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Title: Drosophila melanogaster larvae make foraging choices that minimize developmental time

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Keywords: macronutrient intake; nutritional plasticity; response surfaces; foraging behaviour; oviposition preference

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Abstract: Organisms from slime moulds to humans carefully regulate their macronutrient intake to optimize a wide range of life history characters including survival, stress resistance, and reproductive success. However, life history characters often differ in their response to nutrition, forcing organisms to make foraging decisions while balancing the trade-offs between these effects. To date, we have a limited understanding of how the nutritional environment shapes the relationship between life history characters and foraging decisions. To gain insight into the problem, we used a geometric framework for nutrition to assess how the protein and carbohydrate content of the larval diet affected key life history traits in the fruit fly, Drosophila melanogaster. In no-choice assays, survival from egg to pupae, female and male body size, and ovariole number - a proxy for female fecundity - were maximized at the highest protein to carbohydrate (P:C) ratio (1.5:1). In contrast, development time was minimized at intermediate P:C ratios, around 1:2. Next, we subjected larvae to two-choice tests to determine how they regulated their protein and carbohydrate intake in relation to these life history traits. Our results show that larvae targeted their consumption to P:C ratios that minimized development time. Finally, we examined whether adult females also chose to lay their eggs in the P:C ratios that minimized developmental time. Using a three-choice assay, we found that adult females preferentially laid their eggs in food P:C ratios that were suboptimal for all larval life history traits. Our results demonstrate that D. melanogaster larvae make foraging decisions that trade-off developmental time with body size, ovariole number, and survival. In addition, adult females make oviposition decisions that do not appear to benefit the larvae. We propose that these decisions may reflect the living nature of the larval nutritional environment in rotting fruit. These studies illustrate the interaction between the nutritional environment, life history traits, and foraging choices in D. melanogaster, and lend insight into the ecology of their foraging decisions.

June 23rd, 2015

Dear Dr. Spencer Behmer,

Please find enclosed our revised manuscript, entitled "***Drosophila melanogaster* larvae make foraging choices that minimize developmental time**", for consideration as a research paper in Journal of Insect Physiology. We have addressed review 1's remaining concerns, and hope that you find this manuscript suitable for publication. We have included our response to the reviewer's comments below.

Sincerely,

Christen Mirth and coauthors

Response to Reviewers' comments:

Ms. Ref. No.: IP-D-15-00156

Reviewer #1: I reviewed the earlier version of this manuscript. I found substantial changes were made to this revised version, addressing all the major points raised by the two reviewers. The revised version has become statistically more explicit and better structured than the previous one.

The most important concern I raised earlier was whether the fly larvae actively regulated their target intake of protein and carbohydrate. The authors did some extra statistical analyses to show that larvae seem to regulate the protein intake to some extent, but not carbohydrate intake. The self-selected P:C ratio consumed was thus significantly different among all food choice groups, indicating that these fly larvae have only a limited capacity to defend their preferred amount of protein and carbohydrate eaten (Table 4). These results are now clearly reflected in the revised manuscript (L405-410; 576-577).

In the revised manuscript, the authors have also added the new results of the statistical analysis testing the non-randomness of food selection (Table 3; Figure 2C). The fact that the percentage of higher P:C food in the gut deviates significantly from 50% suggests that the larvae did not select the two choice foods at random. While this provides some circumstantial evidence that the larvae are regulating their macronutrient intake, it still remains questionable whether the insects are really converging their intake ratio close to a specific ratio between 1:4 and 1:2 (Line 575-576; 626-627) because significant

differences in the self-selected P:C ratios were found among food groups. In this regard, I think the conclusion that "larvae regulate their macronutrient intake towards the conditions that minimize development time (Line 660-661)" is rather too strong and hence needs to be toned down. It looks more appropriate that the larvae are selecting more moderate P:C ratios, which cover the ratio at which they exhibit shortest development time when restricted on single diets. I think it is perhaps important to consider focusing on explaining why the larvae ate less of high P:C food.

> Line 574-584: We have changed this section to say "Here, we found that *D. melanogaster* larvae regulate their macronutrient intake towards moderate P:C ratios, which include the ratios that result in the shortest development time on single foods. This suggests that larvae trade-off maximizing other fitness-related traits, like body size and ovariole number, for fast developmental rates. In addition, larvae appear to regulate their protein intake more tightly than their carbohydrate intake. This is reflected in the fact that the amount of carbohydrate consumed differed significantly between more food choice pairs than the amount of protein consumed. Future studies exploring how larvae balance protein versus carbohydrate intake on single foods with varied P:C ratios would clarify whether they prioritize regulating their protein consumption at the cost of misregulating carbohydrate intake."
>
> Line 630-632: We have changed this sentence to "Although larvae regulated their protein and carbohydrate intake towards intermediate P:C ratios, females preferred food containing even lower P:C ratios for oviposition sites."
>
> Line 663-665: We have now changed this sentence to say "Our results highlight that larvae regulate their macronutrient intake towards intermediate P:C ratios, which include those that minimize developmental time."

Apart from this issue, I am generally satisfied with the way in which this manuscript has improved following the revision.

Reviewer #2: The manuscript from Rodrigues et. al., while submitted as a new manuscript, is clearly a revised version of a previous submission. Based on my previous review, I believe the authors have addressed most of my concerns, especially in regard to important additional controls. To this end, I can now recommend the paper for publication.

