypes. Such standing genetic variation in plasticity within populations underpins responses to selection and divergence between populations in response to multiple environmental cues (reviewed in Nussey et al., 2007 and Fox et al., 2019; Bitter et al., 2019). Thus, genetic variation in plasticity within a population affects the ability of plasticity to evolve (Chevin et al., 2010; Dreyer et al., 2016; Lande, 2009; Schlichting & Pigliucci, 1998). Genetic variation in plasticity within populations (Cunningham et al., 2020; Frankino et al., 2019; Lafuente et al., 2018) arises from allelic variation at loci that modulate the plastic response of a trait. Genetic variation at these loci can generate differences in reaction norms within the population, which in turn can ultimately shape population-level variation in plasticity and any subsequent responses to selection imposed by environmental change (Frankino et al., 2019). Therefore, to better predict how populations will adapt to environmental changes, we need to understand the distribution of individual-specific plastic responses within populations.

A powerful approach to quantify genetic variation in plasticity within populations is to use panels of isogenic lines that represent the genetic variation present in natural populations (e.g. *Drosophila* Genetic Reference Panel [DGRP]; Mackay et al., 2012 and the *Drosophila* Synthetic Population Resource [DSPR]; King et al., 2012). Differences in how the lines respond to environmental variation represent genetic differences in plasticity within a population. For example, significant genetic variation in body size and cold tolerance plasticity in response to temperature has been found in the DGRP

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lines (Lafuente et al., 2018; Ørsted et al., 2018, 2019). Significant G×E in adult olfactory behaviour was also found in a study using the DGRP inbred lines that were reared in different nutritional environments (Sambandan et al., 2008).

Understanding the causes of plasticity remains an ongoing challenge. Variation in adult body size of insects in response to both temperature and nutrition is determined primarily by growth during the larval stages (Davidowitz et al., 2004; Davidowitz & Nijhout, 2004; Nijhout, 2003). Larger body sizes are either generated by longer development time or by faster growth rates, both of which depend on the dietary and thermal conditions faced during development (Davidowitz et al., 2004; Diamond & Kingsolver, 2010; Kim et al., 2020; Lee et al., 2015; Lee & Roh, 2010; Lemoine & Shantz, 2016; Neat et al., 1995). Poor nutrition extends developmental time while reducing growth rates, which results in smaller adults (Davidowitz et al., 2004). Lowering the developmental temperature from 25°C to 18°C also extends developmental time and reduces growth rates (Atkinson, 1994; Atkinson & Sibly, 1997; Miller et al., 2009), but results in larger body sizes (McDonald et al., 2018; Nijhout et al., 2010). While we do know something about how genetic variation alters the response of body size traits to multiple environmental stressors (Davidowitz et al., 2004; Diamond & Kingsolver, 2010; Kim et al., 2020; Lee et al., 2015; Lee & Roh, 2010; Lemoine & Shantz, 2016), we know very little about the underlying developmental or physiological mechanisms that contribute to these plastic shifts. For genotypes to vary in their body size plasticity in response to combined nutritional and thermal stress, either the response of development time or growth rates must differ. Understanding the proximate causes of differences in size plasticity allows us to explore how different genetic pathways can contribute to shape individual and population-specific body size plasticity.

In this study, we created a panel of isogenic lines of *D. melanogaster* initiated from a population collected from the central part of its range along the east coast of Australia. This new set of isogenic lines allows us to build on the extensive body of research on the ecology and evolution of *D. melanogaster* along this latitudinal cline (Hoffmann et al., 2005; Hoffmann & Weeks, 2007; Sgro et al., 2010). Using the isogenic lines, we first quantify genetic variation in body size plasticity in response to combined thermal and nutritional stress. We then sought to understand the proximate mechanisms underlying differences in size plasticity between genotypes by asking whether differences in size plasticity might be explained by variation in development time. We simulated changes in nutrient abundance by manipulating caloric concentrations of the diet and measured the impacts of thermal stress by rearing larvae either at the standard 25°C or at the elevated temperature of 28°C (following Kutz et al., 2019; Alton et al., 2020; Chakraborty et al., 2020). We quantified wing area as a proxy for body size (Chakraborty et al., 2020; Kutz et al., 2019; Mirth et al., 2005; Nijhout et al., 2014; Shingleton et al., 2017) because it affects numerous life-history, physiological, and behavioural traits that are closely linked to fitness (Bonner, 2011; Calvo & Molina, 2005; Healy et al., 2014; Lasne et al., 2018; Speakman, 2005), and because it is also sensitive to combinations of temperature and nutrition (Chakraborty et al., 2020; Kutz et al., 2019; Lafuente et al., 2018; Shingleton et al., 2017). We first quantified genetic variation in plasticity of wing area. We then selected five lines that showed the greatest difference in plastic response to temperature and nutrition, and tested whether body size plasticity was genetically correlated with developmental time plasticity across the same diet-temperature combinations. Our study provides a framework for understanding how genotype-specific variation in plasticity contributes to a population's sensitivity to environmental change and sheds light on the proximate cause of this variation.

2 | MATERIALS AND METHODS

2.1 | Generation of the isogenic lines

Two hundred field-inseminated *D. melanogaster* females were collected from a banana plantation and Tropical Fruit World in Duranbah (Queensland), mid-way along the east coast of Australia in January 2018 (28.3°S, 153.5°E). From each wild-caught female, two independent isofemale lines were generated, resulting in 400 lines which were then inbred for 20 generations following Mackay et al. (2012). Some lines were lost as levels of inbreeding increased, such that 81 near-isogenic lines were ultimately established after 20 generations of full-sib mating.

Flies were maintained at a constant temperature of 18°C with a 12-h light/dark cycle in temperature-controlled cabinets and on a yeast-dextrose-potato diet (potato flakes 20 g/L; dextrose 30 g/L; 95 Brewer's yeast 40 g/L; agar 7 g/L; nipagen 6 ml/L; and propionic acid 2.5 ml/L, of P:C ratio of 1:3, 318.42 kcal). We maintained four replicates of each isogenic line for 12 generations at 18°C, after which we kept them at 25°C for two generations immediately prior to the experiments described below. This was done because we were interested in assessing the combined impacts of nutritional stress and elevated temperature on size plasticity; holding all experimental lines at 25°C for two generations ensured that we were able to control for any grandparental and maternal effects carried over from 18°C, and were able to treat 25°C as the 'control' temperature and 28°C as the experimental elevated temperature.

