Title: The proximate sources of genetic variation in body size plasticity: the relative contributions of feeding behaviour and development in *Drosophila melanogaster*

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Abstract

Body size is a key life-history trait that influences many aspects of an animal's biology and is shaped by a variety of factors, both genetic and environmental. While we know that locally-adapted populations differ in the extent to which body size responds plastically to environmental conditions like diet, we have a limited understanding of what causes these differences. We hypothesized that populations could differ in the way body size responds to nutrition either by modulating growth rate, development time, feeding rate, or a combination of the above. Using three locally-adapted populations of *Drosophila melanogaster* from along the east coast of Australia, we investigated body size plasticity across five different diets. We then assessed how these populations differed in feeding behaviour and developmental timing on each of the diets. We observed population-specific plastic responses to nutrition for body size and feeding rate, but not development time. However, differences in feeding rate did not fully explain the differences in the way body

size responded to diet. Thus, we conclude that body size variation in locally-adapted populations is shaped by a combination of growth rate and feeding behaviour. This paves the way for further studies that explore how differences in the regulation of the genetic pathways that control feeding behaviour and growth rate contribute to population-specific responses of body size to diet.

Introduction

Growth is a universal feature of life. All organisms undergo life cycles that incorporate growth, which ultimately determines their size. Body size, in turn, predicts numerous aspects of an organism's biology, including fecundity, lifespan, and stress resistance (Honek 1993; Calder 1984; Calvo and Molina 2005; Speakman 2005; Bonner 2011; Healy et al., 2014; Lasne et al., 2018). Variation in body size is found both within and across populations (Peters 1986, Woodward et al., 2005) and is subject to strong gene by environment interactions. This generates variation in body size plasticity in response to a range of environmental conditions (Nijhout 2003; Davidowitz et al., 2004; Mirth and Shingleton 2012). However, we know very little about the processes contributing to this genetic variation in body size plasticity.

Among all the environmental conditions that affect body size, we understand the most about how variation in nutritional quality and quantity affects body size in insects. The amount of nutrition available during the early-life (larval/nymphal) stages of insects determines the growth of adult structures. The amount of nutrients stored during these stages will sustain further growth and development during metamorphosis (Emlen and Nijhout 1999; Nijhout 2003; Nijhout and Grunert 2010). Both the quality and quantity of

50 nutrition experienced during the larval/nymphal stages generates plastic variation in adult
51 body size (Chown and Terblanche 2006; De Jong et al., 2010; Fischer and Karl 2010; Shama
52 et al., 2011; Kivelä et al., 2012; Sgrò et al., 2016; Chakraborty et al., 2020). For example,
53 larvae reared on low protein diets give rise to smaller adults in a range of insects, including
54 Drosophilid fruit flies (Bakker 1959; Bradshaw and Holzapfel, 2008; Matavelli *et al.*, 2015;
55 Rodrigues *et al.*, 2015; Kristensen *et al.*, 2016; Silva-Soares *et al.*, 2017; Gray et al., 2018;
56 Kutz et al., 2019; Chakraborty et al., 2020) and lepidopterans (Simpson *et al.*, 2004;
57 Davidowitz et al., 2004; Davidowitz and Nijhout 2004; Roeder and Behmer, 2014).
58
59 Genetic variation also contributes to the extent of the plastic response to nutritional
60 conditions, with some genotypes exhibiting greater plasticity in body size than others (Neat
61 et al., 1995; Chakraborty et al., 2020). Genetic variation in body size plasticity to nutrition
62 exists within (Lewis et al., 2012; Thompson 2019) and between genetically-diverse
63 populations, like those found along a latitudinal gradient (Newman 1994; Chakraborty et al.,
64 2020). It is, nevertheless, unclear what happens during the growth phase of insects to give
65 rise to such genetic variation in body size plasticity.
66
67 Genetic variation in body size plasticity could arise due to changes in feeding behaviour.
68 Animals might differ in the time it takes to decide to eat as well as the length of the feeding
69 bouts and speed of ingestion (Reynolds et al., 1986; Mahishi and Huetteroth 2019, and
70 references therein), and changes in any of these behaviours will lead to changes in the
71 amount of food consumed over time. Further, each of these behaviours are known to vary
72 with the quality and quantity of diet available (Simpson and Raubenheimer 1993; Simpson
73 et al., 2004). For instance, animals often will adjust the amount of food they consume

74 depending on the macronutrient composition of the diet. Larvae of several *Drosophila*
75 species decrease the amount of food that they ingest with increasing protein to
76 carbohydrate (P:C) ratios of the food, and with foods higher in caloric content (Carvalho and
77 Mirth 2017, Silva-Soares et al., 2017). Whether genetic variation across populations further
78 shapes the regulation in food intake, ultimately contributing to genetic variation in body size
79 plasticity to nutritional environments, is unknown.
80
81 Alternatively, variation in the developmental programs that lead to organ and body growth
82 could contribute to genetic variation in plasticity. The absorption and assimilation of
83 nutrients, as well as the utilization of nutrient resources for growth and regulation of
84 development time, will also influence body size plasticity across nutritional conditions (Sibly
85 1981). For example, if an organism ingests large quantities of food but assimilates it
86 inadequately or is inefficient in allocating these nutrients to growing tissues, this would lead
87 to a smaller adult body size (Urabe and Watanabe 1991; Neat et al., 1995). The efficiency of
88 food conversion into body mass is dependent on the nutritional environment available to an
89 animal. For example, studies in zooplankton species have shown that growth efficiency
90 changes with the quantity of food (Richman 1958; Mullin and Brooks 1970; Paffenhofer
91 1976), where peak growth efficiency occurs at intermediate food concentrations (Urabe and
92 Watanabe 1991). Furthermore, genetically-diverse, locally-adapted populations of
93 *Drosophila melanogaster* have been shown to differ in their ability to convert nutrients into
94 body mass (James and Partridge 1995). Larger flies from temperate populations are able to
95 convert a set quantity of food into body mass with greater efficiency than smaller flies from
96 tropical populations (James and Partridge 1995). Such genetic variation in nutrient
97 utilization could also contribute to differences in body size plasticity.