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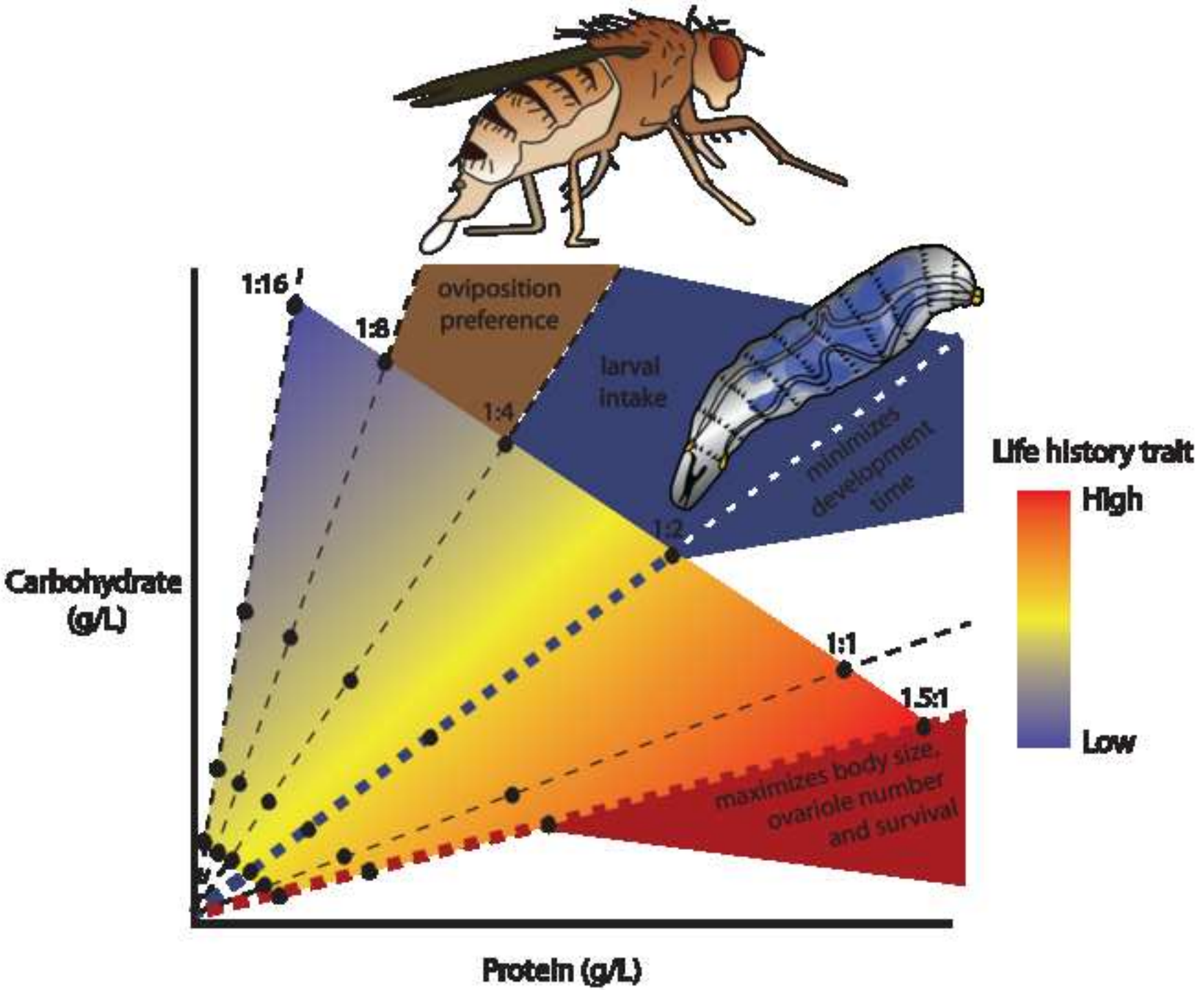
*Graphical Abstract (for review)



***Highlights (for review)**

- Development time is minimized at intermediate protein to carbohydrate (P:C) ratios
- The highest P:C ratios confer maximum body/ovary size and survival
- Larvae regulate their nutrient intake towards intermediate P:C ratios
- Females lay their eggs in low P:C ratios

Drosophila melanogaster larvae make foraging choices that minimize developmental time

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¶ These authors contributed equally to this work

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Summary

Organisms from slime moulds to humans carefully regulate their macronutrient intake to optimize a wide range of life history characters including survival, stress resistance, and reproductive success. However, life history characters often differ in their response to nutrition, forcing organisms to make foraging decisions while balancing the trade-offs between these effects. To date, we have a limited understanding of how the nutritional environment shapes the relationship between life history characters and foraging decisions. To gain insight into the problem, we used a geometric framework for nutrition to assess how the protein and carbohydrate content of the larval diet affected key life history traits in the fruit fly, *Drosophila melanogaster*. In no-choice assays, survival from egg to pupae, female

and male body size, and ovariole number – a proxy for female fecundity – were maximized at the highest protein to carbohydrate (P:C) ratio (1.5:1). In contrast, development time was minimized at intermediate P:C ratios, around 1:2. Next, we subjected larvae to two-choice tests to determine how they regulated their protein and carbohydrate intake in relation to these life history traits. Our results show that larvae targeted their consumption to P:C ratios that minimized development time. Finally, we examined whether adult females also chose to lay their eggs in the P:C ratios that minimized developmental time. Using a three-choice assay, we found that adult females preferentially laid their eggs in food P:C ratios that were suboptimal for all larval life history traits. Our results demonstrate that *D. melanogaster* larvae make foraging decisions that trade-off developmental time with body size, ovariole number, and survival. In addition, adult females make oviposition decisions that do not appear to benefit the larvae. We propose that these decisions may reflect the living nature of the larval nutritional environment in rotting fruit. These studies illustrate the interaction between the nutritional environment, life history traits, and foraging choices in *D. melanogaster*, and lend insight into the ecology of their foraging decisions.

Key-words: macronutrient intake, nutritional plasticity, response surfaces, foraging behaviour, oviposition preference,

1. Introduction

The complex nutritional environment of growing, developing animals contributes to shaping an equally complex suite of life history characters, such as survival,

developmental time, final body size and shape, longevity, and fecundity (Simpson and Raubenheimer, 2012). During their growth phase, juveniles consume target amounts of macronutrients, including protein, carbohydrates, and lipids, in addition to vitamins and minerals to sustain their growth and ensure their survival. To reach these targets, developing animals tightly regulate their foraging behaviour according to their species-specific nutritional needs (Simpson and Raubenheimer, 1993; Simpson et al., 2004). Understanding the relationship between the effects of nutrition on life history traits and the foraging strategy employed by the developing animal remains a fundamental, yet poorly understood, problem in biology.

Dealing with the numerous variables that make up the nutritional environment brings with it a series of challenges. Animals often feed on several food sources to balance their nutritional requirements (Jensen et al., 2012; Simpson and Raubenheimer, 2012; Simpson et al., 2004). Even within a single food, the quality and composition of this resource changes over time as the food source ripens, ages, or rots (Cosgrove et al., 2002; Morais et al., 1995; Raubenheimer, 2011; Starmer, 1981). To pare this nutritional complexity down to manageable size, Raubenheimer, Simpson, and co-authors developed the geometric framework for nutrition (Cheng et al., 2008; Simpson and Raubenheimer, 1993; Simpson and Raubenheimer, 1999; Simpson and Raubenheimer, 2012; Simpson et al., 2004). By varying two nutritional variables at a time across a broad range of nutrient space, nutritional geometry allows us to explore how life history traits change with each variable and how these variables interact. The nutritional landscapes derived by this framework then permit targeted questions about how foraging choices relate to life history traits.

The geometric framework for nutrition has been applied across a wide range of taxa, from slime moulds to humans, to understand how nutrition affects life history

traits and foraging decisions (Berreur et al., 1979; Dussutour et al., 2010; Simpson et al., 2003; Simpson and Raubenheimer, 1993; Simpson et al., 2004). In adult females of both *Drosophila melanogaster* and Queensland fruit flies (*Bactrocera tryoni*), the balance of macronutrients, specifically protein to carbohydrate (P:C) ratios, affects longevity, lifetime egg production, and egg laying rate differently (Fanson et al., 2009; Fanson et al., 2012; Lee et al., 2008). Adult females live the longest in low P:C ratios, lay the most eggs in high P:C ratios, and show the greatest lifetime egg production at intermediate P:C ratios (Fanson et al., 2009; Fanson et al., 2012; Ja et al., 2009; Lee et al., 2008). When offered a choice between two foods, one high in protein and the other high in carbohydrates, females of both species regulate their food intake towards the P:C ratio that maximizes lifetime egg production (Fanson et al., 2009; Lee et al., 2008). Similarly, predatory ground beetles regulate their nutrient intake towards P:C ratios that maximize egg production (Jensen *et al.*, 2012). Thus, in these species adult females choose the macronutrient balance that maximizes reproductive success.