2.2 | Experimental conditions for characterization of plasticity

To test how combinations of stressors affect plasticity, we exposed all isogenic lines to combinations of diet and thermal stress. To simulate changes in quality of food resources predicted due to climate change, we placed *D. melanogaster* larvae on one of three diet dilutions: 12.5% (39.8 kcal), 25% (79.6 kcal), and 100% (318.42 kcal) of standard food. The 100% diet was the standard yeast-dextrose-potato medium with a P:C ratio of 2:3 (318.42 kcal). These diets

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were chosen based on the results of Chakraborty et al. (2020), which showed wing area plasticity to change across these dietary conditions. Further, these diets represent a subset of nutritional conditions found in rotting fruits upon which *Drosophila* flies feed in nature (Matavelli et al., 2015; Silva-Soares et al., 2017), which will likely become more common under climate change (Cross et al., 2015; Rosenblatt & Schmitz, 2016). Each diet was prepared by measuring either 12.5%, 25%, or 100% of the quantities of inactive yeast, dextrose, and potato flakes to the same amount of water and agar.

To impose thermal stress, larvae from all lines for each diet type were reared at one of two temperatures (25°C or 28°C), reflecting the 3°C increase in temperature predicted with climate change (www.bom.gov.au). We have previously (Alton et al., 2020 and Kutz et al., 2019 Functional Ecology) used 28°C for similar reasons.

2.3 | Experiment 1: Assessing within-population genetic variation in body size plasticity

To assess genetic variation in plasticity in response to combined thermal and nutritional stress, we randomly chose 50 isogenic lines from the original 81 lines described above. We obtained eggs from each line for the plasticity assays by acclimating parental flies to egg-laying plates containing standard food with double the amount of agar for 24 h and then allowing them to lay on fresh plates every 12 h for one day. For each line, we transferred eggs into three replicate vials for each of the six diet/temperature treatment combinations. We transferred 20–30 eggs into each vial, which contained 7 mL of the experimental diets. Larvae were left to develop to adult eclosion, and we moved the vials at random within the cabinets every 12 h to remove any biases caused by temperature and light gradients within the cabinets.

2.3.1 | Adult body size measurements

We used wing area as a proxy for body size to assess plastic responses to temperature and nutrition (Chakraborty et al., 2020; Kutz et al., 2019). We collected eclosed flies from all three replicate vials from each line in each treatment combination and preserved them in 70% ethanol/30% glycerol solution (SH solution). We dissected wings for 20–30 female flies for each temperature/diet/line combination, depending on how many females were collected across all replicate vials. We mounted the left wing of each female on a microscope slide and photographed them using a Leica M80 stereo microscope (Leica, Heerbrugg, Switzerland). We only measured female flies because their body size is known to be more sensitive to nutrition compared to males (Shingleton et al., 2017) and because assessing sex-specific plasticity was not the focus of the present study. All images were processed using ImageJ software. Wing area was estimated by calculating the centroid size from landmarks of six vein positions (Figure S1) following Chakraborty et al. (2020).

2.3.2 | Statistical analysis

Testing for significant genotype-by-environment (G×E) interactions

To initially test for significant G×E, we employed linear mixed effect models. The lme4 package in R (Bates et al., 2011) was used to fit sequential models (Table S1) with developmental diet (dilution of 100%, 25% and 12.5%), temperature (25°C and 28°C), and their interactions as fixed factors. Experimental block and isogenic line were included as random effects. Because we are interested in differences in plasticity across the isogenic lines, our analysis focused on differences in slopes and not main effects. Log likelihood ratio tests were used to sequentially test the goodness of fit of each model. The first model,

$$\text{Model 1.1:}\quad y \sim \text{Diet}^{*}\text{Temp} + (1|\,\text{Block}),$$

had diet and temperature as the two fixed factors with experimental block as random effect; this showed the overall population response to combinations of diet and temperature. The second model,

$$\text{Model 1.2:}\quad y \sim \text{Diet}^{*}\text{Temp} + (1|\,\text{Block}) + (1|\,\text{Line})$$

further included line as random effect to account for differences in wing area across the isogenic lines (Table S1).

The third model,

$$\text{Model 1.3:}\quad y \sim \text{Diet}^{*}\text{Temp} + (1|\,\text{Block}) + (1 + \text{Diet:Temp}|\,\text{Line}),$$

further built upon Model 1.2 by adding the interaction between diet, temperature and line as random effects.

Quantifying genetic variance in body size

Bayesian models were then used to determine whether the significant G×E was the result of changes in genetic variance for wing size across diet/temperature combinations, or by genetic correlations across environments whose values were <1 (Falconer, 1952; Via & Lande, 1985). We treated each combination of diet and temperature as a separate trait, resulting in six traits (two temperatures: 25°C and 28°C, and three diet dilutions: 12.5%, 25%, and 100%) following the character-state approach described by Falconer (1952) and Via and Lande (1985). We performed our analysis on the raw data.

To estimate genetic variance in body size within and genetic correlations across environments, we used the package 'MCMCglmm' in R (Hadfield, 2010) to apply the linear mixed effects model

$$\text{Model 2:}\quad y_{ijklm} = D_i \times T_j + l_{k(ij)} + b_{l(kij)} + e_{m(ijkl)}$$

where the only fixed effect was treatment, which was included as the interaction between *i*th diet (D_i) and *j*th temperature (T_j). Random effects included the *k*th isogenic line ($l_{k(ij)}$), block ($b_{l(kij)}$) within the diet and temperature combinations of treatments, and the residual variance ($e_{m(ijkl)}$). For the isogenic line variance component, we estimated random intercepts and slopes across the six treatment combinations,

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which calculates the among-line genetic variance within each treatment as well as the genetic covariance between the treatment, which produces a 6×6 genetic covariance matrix for wing size. To account for block and residual variance within each treatment, we estimated random intercepts (but not slopes) for the block and residual components because different individuals were measured in different blocks and treatments. We used the covariance matrix to estimate genetic correlations across treatment combinations with this analysis.

Overlap in the 95% highest posterior density (HPD) intervals and the density distributions in genetic variances and genetic correlations were used to make inferences about differences in genetic (co) variances across the different treatments.

We applied Model 2 with chains of 800000 Markov Chain Monte Carlo (MCMC) iterations and a burn-in of 100000 iterations. We saved 3200 iterations, which provided the posterior distribution for all parameters that we estimated. We ensured that models converged, and we checked autocorrelation by confirming that effective sample sizes exceeded 85% of the number of saved samples. We used an uninformative parameter-expanded prior, which we checked by changing the scale parameter and ensuring there was no effect on the estimation of the posterior distribution.