Differences in absorption and assimilation of nutrients across diets will ultimately affect body size by altering the length of time an animal spends growing (development time) and/or the rate at which mass accumulates (growth rate) (Atkinson 1994; Partridge and French 1996; Blanckenhorn 1998; Gotthard 1998; Stern 2001; Nijhout 2003; Davidowitz et al., 2004; Davidowitz and Nijhout 2004). Because mature adult body size is ultimately a product of growth rate and developmental timing, by measuring one of these processes we can infer the other. Both growth rate and duration of growth vary with quantity and quality of food in a wide range of developing animals, including the tobacco hornworm, *Manduca sexta*, song sparrows, *Melospiza melodia,* and *D. melanogaster* (Neat et al., 1995; Davidowitz and Nijhout 2004; Searcy et al., 2004; Davidowitz et al., 2004; Nijhout et al., 2006; Nijhout et al., 2010). Low quality foods extend the duration of growth and decrease growth rate, with a net result of generating a smaller adult (Davidowitz et al., 2004). Genetic variation between populations could result in differences in the way animals modulate either growth rate or development time in response to the dietary environment, contributing to variation in body size plasticity.

Here, we aim to understand the extent to which behaviour and development contribute to differences in body size plasticity across populations. We hypothesized that differences in nutritional plasticity across genetically-diverse populations could arise due to differences in 1) food intake, 2) developmental timing, or 3) growth rates (by measuring development time we can infer growth rate) (Figure 1). To test our hypothesis, we used three locally-adapted, genetically-diverse populations of *D. melanogaster* from along the east coast of Australia: a population from Melbourne, one from Ballina, and a third from Townsville.

These populations exhibit population-specific body size plasticity in response to changing nutritional conditions (Chakraborty et al., 2020). We manipulated the nutritional environment by using nutritional geometry (Simpson and Raubenheimer 1993, 1999, 2012; Simpson *et al.*, 2004). We designed five diets that varied in their protein and carbohydrate content. We reared our three populations on all five diets, and measured the response of pupal weight, as a proxy for body size. Because plasticity in body size differed across these populations, we next distinguished how genetic variation in plasticity is shaped by food intake in the last larval stage and the length of the larval period. Our study explores how changes in the responses of feeding behaviour and growth rate across populations can ultimately contribute to differences in body size plasticity.

Methods

Fly Stocks and Maintenance Conditions

We used mass bred populations collected from tropical (Townsville, latitude:19.29S), sub-tropical (Ballina, latitude: 28. 75), and temperate (Melbourne, latitude: 37.73) regions along the east Australian cline (Supplementary Figure 1). The flies to seed these populations were collected in April 2016 and were maintained as mass bred populations at a population size of approximately 1500 flies at a constant temperature of 25ºC on a 12-hour light/dark cycle on standard lab fly food: yeast-dextrose-potato medium of P:C 1:3, 318.42kcal (potato flakes 20g/L; dextrose 30g/L; 95 Brewer's yeast 40 g/L; agar 7g/L; nipagen 6mL/L; and propionic acid 2.5 mL/L), for 65 generations prior to the experiments described below (Chakraborty et al., 2020).

Experimental Diets

Diets were made following a similar protocol to Kutz et al., (2019) and Chakraborty et al., (2020). Five diets were chosen that captured the variation in adult body size shown among these populations (Supplementary Figure 2, Chakraborty et al., 2020). The Reference diet had a protein to carbohydrate ratio (P:C) of 1:3, which contains 79.99g/L of protein and 236.79g/L of carbohydrates (1273.7kcal); it was chosen as the Reference diet because we have previously shown (Chakraborty et al., 2020) that body size varied as the nutritional composition shifted away from this diet (Supplementary Figure 2). We generated two different diet types: one in which protein concentration was varied above and below that found in the Reference diet but carbohydrate concentration was kept constant (Protein_Varies), and a second diet type where carbohydrate concentration was varied above and below the concentration of the Reference diet while protein concentration was kept constant (Carb_Varies) (Table 1, Supplementary Figure 2). We took care to match the P:C ratios across diets, such that they had one of three P:C ratios: 1:5, 1:3 (Reference diet), or 2:3. All diets contained 7g/L of agar, 6mL/L of nipagin, and 2.5 mL/L of propionic acid.

Larval rearing and staging for development time and body size (pupal weight)

All experiments were performed at a constant rearing temperature of 25ºC, because we have previously shown (Chakraborty et al., 2020) that plasticity in development time did not change with temperature. Parental flies from each of the three populations were acclimated to egg laying chambers containing standard food (P:C 1:3, 318.42kcal) for 24 hours, changing the egg plates every 12 hours. Eggs were subsequently collected over a 6-hour laying interval. Approximately 200 eggs from each population were distributed to 55 mm diameter petri dishes containing the Reference diet. Each population had five replicate dishes of ~200 eggs.

Larvae were carefully staged from moult to third larval instar (L3) following Mirth et al., 2005. Briefly, at ~64-74 hours from egg lay, larvae began moulting to the third instar. Following this, larvae were floated out of the food using 20% sucrose solution and all the second-instar larvae were collected and transferred to a new plate with food. Newly moulted L3s were collected every 2 hours. For each population, twenty newly moulted L3s were transferred into vials containing one of five experimental diets, with 4 replicate vials for each diet. Their development time to pupal stage was recorded every 8 hours.

We used pupal weight as a measure of body weight because maximum body size is fixed at pupation, and pupal weight is strongly correlated with adult body size measures (Mirth et al., 2005 ; Nijhout et al., 2014). Individual pharate pupae, as defined by the appearance of dark wings visible through the pupal case, were weighed on aluminium foil boats using Mettler Toledo's XPR Ultra-Mircrobalance.

Food intake

For this experiment, egg lays and egg collections were performed as described above. Around 800 third instar larvae (L3s) from each population were collected at 76 to 88hours from the mid-point of the 6-hour egg lay. These were then randomly placed in petri dishes containing one of the 5 experimental diets described above. Each diet was dyed blue using 5% (v/v) of dye (Queen's blue food colouring dye, batch number: 118106) to quantify the amount of food ingested by the larvae by spectrophotometer (Rodrigues *et al.,* 2015; Carvalho *et al.,* 2017). Larvae were left to feed for 2 hours in the dark (to eliminate visual cues) at 25ºC, after which the assay was stopped by transferring all plates to ice which

causes the larvae to stop eating. After this, larvae were removed from the food, washed with distilled water, and placed inside a 2ml microtube (Sarstedt microtubes) with 350 µl of ice-cold methanol. For each diet and each population, 13 replicate petri dishes with 12 L3s per replicate were obtained. To account for differences in L3 size across populations, we weighed each larval sample (Ultramicrobalance, Mettler Toledo) before processing for spectrophotometry.