Although studies in adult females clearly reveal the links between nutrition, life history traits, and foraging choices, the effects of the nutritional environment are both sex and stage-specific. Nutrition affects reproductive performance differently in males and females in cockroaches, *Nauphoeta cinerea*, field crickets, *Teleogryllus commodus*, and fruit flies, *D. melanogaster*, and this is accompanied by a sex-specific shift in foraging choice (Maklakov et al., 2009; Maklakov et al., 2008; Reddiex et al., 2013; South et al., 2011). Furthermore, many life history characters, like body and organ size, result from the larval nutritional environment (Koyama et al., 2013; Mirth and Riddiford, 2007; Mirth and Shingleton, 2012; Nijhout, 2003a; Nijhout, 2003b; Nijhout et al., 2013; Shingleton et al., 2007; Stern and Emlen, 1999). Finally,

imbalanced larval nutrition affects dietary choices in later larval and adult stages in butterflies (Lee et al., 2012; Mevi-Schütz and Erhardt, 2003). Despite this insight, to date we understand little about how life history traits shaped by juvenile nutrition define their foraging choices.

To explore the relationship between juvenile nutrition, life history traits, and foraging behaviour, we applied the geometric framework for nutrition to larvae of *D. melanogaster*. These larvae live and feed on rotting fruit, obtaining much of their protein and lipid from the yeast communities that aid decomposition (Begon, 1982). The nutrition derived from their food regulates their growth (Bakker, 1959; Tu and Tatar, 2003), their development time (Bakker, 1959; Beadle et al., 1938; Koyama et al., 2013; Mirth et al., 2009; Shingleton et al., 2005; Shingleton et al., 2009; Stieper et al., 2008), and the development of their reproductive organs (David, 1970; Tu and Tatar, 2003). Protein consumption, not carbohydrate consumption, regulates body and tissue growth in larvae (Britton and Edgar, 1998; Colombani et al., 2003), whereas both protein and carbohydrates contribute to variation in developmental timing (Schwarz et al., 2013). The quantity of yeast in the larval diet has profound impacts on adult fitness; larvae raised in food containing a high concentration of yeast show increased fecundity but decreased longevity and starvation resistance with respect to those reared on low-yeast food (Chippindale et al., 1993). Females that are poorly fed as larvae lay larger eggs, and their offspring develop faster on dilute food (Vijendravarma et al., 2010). Furthermore, larvae adapted to poor-quality food, via experimental evolution, metamorphose into smaller adults within the normal developmental time frame when reared on normal food (Kolss et al., 2009). On poor food, the development time and survival of these evolved lines is less affected than that of controls (Kolss *et al.*, 2009). Despite the considerable insight into how

nutrition affects these life history characters, manipulating single nutritional variables for only a few food types cannot generate sufficient resolution to identify differences in the response of life history characters across a nutritional landscape.

Similarly, we have some understanding of how fruit flies regulate their foraging behaviour in response to the protein and carbohydrate composition of the food. In both larval and adult *D. melanogaster*, depriving animals of protein leads them to choose yeast over sucrose (Piper et al., 2014; Ribeiro and Dickson, 2010; Schwarz et al., 2013). Reciprocally, animals deprived of carbohydrates choose sucrose over yeast (Piper et al., 2014; Schwarz et al., 2013). Larvae do not survive on diet that does not contain yeast (Anagnostou et al., 2010; Becher et al., 2012), and prefer strains of yeast that maximize their survival and body size (Anagnostou *et al.*, 2010). Although these studies shed light on how *D. melanogaster* makes foraging choices in relation to single macronutrients, they do not tell us how larvae regulate their intake to achieve the correct balance of proteins and carbohydrates and how this macronutrient balancing relates to life history traits.

In this study, we reared animals from egg to adult on one of twenty-four different diets, which differed in their protein, carbohydrate, and caloric content. We measured five life history traits: survival from egg to pupae, development time, female and male body weight, and ovariole number, a proxy for fecundity in adult females (David, 1970; Tu and Tatar, 2003). Survival, body weight, and ovariole number showed maximum values at the highest P:C ratios (1.5:1), whereas development time was shortest at P:C ratios around 1:2. Next, we assessed whether larvae regulate their P:C intake towards either of these ratios. Finally, we explored where adult females choose to lay their eggs. Our findings provide important insight into how larvae make the

foraging decisions and reveal that larval food choice and oviposition choice do not match.

2. Materials and methods

2.1 FLY STOCKS AND CULTURE MEDIUM

We used an outbred population of *Drosophila melanogaster* from Azeitão, Portugal, maintained at high population sizes (>1000 flies) for more than 50 non-overlapping generations (Martins et al., 2013). Using an outbred population with high genetic diversity increases the probability that the measured effects are features of the population, as they are significant across a range of genetic backgrounds, rather than being specific to a particular inbred genotype. The fly culture media used to maintain these populations contained 45 g of molasses, 75 g of sucrose, 70 g of cornmeal, 10 g of agar, 1100 ml of water, and 25 ml of a 10% Nipagin solution per litre of fly food.

2.2 NUTRITIONAL GEOMETRY AND LIFE HISTORY TRAITS

We reared flies from egg to adult on one of 24 different food types of four caloric values, 0.18, 0.36, 0.72 and 1.44 kcal/ml (45 g/L, 90 g/L, 180 g/L and 360 g/l respectively) and six protein to carbohydrate (P:C) ratios, 1:16, 1:8, 1:4, 1:2, 1:1, 1.5:1 (Lee *et al.*, 2008). Ratios were made up by mixing yeast extract (Pró-vida, Algueirão, Portugal) containing 45% protein and 20% carbohydrate in a 0.5% agar solution with sucrose (Sidul, Santa iria de Azóia, Portugal)/ 0.5% agar solution for each of the caloric values as in Lee *et al*., (2008). Food was autoclaved and to each 500 ml of yeast or sucrose solution we added 5 ml of both proprionic acid (Acros organics, Geel, Belgium) and of 10% nipagen (10% p-hydroxy benzoic acid methyl ester in 95% ethanol) (Apex BioResearch Products).

Approximately 150 flies were transferred into egg laying chambers (100 ml plastic cups) and left to oviposit on 60 mm petri dishes filled with our standard lab food for 4 hours. From these dishes, we separated 30 eggs onto small squares of sterilized paper and randomly distributed each paper square between fly vials that contained 5 ml of treatment food. We replicated each diet 4 times. All replicate cultures were established on the same day. Cultures were maintained at 25°C in a climate-controlled room under 60-70% humidity. These same rearing conditions were used for the nutritional geometry experiments, the larval choice experiments and the oviposition experiments.

We measured survival from egg to pupa, larval development time from egg to pupa (at 8 hour intervals), female and male pharate adult weight, and ovariole number. We used pharate adult weight as a proxy for adult weight, as we have previously found it to be highly correlated with adult wing size (Mirth et al., 2005) and due to the ease of staging and handling this immobile stage. Pharate adults were sexed by examining the first legs for the presence of sex combs, a male-specific feature, before weighing on a SE2 Ultramicrobalance (Sartorius, Goettingen, Germany). To count ovarioles, we mated females and males from the same treatment and replicate, and dissected out the ovaries 3-5 days after mating in phosphate buffered saline.