Quantifying differences in plasticity across isogenic lines – random regression models

Once we established significant G×E in the analyses above, we wanted to identify the genotypes (isogenic lines) that varied the most in their plastic response to nutrition and temperature. We used random regression mixed models (Arnold et al., 2019) composed of two predictors: fixed effects of diet and temperature, that quantified the change in mean of response variable (wing area) across treatments and captured plasticity for the entire population. Random effects included isogenic lines, and how they varied across the treatments, capturing within-population genetic variation in plasticity. This approach effectively characterizes variation in shapes of reaction norms of individual genotypes within a population, allowing us to identify genotypes that vary the most in their plasticity (Arnold et al., 2019).

Random regression models were applied to wing area by using log likelihood ratio tests to determine which model was the best fit for the data, beginning with the simplest linear model:

$$\text{Model 3.1: } y \sim 1 + \text{csDiet} \times \text{Temp} + (1|\,\text{Block})$$

where 1 is the intercept for the overall model, and the two fixed effects are csDiet (mean centred and variance scaled) and Temp is the developmental temperature, where csDiet × Temp models wing area is a population-level reaction norm of the fixed effects of csDiet and Temperature (Table S3). Block is the random effect accounting for experimental blocks. Mean-centred diet was used such that the intercepts and slopes reflected average values (reaction norms) for the population and individual genotypes, respectively (Arnold et al., 2019). This was further variance scaled to obtain model convergence. The above model was built upon by adding additional random terms testing for significant effects of Line, and interactions between Line, Temp, and csDiet (Table S3) until further changes no longer improved the fit, based on AIC values and log likelihood ratio tests, such that the final model was:

$$\text{Model 3.5: } y \sim 1 + \text{csDiet} \times \text{Temp} + (1|\,\text{Block}) + (1 + \text{csDiet} \times \text{Temp}|\,\text{Line})$$

where the addition of the (1 +csDiet*Temp) term to the random effect term (|Line) allows the slopes of the reaction norms for each isogenic line to vary in response to the combined effects of diet and temperature (Table S3). This specifies that now the random component of isogenic line can vary both in slope and intercept in response to the combined effects of diet and temperature. Thus, model 3 tests for variation in plasticity to the combined effects of diet and developmental temperature at the isogenic line level, relative to the population-level fixed effects. A detailed description of each model is provided in Table S3.

We used the final best fitting model to extract the slope of the best linear unbiased predictors (BLUPs) for each genotype (isogenic line) across the combinations of diet and temperature. Using these BLUP slope estimates, we ranked the genotypes in how they differed in reaction norm across the diet and temperature combinations (Arnold et al., 2019) relative to the population mean reaction norm. A negative BLUP slope estimate indicates that the particular line had a steeper slope than the population average, whereas a positive BLUP slope estimate means the line had a shallower slope than the population average (modified from Arnold et al., 2019). From the rankings, we chose three isogenic lines that showed greater change (i.e. steeper slope and thus High plasticity) and two isogenic lines that showed smallest change (i.e. shallower slope, Low plasticity) relative to the population mean.

2.4 | Experiment 2: Identifying mechanisms that shape variation in the plastic response of body size across selected isogenic lines

Variation in adult body size of insects can be determined by the rate of growth and the time an animal takes to develop from egg to adult (Davidowitz et al., 2004; Davidowitz & Nijhout, 2004; Nijhout, 2003). We therefore tested whether differences in body size plasticity between our chosen isogenic lines could be explained by plastic variation in development time.

2.4.1 | Egg-to-adult development time and body size

We measured egg-to-adult development time and body size (female wing area) for the five selected isogenic lines on two diet dilutions (12.5% and 100%) at both 25°C and 28°C, resulting in 4 experimental conditions. Parent flies from each of the five selected lines were acclimated to egg-laying chambers containing standard food for

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24 h, changing the egg plates every 12 h. Eggs were subsequently collected over a 6-h laying interval. Twenty eggs from each line were distributed to four replicate vials per experimental condition.

Egg-to-adult development time was recorded as the time in hours from the mid-point of egg laying to adult eclosion. Once counted, flies were removed from their vial and stored for wing size measurements. Vials were checked every 8 h until 12 consecutive time points yielded no flies, after which vials were discarded.

2.4.2 | Statistical analysis

We used linear mixed effect models within the lme4 package (Bates et al., 2011) to assess the effect of developmental diet and temperature on both traits. Developmental diet (dilution of 100% and 12.5%), temperature (25°C and 28°C), plasticity level (High and Low plasticity), and their interactions were treated as fixed factors. The high plasticity (HP) group contained the three isogenic lines that showed greater change (i.e. steeper slope) across diet/temperature combinations as described in Experiment 1 above. The low plasticity (LP) group contained the two isogenic lines that showed smallest change (i.e. shallower) in slope, as determined from the random regression analysis above. Isogenic lines and replicate vials were included as random effects. Analyses were performed on the full dataset for each trait.

All data were visualized using ggplot2. All statistical analyses were performed in R studio (version 3.4.1, R Development Core Team 157 2017, https://www.r158project.org/). Data and scripts are publicly available at Figshare (https://doi.org/10.26180/17080664).

3 | RESULTS

We aimed to explore genetic variation in body size plasticity in response to the combined effects of nutrition and temperature within a population and to understand the proximate cause of this variation. To this end, we established a new set of inbred isogenic lines and characterized how body size changed in response to combinations of diet and temperature. Genotypes that differed the most in body size plasticity were then used to understand how combinations of thermal and nutritional stress affected one of the two contributors to the final body size and the length of an animal's growth period. Our study considers the role of intra-population variation in plasticity, which may ultimately shape a population's ability to adapt to changing environments.

3.1 | Experiment 1: Assessing within-population genetic variation in body size plasticity

Diet, temperature, and isogenic line all had significant effects on body size (Figure 1; Table S1). On average, flies were smaller at 28°C and on the 12.5% diet compared to 25°C and diets higher in protein (Figure 1). Significant G × E was indicated by the better fit of the model including line and its interaction with diet and temperature as random factors (Model 1.3, Table S1). This means that lines differed in how wing area responded to combined nutritional and thermal stress, resulting in non-parallel reaction norms (Figure 1).

3.2 | Quantifying genetic variance in body size

Because the interactions between genotype and environmental conditions were significant, we next employed Bayesian models to determine whether this genetic variance in plasticity was the result of differences in the amount of genetic variance in wing size across environments, or due to cross-environment genetic correlations being less than one (i.e. an indication that genetic variation in wing area results from partially independent allelic variation across environments). We found significant genetic variance in wing area in all treatments (Figure 2a; Table S2, Figure S2a). However, the amount of genetic variance was similar among treatments (Figure 2a; Table S2). Thus, the variation in wing size plasticity among genotypes was not due to significantly different expression of genetic variance across environments.