Quantification of Food Intake

For the feeding assay, the amount of food ingested was quantified by extracting the dye from larval guts. All 12 L3s from each replicate sample were homogenised in 350ul of methanol using 0.5mm Zirconia/Silica Beads (BioSpec) in a bead-beater tissue-homogeniser (Mini-Beadbeater-96 from Biospec Products). Following this, the samples were centrifuged at 13 g for 10 mins at 4ºC. From each sample, 100 µl of the supernatant was taken and placed in a 96-well plate. As standards, we used eight two-fold serial dilutions (1:2 dilution) of the food dye, using a starting concentration of 5 µl dye/ml of methanol. The amount of food inside the guts of 12 larvae was calculated by measuring the absorbance of each sample at 630nm using a ThermoScientific Varioskan Lux Plate Reader. We used the average weight of seven replicates of 12L3s per population to calculate weight-normalised food intake: these averages were 8.77mg/12L3 for Townsville, 10.66mg/12L3 for Ballina and 9.43mg/12L3 for Melbourne.

Statistical Analysis

Both food intake and pupal weight were fit using linear mixed effect models. Development time was fit using generalized linear models assuming a gamma distribution, due to its long-

tailed distribution. The lme4 package in R was used to fit the above data, using P:C ratio, Diet Type (i.e. either Protein_Varies or Carb_Varies), and Population as fixed factors. The 4 replicates of the Reference diet were split between each of the two diet types, for each population. Replicate vials and experimental block were included as random effects. Analysis was performed on the entire dataset and on the data subset by population and/or diet type, where applicable. Data fit was validated by visual inspection of the residuals and both food intake and pupal weight data met assumptions of normality. All data were visualized using ggplot2.

Analyses were first performed on the full dataset for each trait to determine if there were significant interactions between the fixed factors. To explore significant interaction terms involving population, we employed either 'emmeans' (for the categorical variable Diet Type) to obtain an overall estimate of mean variation in traits, or 'emtrends' (for the continuous variable P:C ratio) to contrast the extent of plastic response of a trait across populations. For feeding intake, we also tested for differences in variance between carbohydrate and protein intake, as in (Carvalho and Mirth 2017). To do this, we calculated $|(x_{ij} \_ x_{i.})/x_{i.}|$ where x_{ij} is the measured variable from the *j*th case from the *i*th group and $x_{i.}$ is the median for the *i*th group for each macronutrient. This generated a data set of normalized differences from the median for each data point. We fit the data with a generalised linear model, assuming a quasipoisson distribution, then used emmeans to test for differences in the variance in macronutrient intake both between populations and within each Diet_Type. All statistical analyses were performed in R Studio (version 3.4.1, R Development Core Team 157 2017, https://www.r158project.org/).

Results

In this work, we aimed to understand the relative contributions of feeding behaviour versus developmental timing to differences in body size plasticity across populations. We chose to use outbred *D. melanogaster* from three populations sampled from along the east coast of Australia that are known to have diverged in mean body size (Lasne et al., 2018), development time (James et al., 1995; James and Partridge 1995), and in their plastic responses to nutrition (Chakraborty et al., 2020). These populations included a tropical population from Townsville, a subtropical population from Ballina, and a temperate population from Melbourne. Nutrition was manipulated using one of two diet types. The first diet type, hereafter called the Protein_Varies diets, contained either low, medium, or high protein concentrations but had a constant carbohydrate concentration. The second diet type, hereafter called the Carb_Varies diets, contained either low, medium, or high carbohydrate concentrations while maintaining a constant protein concentration. This design allowed us to explore how each trait responded to the proportion of dietary macronutrients, and to also examine the variation in trait response to concentrations of dietary protein and carbohydrate independently. For each population, we measured pupal weight as a proxy of body size across each diet. To assess whether differences in size plasticity across populations were due to behaviour, we assessed food intake in third instar larvae (L3) across diets. We measured L3 to pupal development time to account for differences in development. Since we are interested in the plastic response of each trait to nutrition across populations, interactions between population and any element of diet, either diet type or P:C ratio, is suggestive of a population-specific plastic response.

Pupal Weight (as a proxy of body size)

Main effects of diet type, P:C ratio, and population were not significant (Table 2). Interestingly, we found significant interactions between population and both diet type and P:C ratio (Table 2). This means that the plastic response of pupal weight differs in a population-specific manner in response to both the proportion and concentration of protein and carbohydrate in the diet.

To further explore the differences in plasticity among populations, we performed pairwise comparisons for P:C ratio and diet type. We found that populations differed in the way pupal weight responded to the P:C ratio in the diet. For instance, the plastic response of pupal weight in the Ballina population differed significantly from that of the Melbourne population. This difference arose because the Ballina population showed a negative relationship between P:C ratio and pupal weight, whereas pupal weight correlated positively with P:C ratios for the Melbourne population (Figure 2).

Further, we also found that diet type had different effects on pupal weight across populations. Such population-specific differences in pupal weight were largely driven by changes in protein concentration when carbohydrate concentrations were maintained constant. In particular, while Townsville pupae weighed less on the diets in which protein concentration varied than on the diets where carbohydrate concentrations varied, the Ballina population showed the opposite response (Figure 2). Overall, these results confirm our previous findings (Chakraborty et al., 2020) that body size responds differently to diet across these three populations.