2.3 LARVAL FOOD CHOICE ASSAYS

We performed two types of larval food choice assays; in the first we altered the length of time the larvae were left to feed and in the second we varied the rearing conditions of the larvae. In the first assay, larvae were reared from egg to the third instar at a controlled density of 200 eggs/food plate (60 mm diameter petri dishes) in 1:1 food of 0.72 kcal/ml. The assays were performed on the day following the moult

to third instar, as determined by the morphology of the larval anterior spiracles (Bodenstein, 1950).

To alter the rearing conditions, we reared larvae at controlled densities as in the previous experiment, but this time on food with a caloric content of 0.72 kcal/ml and one of three P:C ratios: 1:8, 1:4 or 1:1. One day following the moult to third instar, we offered the larvae a choice of two of the ratios on an assay plate. For both experiments, we replicated each rearing condition at least five times and maintained the cultures at 25°C and 60-70% humidity.

To make the assay plate, we embedded 10 lids from 0.5 ml microcentrifuge tubes in a 60 mm petri dish by filling the plate with a solution of 3% agar up to the edge of the lids. We then distributed the two food types evenly between the lids. For the food choice over time experiment, we offered one of the following food pairs: 1:2 and 1:16 (1:2/1:16), 1:1 and 1:8 (1:1/1:8), 1.5:1 and 1:4 (1.5:1/1:4), or 1.5:1 and 1:8 (1.5:1/1:8). For the altered rearing conditions experiment, we offered one of the following food pairs: 1:2/1:16, 1:1/1:8, or 1.5:1/1:4. To distinguish between the food types, we dyed each food either red or blue with food dye at 4.5% (Rayner, Billingshurst, UK). We controlled for colour preference by dying each food type both colours for each choice assay and by performing experiments in the dark. We assessed larval food choice by spectrophotometry.

To ensure that we can reproducibly recover the correct proportion of dye from the foods, we fed larvae for 1.5 hours on single foods (in 60 mm petri dishes) with one of the following dye combinations– 0% blue/100% red, 25% blue/75% red, 50% blue/50% red, 75% blue/25% red, and 100% blue/0% red – for a total of 4.5 ml of dye in 100 ml of food. We performed this experiment using two food types, either 1:1 or 1:8 diets (both 0.72 kcal/ml). We then tested whether the slope of the regression line

between the percentage of blue in the food and the percentage of blue in the gut had a slope equal to one. The 99% confidence intervals for the slope of the linear models were 0.997-1.031 for the 1:1 food, and 0.996-1.023 for the 1:8 food (Supplementary Fig. S1). These results confirm that we can accurately recover the proportion of dye in the food by measuring the proportion of dye in the larval guts via spectrophotometry.

For the food choice over time assay, ten larvae foraged for 1.5, 3, or 6 hours. For the rearing conditions assay, ten larvae were left to forage on the plates for 1.5 hours. We performed 20-30 replicates of each choice pair for each of the feeding times/ larval rearing conditions.

After the assay, larvae were placed into a 1.5 ml tube with 80 µl of ice-cold methanol, homogenized, and centrifuged at 13, 000 x g for 10 minutes at 4ºC. The supernatant was transferred to a new 1.5 ml tube and the procedure repeated to ensure that we had removed as much dye as possible. As standards, we used a ten-fold dilution (1:2 dilution) series of each dye, using a starting concentration of 4.5 µl dye/100 µl of methanol, to calculate the percentage of each food type, the total amount of food eaten, and the amount of protein and carbohydrate in the larval guts. Finally, we measured the absorbance of 100 µl of each sample at 450 nm for the red dye and at 600 nm for the blue using a Victor3 multilabel plate reader (Perkin Elmer, Waltham, USA).

To determine the extent to which larvae mix foods or choose a single food type, we conducted an additional experiment where we reared larvae in 0.72 kcal/ml, 1:1 food from egg to third instar (L3), then offered them a choice between two foods, 1:1 or 1:8 (both 0.72 kcal/ml). Again we controlled for colour preference by switching the dyes. We then assessed the number of larvae with one food type (one colour) versus two food types (two colours) in their guts after 1.5 hours by eye.

In addition, we assessed whether larvae showed a bias in their first food choice. We again reared larvae in in 0.72 kcal/ml, 1:1 food from egg to L3. After they moulted to L3, we offered them a choice between two foods, 1:1 or 1:8 (both 0.72 kcal/ml). The assay plate for this experiment consisted of two lids from 2 ml eppendorf tubes embedded into a 60 mm petri dish by filling the plate with a solution of 3% agar. We placed ten larvae in the centre of each plate and filmed them over 25 minutes using Firefly MV cameras (Point Grey). We then scored from the film where each larva burrowed first, as our measure of first food choice.

Finally, in the nutritional geometry experiments, we assessed the effects of the larval diet on life history traits from egg to adult eclosion. However, we assayed larval choice in the third instar. To determine whether nutrition in the third instar affects larval development time, we reared larvae from egg to the second instar in 1:1 food of 0.72 kcal/ml. We collected larvae as they moulted to third instar as in (Mirth et al., 2005), and transferred them to vials containing either 1:8, 1:2, or 1.5:1 food (0.72 kcal/ml). We scored the number of larvae pupariating three times daily to estimate developmental time.

2.4 OVIPOSITION ASSAYS

To assay oviposition preference, we used a three-choice design. Animals were reared from egg to eclosion at densities of 30 eggs per vial on 0.72 kcal/ml food with one of three P:C ratios: 1:8, 1:4, or 1:1. All animals were kept at 25°C and 60-70% humidity.

Newly eclosed females were left to mate with males of the same age and rearing condition for four days before the assay. On the fourth day, we placed 20 females and 10 males in the assay chamber and allowed them to lay eggs for 15 hours overnight at 25°C and 60-70% humidity. We gave them a choice between three P:C ratios, 1:8,

1:4, and 1:1, and distinguished between the three ratios by dying the food red, green or blue (4.5 ml of Globo food dye/100 ml of food). The assay chambers consisted of a 100 ml plastic cup placed over a 60 mm petri dish into which we fixed nine 0.5 ml eppendorf lids. The three food types were distributed between the nine lids. We controlled for colour preference by dying each food all three colours and performing the assays in the dark. We replicated each choice combination 27 times. To assess oviposition site preference, we counted the number of eggs laid in each diet.

2.5 STATISTICAL ANALYSIS

We estimated the response of each life history trait to the protein and carbohydrate content of the food following the methods described in (Lee *et al.*, 2008). For survival, we fit a generalized linear model to the data, assuming a quasi-binomial distribution for survival probabilities, thus accounting for over-dispersion of the data, and a logit link function. For all other traits, we fit linear mixed effects models, including replicates as a random effect. Our models included both the linear and quadratic components of protein and carbohydrate and their cross product. We visualized the response of each trait to the nutrient array defined by protein and carbohydrate concentrations in the diet using non-parametric thin plate splines (Blows and Brooks, 2003).

For larval choice experiments, we calculated proportion of each food type in the gut for each choice, rearing condition, or colour, and tested for significance from the null hypothesis – random choice (μ=50% for two-choice tests) – using Wilcoxon signed rank tests. We used Kruskal Wallis rank sum and pair-wise Wilcoxon rank sum tests to test for significant differences between larval macronutrient intake including quantity of protein or carbohydrate or the P:C ratio ingested.