Genetic correlations across all treatments were positive and significantly greater than zero and generally less than 1 (Figure 2b; Figure S2b) (Falconer, 1952; Via & Lande, 1985). Most genetic correlations were less than 1, which implies that there is some level of genetic independence across environments, resulting in significant variation in plasticity among genotypes. Interestingly, we found the lowest genetic correlation (r_g) between 100% diet at 28°C and 12.5% diet at 25°C (r_g = 0.70. 89% HPD = 0.57, 0.85), and the highest genetic correlation between 100% diet at 25°C and 25% diet at 28°C (r_g = 0.93, 89% HPD = 0.88, 0.97, Figure 2b; Figure S2b, Table S2). Thus, the significant G × E in size occurs because genotypes differ in at least some of the alleles that regulate plasticity in wing size in response to combinations of temperature and diet.

3.3 | Quantifying differences in genetic variation in size plasticity across isogenic lines

After quantifying the basis of genetic variance in plasticity for wing area, we then proceeded to identify those lines that differed the most in their plastic response to nutrition across temperature using random regression models (detailed description of each model is given in Table S3). We began with the simplest model, where wing area was modelled as a population-level reaction norm in response to diet and temperature, including experimental block as a random effect (model 3.1, Table 1; Table S3). On increasing the complexity of our models (Table 1; Table S3), we found that the best fitting model was the one that allowed the slopes of the random isogenic lines to vary across both diet and temperature in combination (model 3.5, Table 1). This further confirms the presence of genetic variation in size plasticity to combined stress.

We then extracted BLUP slope estimates and used these to rank the isogenic lines according to their plastic response to the combined effects of temperature and nutrition (Figure 3a). Negative BLUP slope estimates represent steeper plastic responses to combinations

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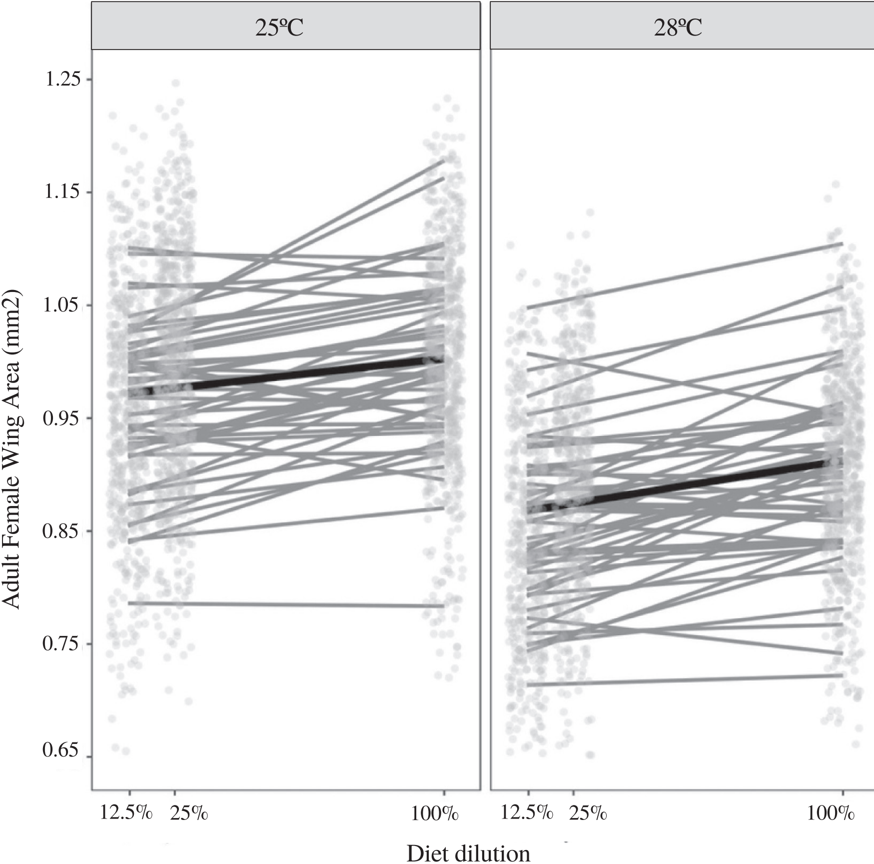

FIGURE 1 Reaction norms for female wing area in 50 independent isogenic lines in response to the combined effects of developmental nutritional and temperature. The solid black line shows the mean population reaction norm. Each isogenic line's reaction norm is given by gray coloured lines. Differences in slopes reveal genetic variation in size plasticity.

of nutrition and temperature, whereas positive BLUP slope estimates represent shallower plastic responses to combinations of nutrition and temperature, relative to the population mean response. Lines varied in their plastic response to developmental diet across developmental temperature relative to the population mean reaction norm (Figure 3a), further emphasizing the fact that the plastic response of individual genotypes does not necessarily predict plasticity across the whole population.

We then chose five isogenic lines that were among the lines that differed the most in their plastic responses to combinations of temperature and nutrition relative to the population mean response. These were chosen from the two extreme ends of the rank order (Figure 3a). Lines 21, 63, and 36 were among the lines with the steepest plastic response, whereas lines 85 and 56 were among the lines with the shallowest plastic response to nutrition at 25°C versus 28°C, relative to the mean population response (Figure 3). Given this diversity of plastic response in wing size across genotypes, we next wanted to better understand how these differences in plasticity across genotypes arose.

3.4 | Experiment 2: Identifying mechanisms that shape variation in the plastic response of body size across selected isogenic lines

We then used the above five lines to explore a proximate mechanism potentially contributing to genetic variation in size plasticity. We grouped the five lines into two groups based on whether they showed a steeper response and hence higher plasticity (HP, lines 21, 63, and 36) or a shallower response and hence low plasticity (LP, lines 56 and 85). We used the two extreme dietary dilutions of 12.5% and 100%, at either 25°C or 28°C for the subsequent experiments. Because final adult body size is a product of how much time an animal takes to develop and the rate at which it grows (Davidowitz et al., 2004; Davidowitz & Nijhout, 2004), we measured egg-to-adult development time and wing area for these two plasticity groups (HP and LP) across four nutritional/thermal conditions.

For wing area, we found that diet, temperature, and their combination had a significant effect on the plastic response. This second experiment further confirmed that the presence of genetic variance in size plasticity, as indicated by the significant three-way interactions between diet, temperature, and plasticity level (Table 2, Figure 4a). This was driven by the high plasticity group (HP, lines 21, 36, and 63), which changed their extent of nutritional plasticity in wing area depending on the rearing temperature, compared to the low plasticity group (LP, lines 56 and 85).