Larval Food Intake

289 Having identified differences in body size plasticity across populations, we next sought to
290 identify whether these changes arose due to differences in feeding behaviour or differences
291 in the developmental processes known to be regulated by food. To do this, we first assessed
292 food intake on each of the diets for the three populations. We chose to examine food intake
293 specifically in L3, as first and second instar larvae are too small to measure accurately using
294 spectrophotometry.
295
296 In general, Townsville consumed more across all diets, whereas Ballina consumed the least
297 (Table 2, Figure 3). A significant two-way interaction between diet type and P:C ratio implied
298 that the response of food intake to the P:C ratio of the diet depended on whether that diet
299 varied in protein or in carbohydrate (Table 2). Specifically, consumption decreased with
300 increasing P:C ratio on diets varying in protein concentrations (Protein_Varies diet). On diets
301 with varying carbohydrate concentrations (Carb_Varies diet), larvae consumed similar
302 amounts across all three P:C ratios (Figure 3). While they differed in total intake,
303 populations did not differ in the way they respond to either the P:C ratio or the diet type.
304 These results suggest that population-specific differences in food intake cannot explain the
305 variation in body size plasticity across the three populations.
306
307 While we did not observe population-specific differences in food intake across diet types,
308 our diets are not calorically matched, and differences in the response to caloric content in
309 the diet could drive variation across populations. One way of defining how larvae relate
310 their food intake is to examine their macronutrient balancing strategies using food intake
311 arrays (Simpson and Raubenheimer 1993, 1999, 2012; Simpson *et al.*, 2004). These arrays

tell us whether animals regulate their food intake by ingesting to fulfill protein, carbohydrate, or caloric targets.

D. melanogaster larvae are known to tightly regulate their protein intake at the expense of over- or under-consuming carbohydrates (Carvalho and Mirth 2015), a macronutrient balancing strategy known as protein leveraging (Simpson and Raubenheimer 2005). To assess whether the three populations macronutrient balance in the same manner, we examined the variation in macronutrient intake for protein and carbohydrate across populations and diet types (Figure 3b, Table 3). We observed significantly greater variation in carbohydrate intake than protein intake, indicative of protein leveraging, for the Townsville and Ballina populations. However, for the Melbourne population, variance in carbohydrate intake did not differ significantly from the variance in protein intake (Figure 3b, Table 3). This suggests that Melbourne could use less stringent protein leveraging strategies across diet types than the other two populations, potentially contributing to the difference in nutritional plasticity for body size observed in the Melbourne population.

L3 to Pupal Development Time

We next explored whether the differences in body size plasticity could be explained by differences in developmental timing. We reasoned that since body size is ultimately the product of growth rate and developmental timing, by measuring one we can infer the other. Over 80% of body growth occurs in the third instar, and final body size is primarily a function of growth rates and the length of time spent growing during this last larval stage (Shingleton et al., 2008). Thus, we measured L3 to pupal development time to understand how it contributes to variation in body size plasticity.

Overall, development time decreased with increasing P:C ratio, but did not differ across populations or diet type (Table 2, Figure 4). This means that L3-to-pupal development time did not respond differently to diet across populations. Neither food intake nor developmental time showed the same population-specific patterns of variation as body size plasticity. Thus, we propose that differences in growth rate contribute to variation in body size plasticity across populations.

Discussion

Body size is a key life-history trait, which exhibits plastic variation in response to environmental conditions like temperature and nutrition (De Jong et al., 2010; Fischer and Karl 2010; Shama et al., 2011; Kivelä et al.,2012; Sgrò et al., 2016). Variation in body size plasticity can be found both within and across populations, indicating the presence of genetic variation in plasticity (Chakraborty et al., 2020). Such variation in plasticity can arise via changes in feeding behaviour, growth rate, and/or growth duration, however we know relatively little about the relative contribution of these mechanisms to such variation in size plasticity. To this end, we aimed to understand the extent to which developmental timing and feeding behaviour contribute to genetic variation in body size plasticity.

Previous studies have revealed genetic differences in body and organ size plasticity in response to the proportion of carbohydrate and protein in the diet (P:C ratio) across (Matavelli et al., 2015; Silva Soares et al., 2017) and within (Chakraborty et al., 2020) *Drosophila* species. For example, in *Zaprionus indianus* ovariole number was maximised when larvae were reared on intermediate P:C diets, whereas *Drosophila simulans* had more

ovarioles when reared on high P:C and high calorie diets (Matavelli et al., 2015). Similarly, female body weight was highest on diets with low to intermediate protein concentrations and high P:C ratios in *Drosophila suzukii,* but was highest on high protein and high P:C diets in *Drosophila biarmpes* (Silva Soares et al., 2017). Within species, Chakraborty et al (2020) found that the response of body size to nutrition varied across locally-adapted populations, such that sub-tropical flies were larger when reared on diets with intermediate to high P:C ratios and calories , whereas size was maximised in tropical flies on diets with intermediate P:C ratios and calories.

The results of the current study are consistent with this previous work. Namely, we found population differences in size plasticity in response to nutrition that was shaped by two elements of nutrition: the P:C ratio and the concentration of dietary protein or carbohydrate. Size decreased with increasing P:C ratio in the Ballina population, whereas the opposite was true in the Melbourne population. On the other hand, pupae were smaller on diets varying in protein compared to diets that varied in carbohydrate in the Townsville population, but the opposite was true of the Ballina population. This is in line with our previous study (Chakraborty et al., 2020), wherein protein concentration was the major determinant of larger wing area in Ballina flies, while Townsville flies showed the largest wing sizes at intermediate P:C ratios.

Next, we wanted to establish whether population-specific differences in feeding behaviour (food intake) across nutritional environments could explain the observed genetic variation in body size plasticity. Previous studies have found mixed evidence for differences in feeding rates across populations. For example, high latitude populations of Atlantic silverside,

384 *Menidia menidia*, were shown to ingest more food compared to lower latitude populations
385 (Present and Conover 1992; Billerbeck et al., 2000), while the opposite pattern was found in
386 populations of *Rana tempraria* (Lindgren and Laurila 2005). In contrast, no difference in
387 larval feeding rate was found among populations of *D. melanogaster* sampled from a
388 latitudinal gradient (Robinson and Partridge 2001). While these studies tested for variation
389 in feeding rate between populations, none did so in the context of changed nutritional
390 environments. This is despite the fact that animals have been shown to differ in the way
391 they regulate their food intake in response to macronutrient composition (Behmer et al.,
392 2001; Lee, K., et al 2002; Raubenheimer and Simpson 2003), such that many animals
393 increase feeding rates on low protein diets in order to meet their protein targets
394 (Raubenheimer and Simpson 2003; 1993; Carvalho and Mirth 2017, Silva-Soares et al.,
395 2017).
396
397 Our results are consistent this work; larvae increased their food intake as dietary protein
398 concentration decreased, whereas food intake was insensitive to varying levels of
399 carbohydrate. We also found that the three populations differed in their overall total food
400 intake, such that the low-latitude Townsville population consumed the most, and the mid-
401 latitude population Ballina consumed the least amount of food. Finally, we found evidence
402 for differences in macronutrient balancing strategies, with the Melbourne population
403 showing lower variance in total carbohydrate consumed. While these differences in feeding
404 rate could explain some of the observed population-specific plastic responses of size to diet,
405 they do not explain all the differences.
406