We tested whether the proportion of larvae mixing foods was greater than those choosing single foods by fitting generalized linear models assuming a binomial distribution and a logit link function. To test for significant biases in the food into which larvae first burrowed, we used generalized linear models assuming a quasibinomial distribution, to account for the overdispersion of the data, and a logit link function. In both cases, we then compared across P:C ratios using a null distribution of $\mu=0.5$ (no choice between two food types) and compared the least squared means between P:C ratios. We adjusted the *p*-values for tests involving multiple comparisons using sequential Bonferroni (Holm) correction.

We tested for effects of third instar larval diet on development time using Kruskal Wallis tests with post hoc pairwise Wilcoxon tests. We adjusted the p-values for multiple comparisons using Holm correction.

Finally, to test between differences in the proportion of eggs laid in each diet or colour, we fit the data with generalized linear models, using a quasibinomial distribution to account for the overdispersion of the data. We then compared the proportion of eggs laid in each P:C ratio against a null distribution of $\mu=0.33$ (no choice between all three food types) and compared the least squared means for each P:C ratio. We adjusted the p-values for tests involving multiple comparisons using Holm correction.

All statistical analyses were performed in R using the nlme, lme4, lmmfit, lsmeans, stats, and fields packages, and plotted using ggplot2. The complete datasets along with the R scripts can be found in Dryad (reference number to be provided).

3. Results

3.1 SURVIVAL FROM EGG TO PUPA

Survival from egg to pupa varied negatively with the linear component of carbohydrate and positively with the linear component of protein (Fig. 1A, Table 1). In addition, we observed a significant negative correlation with the quadratic effects of protein. Neither the quadratic effect of carbohydrate nor the cross product of carbohydrate and protein significantly affected survival. Overall, this resulted in a peak in survival at low carbohydrate levels and intermediate levels of protein, with the isoclines dropping away from the peak at both lower and higher protein concentration. The peak centred around the highest P:C ratios, 1:1 and 1.5:1.

3.2 DEVELOPMENT TIME

The full model, including the linear and quadratic components of carbohydrate and protein and their cross product, explained 68.7% of the variance in development time. Development time increased with the linear component of carbohydrate and decreased with the linear component of protein (Fig. 1B, Table 1). The quadratic component of protein showed a significant positive relationship with development time. Neither the quadratic component of carbohydrate nor the cross product of carbohydrate and protein significantly affected development time. This resulted in a minimum development time at P:C ratios around 1:2, with development time increasing as protein levels either increased or decreased from this minimum.

3.3 BODY SIZE

Large-sized flies frequently have higher fecundity, higher courtship success, and better survival (Ewing, 1961; Ewing, 1964; Partridge and Farquhar, 1983; Partridge and Fowler, 1993) making body size a relevant fitness-related trait. To determine the effects of nutritional composition on adult body size, we sexed and weighed individuals at the pharate adult stage. We analysed the data for males and females separately.

The full model explained 15.1% of the variation in female weight. The linear component of carbohydrate showed negative correlation with weight, whereas the correlation between weight and the linear component of protein was positive (Fig. 1C, Table 1). The quadratic component of protein also showed a significant negative correlation. This resulted in a peak in body size at intermediate protein concentrations around the highest P:C ratios of 1:1 and 1.5:1. The isoclines dropped as protein concentration decreased or increased from this intermediate range.

For male weight, the full model explained 2.8% of the variation in the data. The response surface for male weight was similar to that of female weight, with the exception that the linear component of carbohydrates did not show a significant effect on body size (Fig. 1D, Table 1). Body weight for males reached a maximum at the highest P:C ratios of 1.5:1.

3.4 OVARIOLE NUMBER

In *D. melanogaster* females, as in all insects, the ovaries are composed of strings of egg chambers known as ovarioles. The number of ovarioles per ovary is defined during larval development (King et al., 1968) and positively correlates with the number of eggs laid (David, 1970; Tu and Tatar, 2003). We assessed ovariole number as a proxy of female fecundity.

Female body size is known to correlate with ovariole number in *Drosophila* (David, 1970; David et al., 1994; Delpuech et al., 1995). To assess the effects of larval diet on ovariole number independent of body size, we used female body weight as a covariate in our linear mixed-effects model. The full model explained 12.5% of the variation in ovariole number (Table 1). Ovariole number correlated positively with the linear component of protein and negatively with its quadratic component (Fig. 1E, Table 1). This resulted in ovariole number showing maximum values at the highest

P:C ratio, 1.5:1, but at intermediate values of protein. Ovariole number decreased as protein both decreased and increased away from these values.

3.5 COMPARISONS BETWEEN RESPONSE SURFACES

We next compared the nutritional response surfaces for each of the larval traits, to determine if there were significant differences between their shapes. To eliminate the differences in scale between life history traits, we first standardized the dependent measures to have means of zero and unit standard deviations before fitting the models for the partial F tests. In relation to the four other traits examined, the response surface for development time was inverted, decreasing with increasing protein. Thus, we used the inverse values for development time.

We found that the shape of the inverted response surface for development time differed significantly from all other traits (Table 2). The other four traits – survival, female body size, male body size, and ovariole number – were statistically indistinguishable. Thus, there appeared to be two classes of nutritional response surfaces, one for development time showing peak values at P:C ratios around 1:2 and a second for the remaining traits showing peak values at P:C ratios around 1.5:1.

3.6 LARVAL FOOD CHOICE

Given the differences in response surface shape between life history traits, we sought to determine whether larvae make foraging decisions to maximize survival, body size, and ovariole number, or to minimize development time. We reared larvae at controlled densities from egg to the third instar on 0.72 kcal/ml food with a P:C ratio of 1:1. On the day after the moult to third instar (L3), we offered larvae a choice between two foods equal in their caloric content (0.72 kcal/ml), but differing in their P:C ratios (Fig. 2A). For this assay, larvae were offered one of four choice pairs: 1:2/1:16, 1:1/1:8, 1:1.5/1:4, and 1:1.5/1:8. Larvae were left to feed for 1.5, 3, or 6

hours and, after the defined intervals, we assessed the quantity of food eaten by quantifying the colour in the gut by spectrophotometry (Fig. 2B). Larvae showed a slight, but significant, preference for blue in one of the four choice pairs (1:2/1:16) in this assay (Supplementary Fig. S2A, Supplementary Table S1).

For all four of the choices examined, we found that larvae ate significantly less of the higher P:C ratio (Fig. 2C, Table 3), indicating that larvae choose between foods of different P:C ratios. For three of the four choice combinations, larvae ate P:C ratios close to 1:4 and 1:2 (Fig. 2E). The amount of protein eaten was statistically indistinguishable between most choices, with the exception that larvae offered a choice between 1.5:1 and 1:8 food ate significantly less protein than larvae offered any other choice (Fig. 2E and Table 4). Both the amount of carbohydrate and the P:C ratio consumed differed significantly between all choice combinations (Fig. 2E, Table 4, and Supplementary Table S2).