We then examined the extent of plasticity in developmental time across these five lines. Overall, faster development time was found when animals were reared at 28°C and 100% diet (Figure 4b). The two plasticity groups also significantly varied in their plastic response to combinations of nutrition and temperature (Table 2, Figure 4b), resulting in a significant three-way interaction between diet, temperature, and plasticity level. This was again driven by the high plasticity group (HP, lines 21, 36, and 63) which changed their nutritional plasticity depending on the rearing temperature, compared to the

FIGURE 2 (a) Estimates of genetic variance and (b) genetic correlations, in adult female wing area across the six combinations of diet dilution and developmental temperature treatments. Error bars represent 95% highest posterior density (HPD) intervals.

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TABLE 1 Random regression mixed models testing for genetic variation in plasticity of adult female wing area, in response to the combined effects of developmental diet and temperature.

<table>
<tr><th>Model number</th><th>Wing area (y) model</th><th>R^2</th><th>AIC</th><th>Likelihood ratio test (p value)</th></tr>
<tr><td>3.1</td><td>y ~ 1 + csDiet*Temp + (1|Block)</td><td>0.45</td><td>−7692.9</td><td>–</td></tr>
<tr><td>3.2</td><td>y ~ 1 + csDiet*Temp + (1|Block) + (1|Line)</td><td>0.57</td><td>−8911.9</td><td>1.1–1.2[a]: <0.0001</td></tr>
<tr><td>3.3a</td><td>y ~ 1 + csDiet*Temp + (1|Block) + (1 + csDiet|Line)</td><td>0.595</td><td>−9004.9</td><td>1.2–1.3a[a]: <0.0001</td></tr>
<tr><td>3.3b</td><td>y ~ 1 + csDiet*Temp + (1|Block) + (1 + Temp|Line)</td><td>0.599</td><td>−9018.7</td><td>1.2–1.3b[a]: <0.0001</td></tr>
<tr><td>3.4</td><td>y ~ 1 + csDiet*Temp + (1|Block) + (1 + csDiet|Line) + (1 + Temp|Line)</td><td>0.62</td><td>−9110.8</td><td>1.3a–1.4[a]: <0.0001
1.3b–1.4[a]: <0.0001</td></tr>
<tr><td>3.5</td><td>y ~ 1 + csDiet*Temp + (1|Block) + (1 + csDiet*Temp|Line)</td><td>0.63</td><td>−9150.0</td><td>1.4–1.5[a]: <0.0001</td></tr>
</table>

Note: Mean population-level phenotypic response to the combined effects of diet and temperature is assessed by model 3.1, while variation at the individual genotype level is assessed in models 3.2 onwards, whereas Line (genotype) is included as a random effect. *csDiet* represents the fixed effect of diet that has been standardized by means and variance, that is both centred (*c*) and scaled (*s*). *Block* represents the random effect accounting for the differences between measures across experimental blocks. Detailed description of each model is given in Table S3. Model fit improves with each modification, as indicated by an increase in R^2 and decrease in AIC values. To determine significance of the improved fit of each model, likelihood ratio tests were performed (*p* value of test displayed).

[a]The models being compared in the log likelihood ratio tests.

low plasticity group (LP, lines 56 and 85). Thus, we found evidence for significant genetic variation in the plastic response of development time to combinations of diet and temperature.

Given that development time is an important proximate mechanism for determining adult body size characters, we next asked whether plasticity in development time could predict plasticity in wing size across the two plasticity groups (HP and LP). Strong correlations between these two traits would indicate that the genotype by environment interactions of both traits share a common genetic mechanism.

To better understand the extent to which plasticity in development time can predict plasticity in body size, we estimated the correlation between egg to adult development and adult female wing area, across the five chosen lines. Pearson's correlation between the two traits across the entire dataset was significant and negative ($r = -0.2$, $p = 0.00028$); shorter development time was associated with larger body size (Figure 5a). However, when we examined lines individually, we found that only lines 36 (HP) and 56 (LP) displayed a significant negative correlation between development time and body size. This means that development time was not a reliable predictor of body size for all lines across the high and low plasticity groups over the environmental conditions used in this study. Thus, plasticity in development time on its own could not explain the differences in the wing area plasticity across the high plastic and low plastic groups. In other words, genetic variation underpinning plasticity in wing size and development time is at least partially independent (Figure 5b).

4 | DISCUSSION

The ability of organisms to immediately adjust to environmental changes via phenotypic plasticity has been well studied (Bonamour et al., 2019; Sgro et al., 2016). Animals in the wild are regularly challenged by changes in their thermal environment and in the quality and quantity of food available to them (Long et al., 2014; Rosenblatt & Schmitz, 2016). Differences in the way that individuals within a population respond to shifts in diet and temperature may shape the capacity for a population to adapt and persist in the face of environmental change (Chevin et al., 2010; Dreyer et al., 2016; Frankino et al., 2019; Lande, 2009; Schlichting & Pigliucci, 1998). For this reason, understanding intrapopulation variation in plasticity to combinations of environmental stress is important, since it may either limit or facilitate adaptation to global change.

Multiple studies have shown plasticity to differ across locally-adapted, genetically-diverged populations (Chakraborty et al., 2020, 2021; Kivelä et al., 2012; Liefting et al., 2009; Rohr et al., 2018; Sgro et al., 2010; Sørensen et al., 2016). However, a population is composed of individuals that vary in genotype. Studies across a wide range of taxa have shown within-population variation in plasticity for numerous life-history and stress resistance traits (Camus et al., 2017; Cunningham et al., 2020; Frankino et al., 2019; Lafuente et al., 2018; Ørsted et al., 2017, 2018; Sambandan et al., 2008). For example, Lafuente et al., 2018 showed that genotypes within a population differed in thermal plasticity of abdomen and thorax size. Similarly, genotypes differed in their plastic response of fecundity to the concentration of protein and carbohydrates in diet (Camus et al., 2017). The above-mentioned studies demonstrate that within a population, genotype-specific plastic responses to single environmental factors like nutrition or temperature can differ from population-level plastic responses.