The higher food intake in Townsville population did not translate into bigger body size, since Townsville flies exhibited similar average body size to that of the Ballina and Melbourne populations. This suggests that perhaps the Townsville population is less efficient in food assimilation and/or absorption. This idea is supported by previous studies that have shown that high latitude clinal populations and cold-adapted laboratory populations are more efficient at converting food into size than their tropical/warm-adapted counterparts (Partridge et al 1994; James and Partridge 1995; Neat et al., 1995; Robinson and Partridge 2001). It is likely that this is a result of metabolic costs associated with greater efficiency of nutrient assimilation in a warmer climate. High latitudes with lower temperatures increase the potential for growth, making it easier to achieve higher efficiency (Robinson and Partridge 2001).

In principle, shifts in development time with nutrition may also contribute to variation in size plasticity. Larval to pupal development time across species has been shown to be fastest when larvae are reared on diets with intermediate to high protein concentrations (high P:C ratios) and calories (Matavelli et al., 2015; Rodrigues et al., 2015; Silva Soares et al., 2017; Chakraborty et al., 2020). Our results reveal that development time decreased with increasing P:C ratio, but did not differ across populations or diet type. These results are not consistent with our earlier work (Chakraborty et al., 2020) where we found population-specific plastic shifts in development time in response to nutrition. This discrepancy could reflect the fact that our earlier study used a much broader range of diets, and examined egg-to-adult development time, rather than L3-to-pupal development time. Overall, our results suggest that differences in growth duration are unable to explain the

differences in body size plasticity observed across the three populations of the current study.

We have shown that locally-adapted, genetically-diverged populations differ in body size plasticity in response to nutrition, consistent with our previous study (Chakraborty et al., 2020). Given that neither differences in food intake nor developmental time could explain the observed population-specific body size plasticity, the proximate source of this genetically-based variation in body size plasticity is likely to arise from differences in growth rate across populations.

Differences in growth rate across populations sampled from along latitudinal gradients have been reported in a wide range of taxa; populations of insects, fish, and frogs from higher latitudes have been reported to show higher intrinsic growth rate than their low latitude counterparts (Conover and Present 1990; James & Partridge 1995; Neat et al., 1995; Billerbeck et al., 2000; Laugen et al., 2003; Blanckenhorn and Demont, 2004; Lindgren and Laurila, 2005; Yamahira and Takeshi 2008; Lindgren and Laurila 2009). Seasonal variation in temperate high latitude regions can select for faster growth rate, enabling organisms to take full advantage of shorter growing seasons (James and Partridge 1995). Previous work (Neat et al., 1995 and Robinson and Partridge 2001) suggests that latitudinal variation in body size in *D. melanogaster* may be explained by differences in nutrient absorption and assimilation, such that high latitude populations are more efficient at converting nutrients consumed into increased size. Such differences in growth efficiency could contribute to the variation in body size plasticity across populations observed in the current study.

Assimilation rates have also been shown to vary between populations selected on nutritionally poor diets (Cavigliasso et al., 2020), which are likely to be mediated by differences in post-ingestive dietary compensation. The locally-adapted populations used in our study might differentially regulate post-ingestive processes including the production of digestive enzymes, nutrient absorption and transport across the gut, and processing and allocation of macronutrients (Cavigliasso et al., 2020). Differences in any of these processes would contribute to population-specific body size plasticity in response to nutrition. Future studies measuring growth rate and quantification of nutrient assimilation and excretion rates across latitudinal populations would elucidate the extent to which differences in food absorption/utilisation contribute to population-specific plastic shifts in response to nutrition.

While in the current study, we ascribe differences in pupal mass to differences in body growth, differences in mass can also arise due to differences in body composition (Musselman et al., 2011; Pasco and Léopold 2012). Differences in the relative amounts of trehalose, glycogen, protein, and triglycerides stored within the body's tissues can vary with dietary quality and quantity (Chng et al., 2017). Specifically, lipid (triglycerides) storage tend to increase when *D. melanogaster* are reared on high carbohydrate or low P:C ratio diets (Musselman et al., 2011; Pasco and Léopold 2012). It would be interesting to know if our populations differ in their body composition when reared on the different diet types.

Future studies comparing growth dynamics over the entire larval period across populations would elucidate how the degree of variation in growth rates among populations contributes to population-specific body size plasticity. Subsequent studies focussing on underlying

signalling pathways that regulate growth and development in response to different environmental factors, such as the insulin signalling pathway (reviewed in Cobham and Mirth 2020), across genetically-diverged populations, would also elucidate how differences in signalling activities in these key pathways might lead to population-specific variation in body size plasticity.

Author Contributions

AC collected all experimental data. All authors contributed to the experimental design, data analysis, and final manuscript preparation.

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Data Archiving

All data and scripts are available on Figshare (DOI: https://figshare.com/s/812840e075cc89f6fb2b) and all materials are available on request.

Declaration of Interests

The authors declare no potential competing interests.

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712

713 **Tables and Figures**

714

Diet Type	*Protein g/L*	*Carbohydrates g/L*	*Ratio*	*Total Calories kcal*
Reference (PC_CC)	79.99	236.79	1:3	1273.7
Carb_Varies (PC_HC)	79.49	397.09	1:5	1913.3
Carb_Varies (PC_LC)	79.75	119.21	2:3	803.0
Protein_Varies (CC_HP)	158	236.66	2:3	1592.5
Protein_Varies (CC_LP)	47.25	236.05	1:5	1136.6

715

716 Table 1: Protein and carbohydrate concentrations and total calories in each experimental
717 diet and their corresponding ratios. Reference diet ; Carb_varies: Protein constant PC with
718 High and Low Carbohydrate (_HC or _LC, respectively) compared to the reference;
719 Protein_varies: Carbohydrate constant with High and Low Protein (CC with _HP or _LP,
720 respectively) compared to the reference.