Because in our assays we pool all larvae together to calculate the proportion of each food type eaten, larvae could show preferences towards eating the lower P:C food either because 1) individual larvae eat only one food but fewer choose to eat the higher P:C food, or 2) larvae sample both foods but eat less of the higher P:C food. To determine whether larvae ate single foods or mixed both foods, we examined the proportion of larvae that had either one or two colours of food in their guts when offered a choice between 1:1 and 1:8. We found a greater proportion of larvae with two foods, rather than one, in their guts (Supplementary Fig. S3A, χ^2=11.19, p value=0.00082), indicating that larvae tend to sample both foods but consume less of the higher P:C food.

Even if larvae show a tendency to mix between foods, they may show a bias in the amount of food they eat of each type depending on the order in which they eat it. We

next determined whether larvae burrowed first into higher versus lower P:C ratios. To do this, we offered larvae a choice between 0.72 kcal/ml food with P:C ratios of either 1:1 or 1:8 and scored their first burrowing site. A higher proportion of larvae burrowed first into the 1:1 food (Supplementary Fig. S3B, χ^2=13.14, p-value: 0.00029). This suggests that larvae tend to eat less of the food into which they first burrow. Larvae did not show a significant preference for either blue or red in this assay (χ^2=-1.28e-14, p-value: 1).

Next, we explored whether the larval rearing conditions affected their macronutrient intake. To assess this, we first reared larvae from egg to third instar on 0.72 kcal/ml food containing P:C ratios of either 1:8, 1:4 or 1:1. Then, we performed two-choice tests where larvae were offered two foods with caloric contents of 0.72 kcal/ml that differed in their P:C ratios. One of three choice pairs were offered: 1:2/1:16, 1:1/1:8, and 1.5:1/1:4. Larvae fed for 1.5 hours and their food choice was determined by spectrophotometry. Larvae showed a slight, but significant, preference for blue for 2 of the three choices (Supplementary Fig. S2B, Supplementary Table S1). Similar to the previous assay, we found that larvae ate less of the higher P:C food (Fig. 2D, Table 3).

When we calculated the protein and carbohydrate content of the food found in the larval gut, we found that for two of the three choices larval macronutrient intake centred around P:C ratios of 1:4 and 1:2 (Fig. 2F). The amount of protein or carbohydrate consumed depended on the choice larvae were offered. Although the amount of protein consumed by larvae offered either 1:2/1:16 and 1.5:1/1:4 was indistinguishable, larvae offered the 1:1/1:8 choice pair ate significantly more protein than the other two choice pairs (Table 5). The amount of carbohydrate consumed also differed significantly between choice pairs, with larvae offered 1.5:1/1:4 consuming

significantly less carbohydrate than those offered the other two choice pairs (Fig. 2F, Table 5). Finally, the P:C ratio consumed differed significantly between all three choice pairs (Fig. 2F, Supplementary Table S3).

The diet on which larvae were reared also affected the amount of protein and carbohydrate consumed. Larvae reared on P:C ratios of 1:8 ate significantly less protein and carbohydrate than larvae raised on the other two diets (Fig. 2F, Table 6). Furthermore, larvae reared on 1:8 food ate slightly, but significantly, lower P:C ratios than larvae reared on 1:1 food (Supplementary Fig. S4, Supplementary Table S4). Finally, although larvae reared on 1:1 ate significantly more protein than those reared on 1:4, they ate less carbohydrate (Fig. 2F, Table 6).

Our data suggest that larvae may regulate their macronutrient intake towards P:C ratios between 1:4 and 1:2, ratios similar to those that minimized developmental time. However, in the nutritional geometry experiments, larvae were fed on each of the diets from hatch until the end of larval development, whereas our behavioural experiments were conducted only on L3 larvae. To determine whether altering the P:C ratio of the diet in the L3 could still affect developmental time, we reared larvae from egg to the moult to L3 on 1:1, 0.72 kcal/ml food. After the moult to L3, we transferred them to one of three P:C ratios, 1:8, 1:2, or 1.5:1 and measured the time from the moult to the onset of pupariation. Larvae transferred to 1:8 food took significantly longer to pupariate than larvae transferred to 1:2 and 1.5:1 food (5 and 7 hours longer respectively, Table 7).

3.7 OVIPOSITION SITE CHOICE

Finally, we assessed whether females chose to lay their eggs on P:C ratios that maximized larval performance or reproductive potential and whether the larval rearing diet affected this choice. To do so, we reared larvae on 0.72 kcal/ml food

containing one of three P:C ratios: 1:8, 1:4 and 1:1. Upon eclosion, we selected 20 females and 10 males of the same rearing regime and allowed them to mate over three days. We then offered each group a three-way choice of 1:8, 1:4, and 1:1 food (Figure 3A). Oviposition choice was determined by counting the number of eggs laid on each food type. Females did not show a significant colour preference for oviposition site (Supplementary Fig. S2C, Supplementary Table S5).

Females laid significantly more eggs in higher carbohydrate food; they preferred P:C ratios of 1:8 over 1:4, and of 1:4 over 1:1 (Fig. 3B, Table 8). Larval rearing conditions showed a slight, but significant, effect on oviposition site choice, with females reared in 1:4 laying significantly more eggs laid in 1:1 food than females reared in 1:8 (Supplementary Table S6).

4. Discussion

In this study, we sought to understand how the response of life history characters to the macronutrient content of the larval diet correlated with larval foraging decisions and female oviposition choice. To address this, we characterized the response of five life history traits to the protein and carbohydrate content of the larval diet in *D. melanogaster*. We then explored whether larval macronutrient consumption and adult female oviposition site choice corresponded to peak values for life history traits.

4.1 THE EFFECTS OF LARVAL DIET COMPOSITION ON LIFE HISTORY TRAITS

Our data demonstrate that survival from egg to pupa, pharate weight, and ovariole number respond similarly to the protein and carbohydrate composition of the larval food, with maximum values for each reached at the highest P:C ratios. Development time, on the other hand, differs in its response to macronutrient composition of the

food. The fastest development times occurred at intermediate P:C ratios around 1:4-1:2. For the larva, this means it would be impossible to simultaneously minimize development time, thought to reduce both predation risk, competitive ability, and the risk of resource exhaustion (Krijger et al., 2001; Roff, 2002), and maximize fitness-related traits such as body size and ovariole number.

Trade-offs in the responses of life history traits to the macronutrient content of the food appear common. In male *Telostylinus angusticollis* neriid flies, survival and body size differ in their response to the protein to carbohydrate composition of the larval diet (Sentinella et al., 2013). Like in *D. melanogaster* larvae, body size in neriid flies is maximized at high P:C ratios. In contrast, survival from egg to adult decreased with increasing protein (Sentinella *et al.*, 2013). In adult females of both *D. melanogaster* and Queensland fruit flies *Bactrocera tryoni*, the protein and carbohydrate conditions that maximize egg production rate, lifetime egg production, and lifespan differ with each trait (Fanson et al., 2009; Lee et al., 2008).