However, in nature animals need to adjust to combinations of both nutrition and temperature (Rosenblatt & Schmitz, 2016). A growing body of work highlights the degree to which temperature can modify the response of animals to other environmental stressors like nutrition (Chakraborty et al., 2020; Clissold & Simpson, 2015; Kim et al., 2020; Kutz et al., 2019; Lee et al., 2015; Lemoine & Shantz, 2016). In particular, temperature can change the dietary conditions that maximize life-history traits like body size, fecundity, lifespan, and viability. For instance, higher temperatures significantly decreased lifespan but increased developmental rate with increasing dietary P:C ratio compared to cooler temperatures (Kim et al., 2020). The current study adds to these insights by showing that individuals within a population can vary in the extent and/or direction of body size plasticity in response to combinations of diet and temperature,

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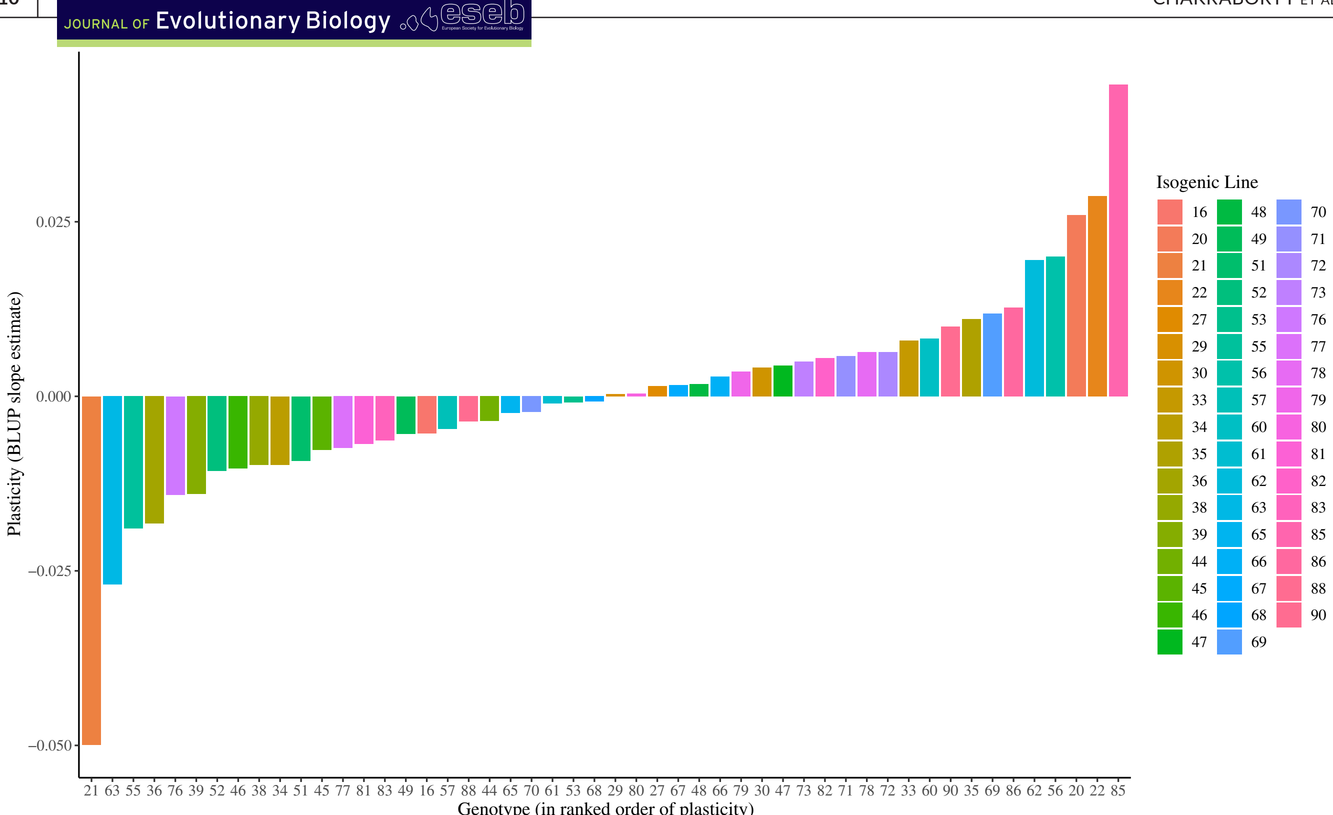

FIGURE 3 Rank order of body size plasticity of isogenic lines in response to the combined effects of rearing temperature (25°C and 28°C) and diet dilution. Lines are ranked from most to least plastic based on their BLUP slope estimates. The average population-level plasticity is depicted by zero on the *Y*-axis (BLUP slope estimate), and the degree of variation in plasticity across isogenic lines is represented by deviation from zero; a more negative BLUP slope estimate represents a steeper plastic response compared to the population mean, whereas a more positive BLUP slope estimates represent a shallower plastic response compared to the population mean, across combinations of diet and temperature.

TABLE 2 Effects of developmental diet dilution (100% or 12.5%), temperature (either 25°C or 28°C), plasticity level and their products on body size and egg-adult development time assessed for the five selected isogenic lines grouped into either high plasticity (HP) or low plasticity (LP).

Traits	Egg-adult development time	Adult female wing area	d.f.
Terms	Chisq	Chisq	–
Diet	806.753***	101.311***	1
Temp	270.086***	38.360***	1
Plasticity Level	1.379	0.3122	1
Diet × Temp	3.712	8.440*	1
Diet × Plasticity Level	13.161***	1.218	1
Temp × Plasticity Level	0.481	3.250	1
Diet × Temp × Plasticity Level	5.152*	4.308*	1

***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

and that this variation is not predicted from the population-level plastic response. This is important, because it means that even though population-level reaction norms may look very similar across populations, the underlying patterns of genotype-level plasticity could differ significantly (Dreyer et al., 2016; Frankino et al., 2019). This distribution of genotype-specific reaction norms within populations matters because it determines how a population responds to selection pressures that accompany global change (Dreyer et al., 2016).

The observed G × E interactions within a population can be caused either by differences in genetic variance across environments and/or cross-environment genetic correlations that are less than one (Falconer, 1952; Hoffmann & Merilä, 1999; Ørsted et al., 2017, 2018; Sgrò & Hoffmann, 2004; Via & Lande, 1985). When considering a single stressor, one might expect plasticity (if adaptive) to reduce genetic variation in the trait (Arnold et al., 2019; Ghalambor et al., 2007; Noble et al., 2019). However, in the context of combined stressors perhaps similar levels of genetic variation across environments might increase the capacity of a population to be able to respond to a continuously varying, and perhaps unpredictable, environment. While we found significant genetic variance for wing area across all combinations of diet and temperature, the expression of genetic variance did not vary with environment. Consistent with previous studies (Ebert et al., 1993; Etges, 1993; Ørsted et al., 2018; Windig, 1994), we found genetic correlations to be above zero, and generally less than one, across the diet and temperature combinations. This indicates that the alleles regulating the plastic response of wing size across diets and temperatures are at least partially independent.