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Traits	***Pupal Weight***	***Larval Food Intake***	***L3-Pupal Development Time***	***Df***
Terms	Chi-square	Chi-square	Chi-square	-
Diet Type	0.363	1.580	0.497	1
P:C Ratio	1.452	13.306 ***	11.42 ***	1
Population	2.459	7.238 *	1.549	2
Diet Type x P:C Ratio	0.009	15.290 ***	1.421	1
Diet Type x Population	9.855 **	0.582	0.463	2
P:C Ratio x Population	8.710 *	0.293	0.320	2
Diet Type x P:C Ratio x Population	2.188	0.514	3.423	2

724 Table 2: Effects of Diet Type (either Protein_Varies = concentration of carbohydrates
725 same as that of Reference diet with increasing or decreasing concentration of proteins
726 (CC_HP and CC_LP) or Carb_Varies = concentration of protein same as that of Reference diet
727 with increasing or decreasing concentrations of carbohydrates(PC_HC and PC_LC)), Protein:
728 Carbohydrate (P:C) Ratio, Population and their products in the three traits

measured in this study. Chi-square = the chi-square value obtained from mixed linear models for each trait and Df represents the 'degrees of freedom'.

*** P < 0.001; ** P < 0.01; * P < 0.05

Groupings	emmean	Group
Townsville_Carb	-0.159	2
Townsville_Prot	-0.752	1
Ballina_Carb	-0.245	2
Ballina_Prot	-0.787	1
Melbourne_Carb	-0.332	12
Melbourne_Prot	-0.783	1

Table 3: Differences in macronutrient intake across populations, using emmeans on median-normalised data. Confidence level used = 95%. P value adjustment using tukey method for pairwise comparisons, significance level used $\alpha = 0.05$. Significant differences in variances across macronutrient type and population were found in the generalised linear model (Chi-square 6.224e-06 ***, degrees of freedom 5).

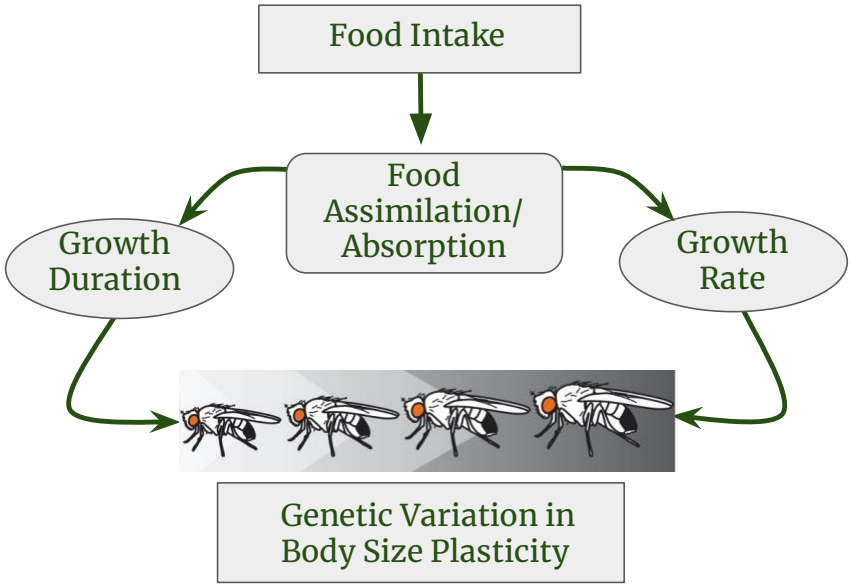

Figure 1: The different ways in which plastic variation in body size can be generated across organisms.

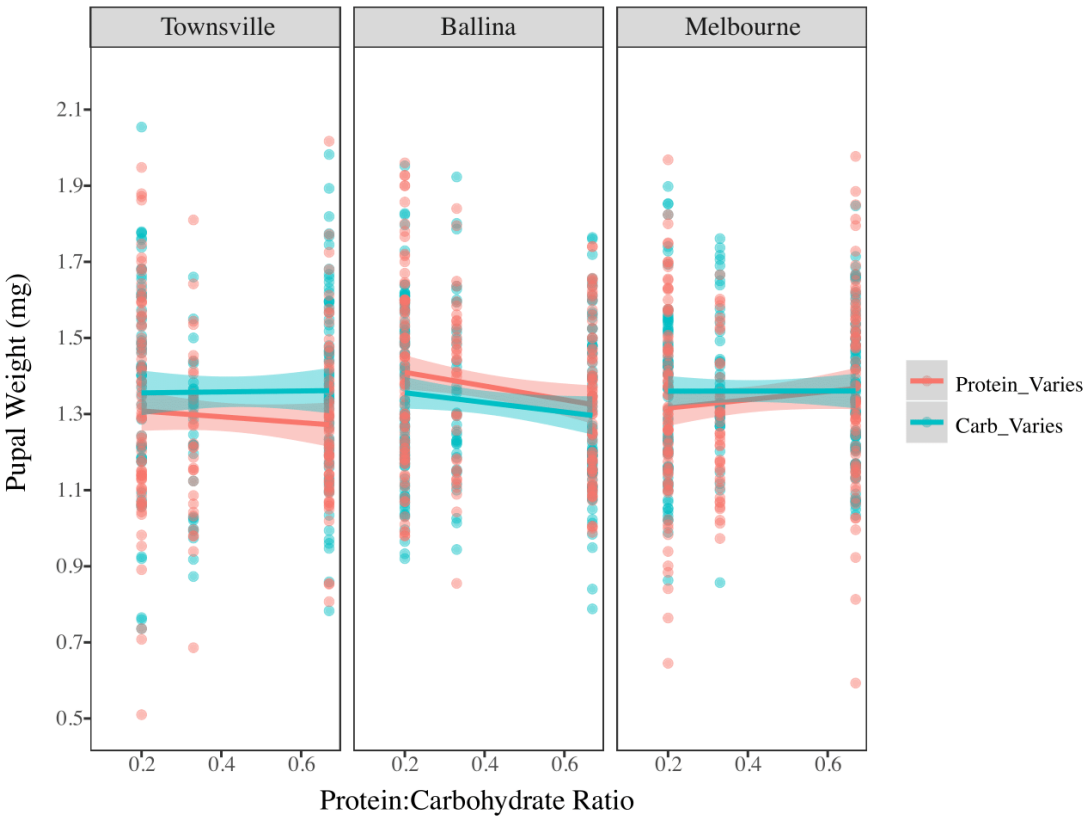

Figure 2: Variation in pupal weight with P:C ratio across three populations on diets that vary either in their protein concentration (Protein_Varies) or carbohydrate concentration (Carb_Varies) . Protein_Varies = constant concentration of carbohydrates with varied protein concentration; Carb_Varies = constant concentration of proteins with varied carbohydrate concentrations.