In addition to differences in the shapes of the response surfaces, the carbohydrate and protein content of the larval diet explained a different proportion of the variance in the observed response for each trait. The magnitude of the effects of diet composition on a given life history trait can depend on their environmental or genetic contexts. In the caterpillar *Spodoptera littoralis*, viral infection alters the effects of macronutrient composition on survival (Lee et al., 2006). In control animals, survival did not correlate with the P:C ratio of the diet within the range tested. When infected with nucleopolyhedrovirus, caterpillars fed on the highest P:C ratios showed the best survival (Lee *et al.*, 2006).

In the current study, protein and carbohydrate content of the food explained only a small proportion of the observed response in adult body size measures: female/male

pharate adult weight and ovariole number. Because we used an outbred population, this could be due to a high degree of genetic diversity both in the traits themselves and in the reaction norms of these traits to the protein and carbohydrate content of the larval diet. Taken together, we expect that a wide range of factors – be they environmental or genetic – alter the shape of nutritional response surfaces for life history traits.

For traits such as body and organ size, where the size of adult organs is determined primarily by their growth in larval stages, adult nutrition cannot change the outcome of poor larval nutrition. Nevertheless, it seems possible that other types of traits, such as those related to metabolic functions like fat storage and the rate of egg production, might be determined by adult diet. In the cockroach, *Nauphoeta cinerea*, nymphal nutrition alters the metabolic state of the adult. Female *N. cinerea* cannot overcome the negative effects of poor nymphal nutrition on fat and ovarian mass with good adult nutrition, although they show significant increases in both traits on rich adult diets (Barrett et al., 2009). Larval nutrition can also directly contribute to the energy stores available for flight and reproduction. In *D. melanogaster,* the degeneration of the larval fat body during metamorphosis contributes over half of the energy sequestered into the ovary in the first two days of adult life, thereby playing an important role in regulating egg production rate (Aguila et al., 2013). Thus it appears that the effects of larval diet go beyond constraining the size of adult structures, and contribute significantly to programming adult metabolic functions.

4.2 COMPARISSONS BETWEEN LARVAL AND ADULT DIET COMPOSITIONS ON LIFE HISTORY TRAITS

In insects, nutritional requirements are expected to differ between juveniles and adults due to the differences in the metabolic costs of growth in the juvenile stages

versus flight and reproduction in adults (Simpson et al., 2002). Since we employed a protocol similar to that designed by Lee *et al.,* (2008) to explore the effects of nutritional geometry on adult female life history traits in *D. melanogaster*, our data present a unique opportunity to compare the effects of diet at different life stages on life history traits. In adult females, the nutritional conditions that maximize egg production rate and lifetime egg production centred around P:C ratios of 1:2 and 1:4 respectively (Lee et al., 2008). On the other hand, lifespan for adult females was highest around P:C ratios of 1:16 (Lee et al., 2008). This suggests that traits related to development and growth, like egg production, tend to be maximal in intermediate to high P:C ranges, whereas those relating to metabolic function, such as longevity, show the opposite pattern.

For larvae, all traits relate to development and growth. This may explain why their maxima occupy the intermediate to high P:C ranges. Interestingly, the P:C ratios that maximize egg production and fecundity in adults occupy the same range as those that minimize development time in larvae. Other growth-related traits in larvae, such as body size, ovariole number, and survival, are maximized at higher P:C ratios. Why growth-related traits occupy different P:C ranges remains unclear.

4.3 FEEDING BEHAVIOUR AND THE REGULATION OF MACRONUTRIENT INTAKE

The relative importance of trade-offs between the nutritional response surfaces of life history traits becomes apparent when assessing the foraging decisions animals make. When offered a choice between two foods containing different concentrations of protein and carbohydrate, both *D. melanogaster* and *B. tryoni* females combine these foods to ingest the P:C ratio that maximises lifetime egg production (Fanson et

al., 2009; Lee et al., 2008). These studies elucidate how the nutritional response of life history traits shape foraging decisions in these insects.

Here, we found that *D. melanogaster* larvae regulate their macronutrient intake towards moderate P:C ratios, which include the ratios that result in the shortest development time on single foods. This suggests that larvae trade-off maximizing other fitness-related traits, like body size and ovariole number, for fast developmental rates. In addition, larvae appear to regulate their protein intake more tightly than their carbohydrate intake. This is reflected in the fact that the amount of carbohydrate consumed differed significantly between more food choice pairs than the amount of protein consumed. Future studies exploring how larvae balance protein versus carbohydrate intake on single foods with varied P:C ratios would clarify whether they prioritize regulating their protein consumption at the cost of misregulating carbohydrate intake.

In their natural habitat, larvae live in a complex, living environment that changes with time. As it rots, fruit is colonized by a succession of yeast species and other microbiota that decomposes it until finally the fruit tissue is exhausted (Morais *et al.*, 1995). Due to their preference for particular yeast species (da Cunha et al., 1951; da Cunha et al., 1957; Morais et al., 1995), different species of Drosophilids are known to colonize fruit at different phases of the ripening/rotting process (Lachaise et al., 1982; Nunney, 1990). Some species, such as those from the subgenus *Zaprionus,* colonize fruit while it is ripe, while others, like those from the *Drosophila fima* subgroup, colonize fruit at advanced stages of decay (Lachaise *et al.*, 1982). This preference for particular stages of decomposition may affect how larvae make their foraging decisions. Larvae that colonize fruit at more advanced stages of decay may opt to make foraging decisions that minimize developmental time to avoid exhaustion

of the substrate. Other species that colonize earlier stages might make foraging decisions that maximize other life history traits, as developmental time might not be as limiting.

Although yeast is the primary source of protein for larval and adult *D. melanogaster*, and protein is required for larval growth and development (Britton and Edgar, 1998), it also contains a complex combination of other micronutrients and macronutrients not accounted for in this study. To generate our nutrient space, we used a semi-synthetic medium composed of sucrose and yeast extract. However, life history traits are likely to respond not only to the protein and carbohydrate composition of the diet, but also to complex interactions between these other nutritional elements. To dissect out the effects of these other nutrients requires a more delicate approach using a holidic medium, similar to that recently developed by (Piper et al., 2014). Although this medium is reported to considerably extend larval development time (Piper et al., 2014), its use would determine the contribution of other macronutrients in the larval diet to life history characters.

4.4 LARVAE DO NOT COMPENSATE FOR LONG-TERM NUTRITIONAL DEFICITS

Previous studies in locusts and *D. melanogaster* larvae showed that animals fed for a short time on a diet lacking either protein or carbohydrate later preferred the diet containing the nutrient(s) for which they were deficient (Schwarz et al., 2013; Simpson et al., 1991). Similarly in adult *D. melanogaster,* feeding females on sucrose alone for several days increases their preference for yeast (Ribeiro and Dickson, 2010). Given these findings, we expected larvae to selectively eat foods that would complement their nutritional deficit.

In our assays, larvae reared in 1:8 food did not compensate for low protein by consuming higher P:C ratios. Instead, the food in their gut was slightly lower in P:C ratio when compared to larvae reared in 1:1 food. When faced with nutritional deficits, locusts modify the production of digestive enzymes to balance macronutrients post-ingestion (Clissold et al., 2010). Under long-term nutritional deficit, changes in the gastrointestinal tract and/or in metabolism might play a more important role in balancing macronutrients than behaviour.