Any variation in body size plasticity arising from either plastic responses or genetic differences must arise from changes in growth

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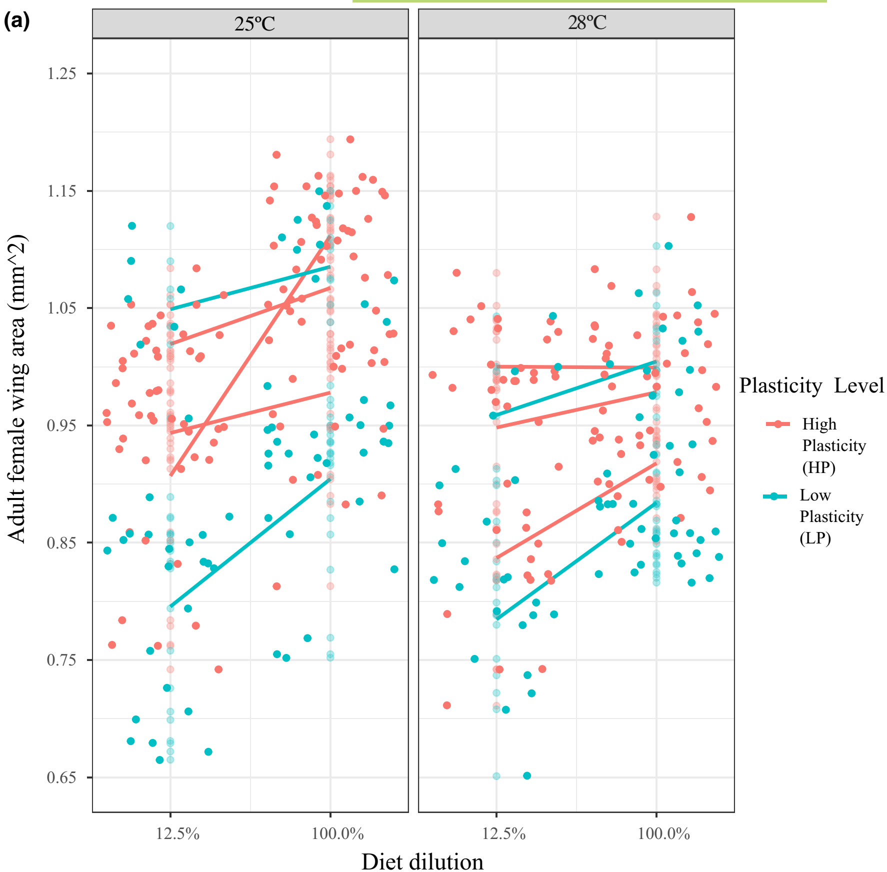

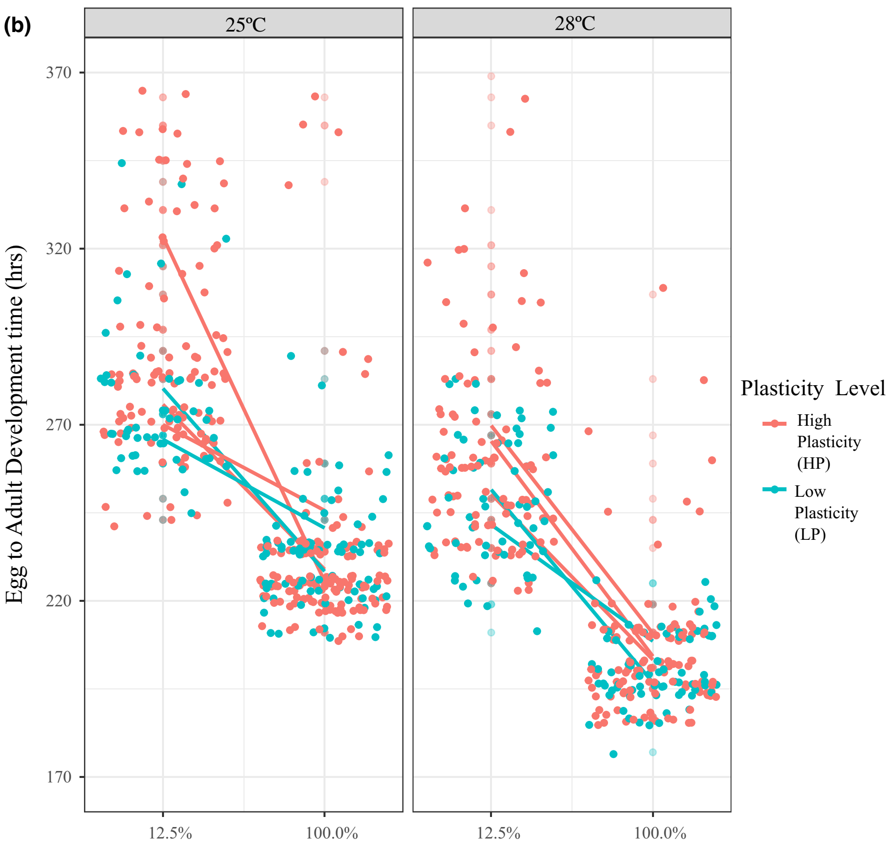

FIGURE 4 Plastic response of the five selected lines grouped as either high (lines 21, 36, 63) or low (lines 56 and 85) plasticity for (a) female wing area and (b) egg to adult development time, in response to developmental diet dilution (12.5% or 100%) and rearing temperature (25°C or 28°C).

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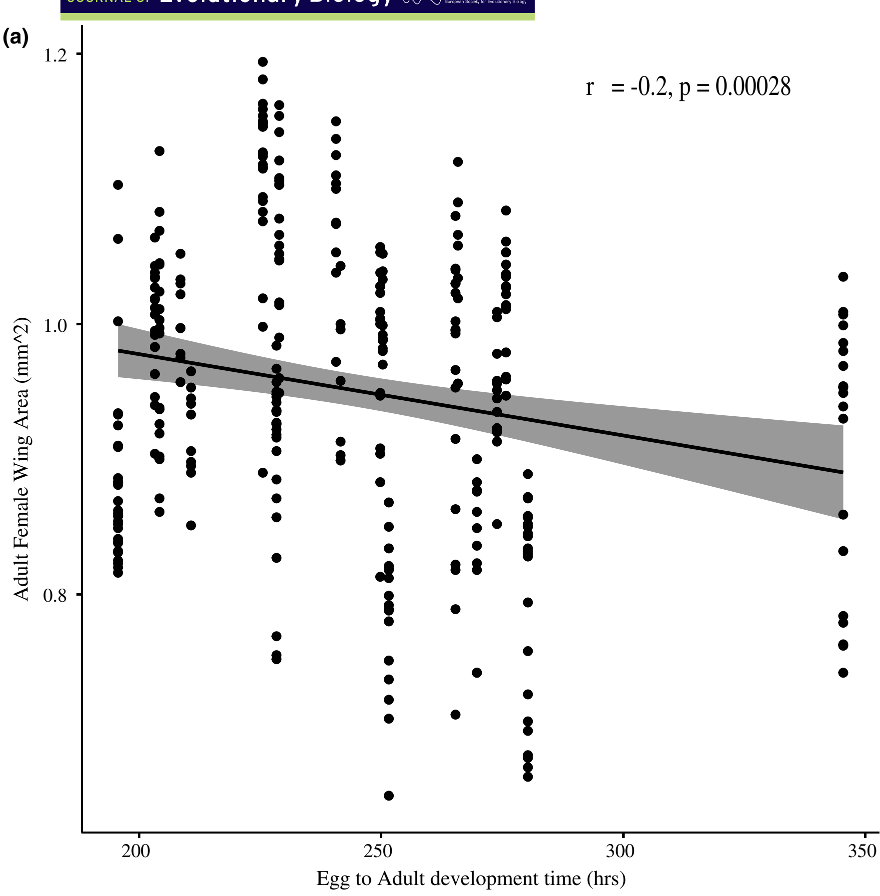