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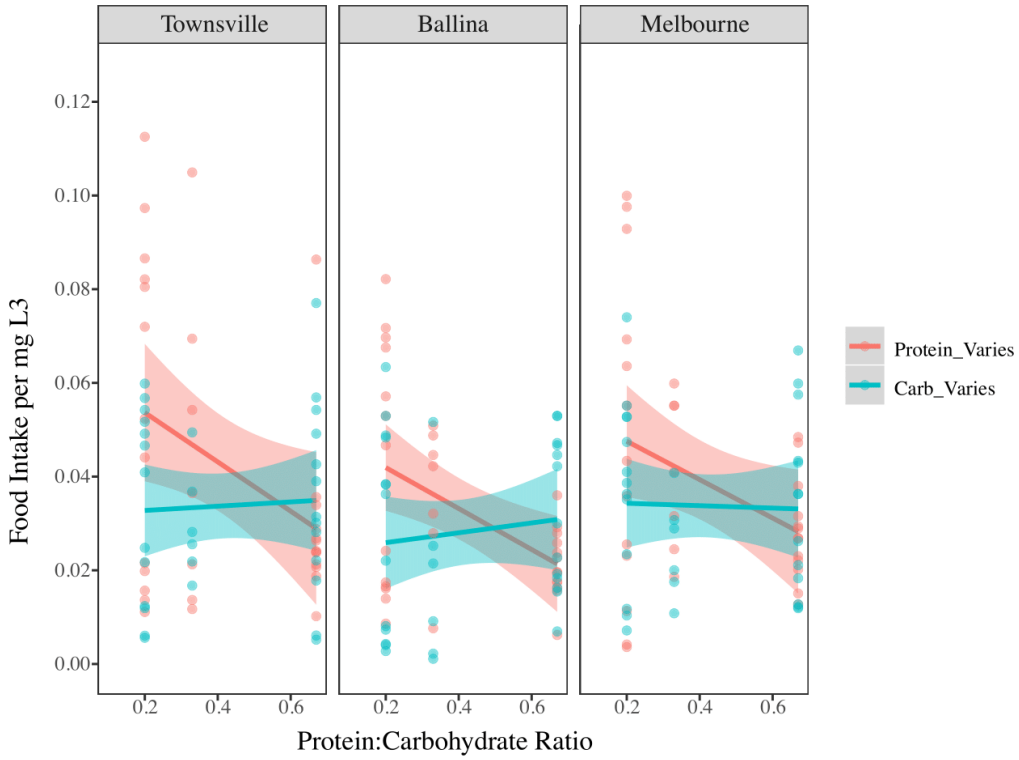
779 a)



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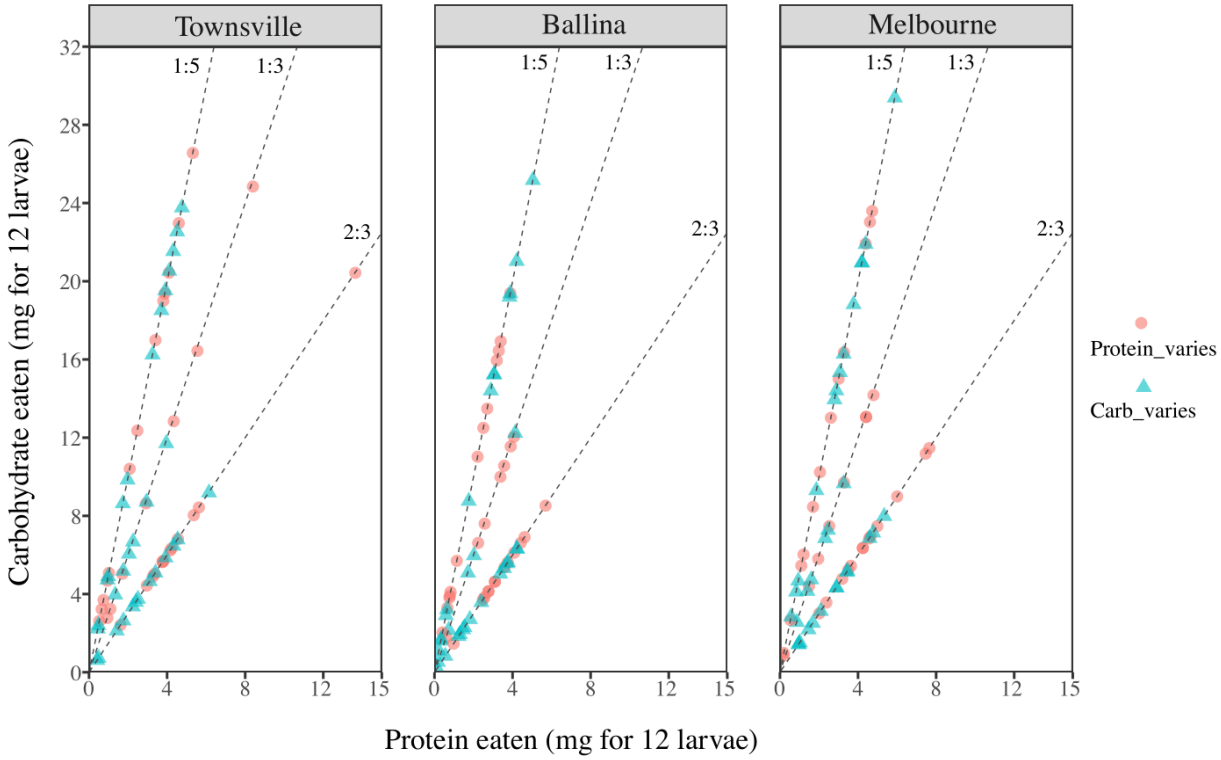
781 b)



782

783 Figure 3: Feeding behaviours across populations vary. a) Variation in total food intake with

784 P:C ratio across three populations on diets that either vary in their protein concentration or

785 carbohydrate concentration. b) Intake arrays show variation in macronutrient intake across

786 the three populations on two diet types. Dashed lines show the P:C ratios for each of the

787 diets. Protein_Varies = constant concentration of carbohydrates with varied protein

788 concentration; Carb_Varies = constant concentration of proteins with varied carbohydrate

789 concentration.