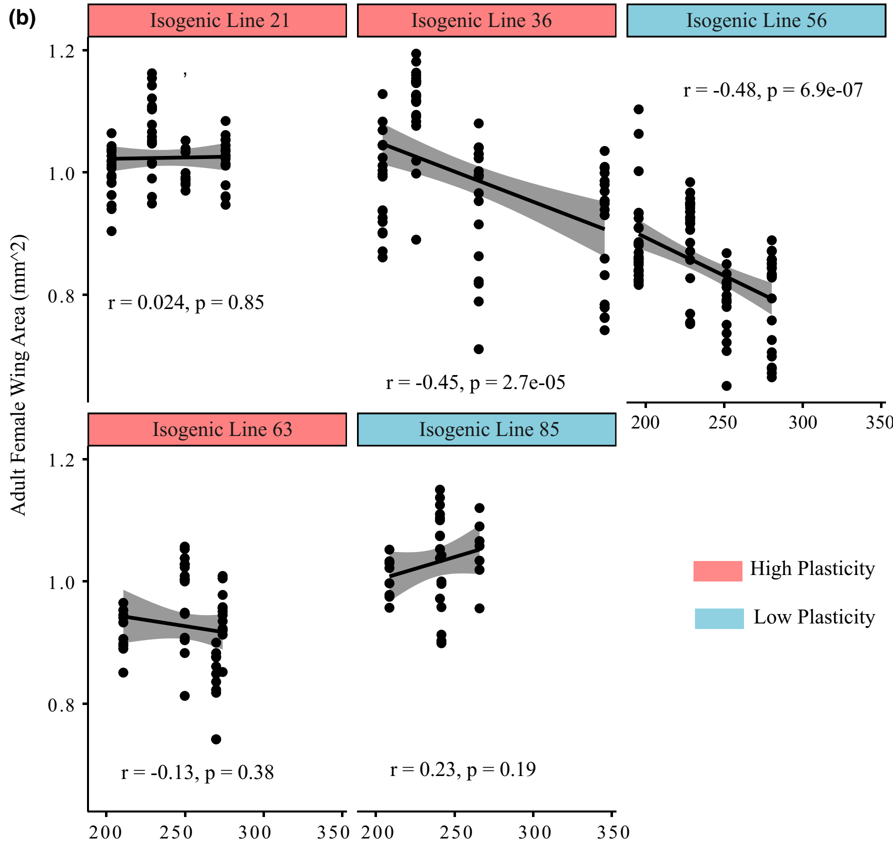

FIGURE 5 Correlation between egg-to-adult development time and adult female wing area (a) combining all five lines from the high and low plasticity groups and (b) for each line coded by high or low plasticity, across the combined developmental conditions of diet dilution and temperature. Red denotes high plasticity lines (lines 21, 36 and 63) while blue denotes low plasticity lines (lines 56 and 85). All correlations are estimated using Pearson's correlation coefficient. The grey shading around the fitted black lines represents the 95% confidence interval around the line of best fit.

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rate and/or growth duration (Nijhout, 2003; Nijhout et al., 2010). However, little is known about how much each process contributes to genetic variation in size plasticity. We reasoned that if the plastic responses of development time and body size are genetically correlated, then it is likely that genetic variation underpinning plasticity in body size and development time is shared. If not, they are at least partially independent. We found genotype-specific differences in both development time and body size plasticity in response to combinations of diet and temperature. Furthermore, while development time and wing size were moderately correlated for some genotypes, this was not the case for all genotypes in this study. This suggests that the plastic responses of these two traits are regulated by at least partially independent sets of alleles (Bergland et al., 2008; Lafuente et al., 2018; Norry & Gomez, 2017), although a larger sample size is needed to determine this unequivocally. Nonetheless, our results suggest that differences in the plastic responses of growth rate to thermal and nutritional stress may play an important role in determining genetic differences in body size plasticity (Chakraborty et al., 2021).

The proximate causes of genetic variation in plasticity are poorly understood. While final body size is the product of growth rate and development time, variation in either of these traits is ultimately determined either by differences in feeding rates or by differences in the assimilation and absorption rates of nutrients across genotypes (Chakraborty et al., 2021). Furthermore, the signalling pathways that are necessary to ultimately regulate growth and developmental time are likely to vary across genotypes and generate differences in plastic responses. Genetic pathways controlling growth and development in insects like *Drosophila melanogaster* primarily involve the ecdysone hormone pathways and the insulin/TOR signalling systems (Hyun, 2018; Koyama & Mirth, 2016; Nijhout et al., 2014; Nijhout & Callier, 2015). To date, we have a limited understanding of how genetic variation in plasticity impacts the proximate mechanisms regulating body size plasticity, and how they impact the activities of key signalling pathways to change responses to diet and temperature. These types of approaches, integrating the proximate causes of plasticity with genetic variation in these responses, would provide substantial insight into what generates genotype-specific plasticity within populations.

AUTHOR CONTRIBUTIONS

AC collected all experimental data. AC and ANA generated and maintained the panel of isogenic lines used in this study. GMW provided R scripts and helped with Bayesian analysis along with comments on final manuscript. KM helped with Random regression analysis and part of Bayesian analysis. AC, CKM and CMS contributed to the experimental design, data analysis and final manuscript preparation.

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CONFLICT OF INTEREST

The authors declare no potential competing interests.

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DATA AVAILABILITY STATEMENT

All data and scripts are publicly available on Figshare (https://doi.org/10.26180/17080664).

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