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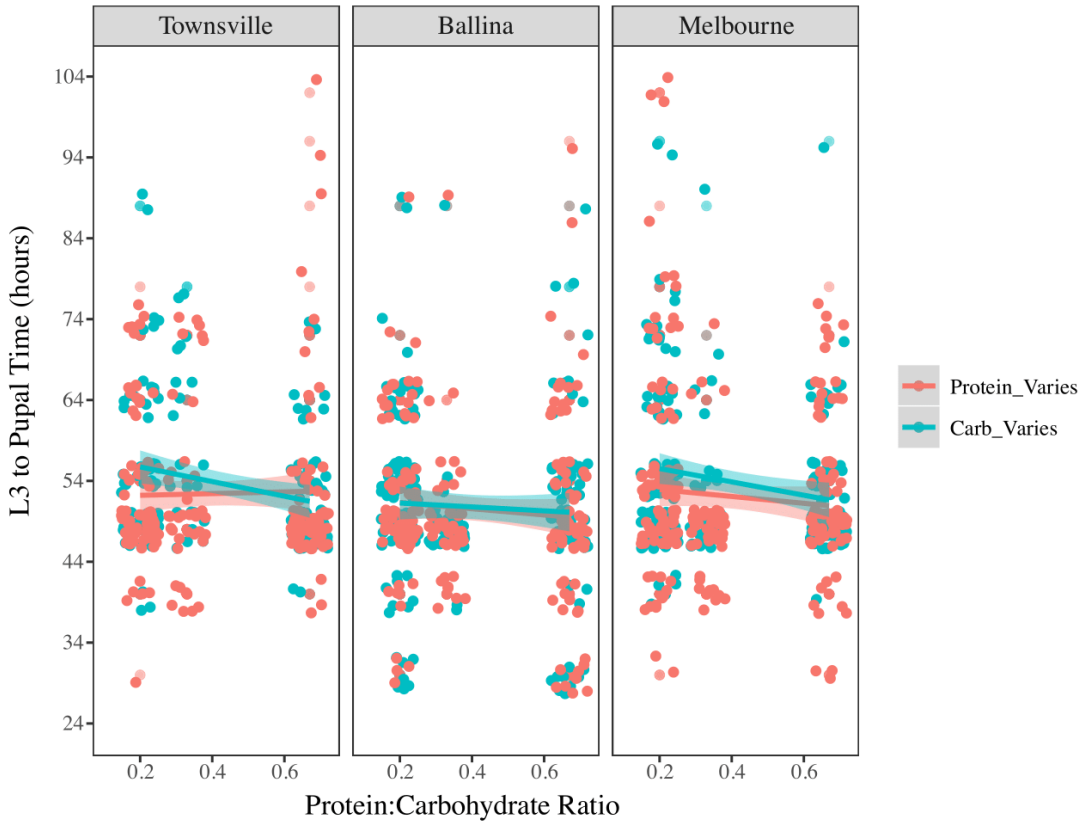

792

793 Figure 4: Development time from third-instar larvae (L3) to pupal stage across P:C ratios for

794 three populations on that either vary in their protein concentration or carbohydrate

795 concentration. Protein_Varies = constant concentration of carbohydrates with varied

796 protein concentration; Carb_Varies = constant concentration of proteins with varied

797 carbohydrate concentration.

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Supplementary Figures

Figure 1: Map of eastern Australian coast indicating the three sites, Townsville, Ballina, and Melbourne, from which *Drosophila melanogaster* used in this study were collected.

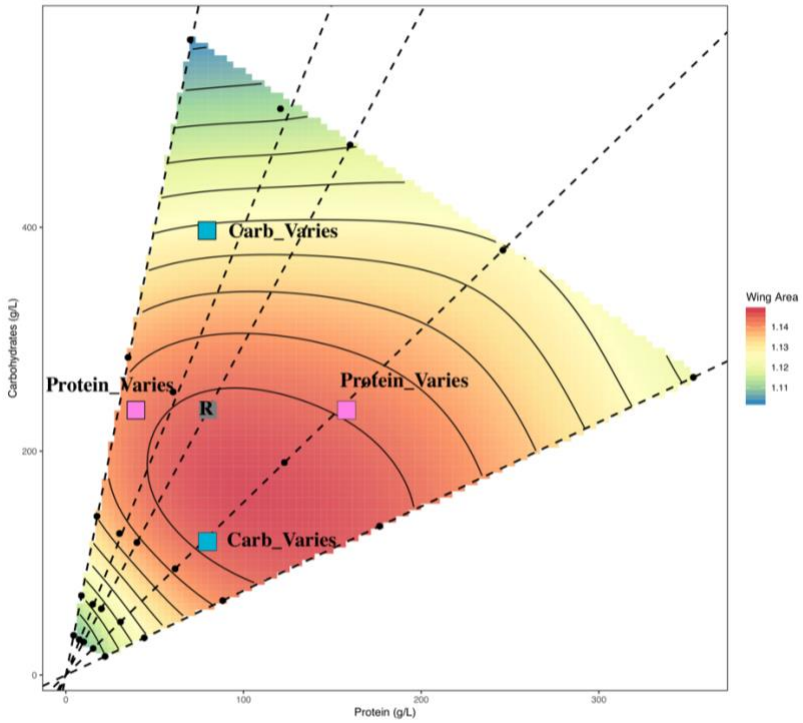

Figure 2: Experimental diets capturing the variation in body size (wing area) as seen on the multidimensional nutritional surface from Chakraborty et al 2020. Diet R: Reference diet was chosen since it results in body size that falls in the mid-point of the four paired experimental diets. A pair of Protein_Varies diets with carbohydrate concentration constant but varying protein concentration (high or low) relative to diet R. A pair of Carb_Varies diets with protein concentration constant and varying carbohydrate concentration (high or low) relative to diet R.