4.5 OVIPOSITION SITE CHOICE AND MACRONUTRIENT COMPOSITION OF THE SUBSTRATE

Although larvae regulated their protein and carbohydrate intake towards intermediate P:C ratios, females preferred food containing even lower P:C ratios for oviposition sites. At first glance, this suggests that females choose oviposition sites that are suboptimal for larvae. However, P:C ratios presumably signal the stage of fruit decay, as yeast populations, the primary source of protein for larvae, increase with fruit decomposition (Morais et al., 1995). Thus foods with high P:C may be too advanced in the decay process to sustain larvae throughout their development. When offered fruit that has been rotting over a range of time, *D. melanogaster* females prefer to lay eggs in fruit with intermediate levels of decay (Hoffmann, 1985). Furthermore, they prefer to lay eggs close to, but not in, yeast sources (Miller et al., 2011). Potentially, females may select oviposition sites with lower P:C ratios as they will provide better feeding sites in the future. Because different species prefer different stages of fruit decay for oviposition (Lachaise et al., 1986; Nunney, 1990), this hypothesis could be tested by surveying a range of species for a correlation between their decay stage preference and their P:C preference for oviposition.

Much like foraging choice, *D. melanogaster* females appear to integrate cues from the environment with their internal state when choosing oviposition sites. In addition to their preference for particular P:C ratios, females are attracted to egg laying sites by a cocktail of odour cues (Stensmyr et al., 2003). Substrate texture, substrate temperature and the presence of larval residues also affect oviposition site choice (Chess and Ringo, 1985; Fogleman, 1979). Learning and prior experience adds additional layers of complexity to the mechanisms that modulate oviposition preferences (Hoffmann, 1985; Mery and Kawecki, 2004; Mery and Kawecki, 2005). Finally, adult females engage in niche construction, actively inoculating the fruit substrate with yeast during oviposition (Buser et al., 2014; Chandler et al., 2012; da Cunha et al., 1957). Because they bring the protein source with them, females may use qualities of the decomposing fruit other than its P:C content to determine oviposition site preference. Taken together, the unexpected mismatch between female oviposition preference and larval nutritional responses and foraging choices may be explained by a multitude of processes that call for further dedicated experiments.

4.6 CONCLUSIONS

Nutrition geometry is a powerful, established framework for assessing the effects of macronutrient composition in the diet on a wide range of traits. Further, it provides a tool for determining how organisms regulate their feeding behaviour. Our results highlight that larvae regulate their macronutrient intake towards intermediate P:C ratios, which include those that minimize developmental time. However, the adult females choose to lay their eggs in foods that were suboptimal for all traits measured. These studies provide valuable insight into how life history traits shape foraging decisions, and provide an inroad to explore the relationship between larval nutritional ecology and foraging choice of both larval and adult *D. melanogaster*.

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Figures:

Figure 1: The effects of protein and carbohydrate content of the larval diet on life history traits. The fitted response surfaces of the effects of 24 different diets varying in the protein, carbohydrate and caloric composition for (A) proportion of larvae surviving from egg to pupae, (B) development time from egg to pupae, (C) female pharate adult weight (mg), (D) male pharate adult weight (mg) and (E) female ovariole number. Dashed black lines indicate the P:C ratios. Filled black circles represent each of the 24 foods.

Figure 2: Larval food preference and intake target. Ten larvae were offered a choice between two protein to carbohydrate (P:C) ratios (A). Larval food preference was first assayed by eye (B) and then quantified by spectrophotometer. (C) Proportion of the higher protein food in the guts of larvae offered one of four food pair choices (1:2/1:16, 1:1/1:8, 1.5:1/1:4 and 1.5:1/1:8). The data is pooled from larvae left to forage for 1.5, 3, and 6 hours. The dashed line represents the no choice (50%) scenario. (D) Proportion of the higher protein food in the guts of larvae offered one of three food pair choices (1:2/1:16, 1:1/1:8, and 1.5:1/1:4) for 1.5 hours. The data is pooled from larvae reared on 1:8, 1:4, or 1:1 foods. The dashed line represents the no choice (50%) scenario. (E) Larval target intake over time (1.5, 3 and 6 hours) for four food choices (1:2/1:16, 1:1/1:8, 1.5:1/1:4 and 1.5:1/1:8). The error bars are 95% confidence intervals of the means. We replicated each choice 20-30 times for three times sampled, with a total of 60-80 replicates/choice. (F) Larval target intake changed with rearing conditions (1:8, 1:4 and 1:1) and the food choice (1:2/1:16, 1:1/1:8, and 1.5:1/1:4). The error bars are 95% confidence intervals of the means. We replicated each choice 19-20 times for all three rearing conditions, with a total of 59-

60 replicates/choice. The blue dashed line in E and F indicates the P:C ratio that minimized development time. The red dashed line in E and F indicates the P:C ratio that maximized survival, female/male body size and ovariole number.

Figure 3: Female oviposition site preference. Females were offered three protein to carbohydrate (P:C) ratios for oviposition (A). (B) The oviposition site preference index (PI) was calculated as follows: (# eggs in food 1- # eggs in food 2 - # eggs in food 3)/total # eggs. Asterisks in (B) indicate PI significantly different from μ=-0.33 (no choice) by Wilcoxon signed rank test ($p<0.0001$). Letters in (B) indicate significant difference between food types as determined by a pair-wise Wilcoxon Rank Sum Test ($p<0.0001$). We replicated the assay 27 times/choice combination.

Supplementary Figure S1: The relationship between the percentage of blue dye in the food and the percentage of blue dye measure in the larval guts by spectrophotometer on two different diets (0.72 kcal/ml food with P:C of either 1:1 or 1:8).

Supplementary Figure S2: Control for colour preference in the larval feeding and adult oviposition assays. (A) The percentage of larvae choosing red versus blue food for four choice conditions (all time points combined). (B) The percentage of larvae choosing red versus blue food for each choice condition under three P:C conditions (all rearing conditions combined). The asterisk corresponds to a significant preference between the two colours, as determined by a Wilcoxon signed rank test. (C) *$p<0.05$

Supplementary Figure S3: A) The proportion of larvae with one versus two foods in their gut. Significantly more larvae had two foods in their gut then one food as determined by ANOVA of the data fit with a generalized linear model assuming a binomial distribution and a logit link (χ^2=11.19). B) The proportion of larvae burrowing first into 1:1 versus 1:8 food. Significantly more larvae burrow into the 1:1 food, as determined by ANOVA of the data fit with a generalized linear model assuming a quasibinomial distribution and using a logit link. Dashed lines indicate equal proportion for both conditions. **p<0.01, *** p<0.001

Supplementary Figure S4: The effect of larval rearing conditions on the protein to carbohydrate (P:C) ratio consumed. Larvae reared on P:C ratios of 1:8 ate lower P:C ratios than those reared on 1:1 as determined by Kruskal Wallis Tests and post hoc Wilcoxon rank sum pair-wise comparisons. ** p<0.001

Tables

Life History Trait		C	P	C^2	P^2	C x P	R^2
Survival	β	**-0.016**	**0.087**	2.92e-05	**-5.79e-04**	5.59e-05	
	t value	**-3.012****	**6.90*****	1.98	**-8.15*****	0.922	
Development Time	β	**0.472**	**-2.19**	-0.00046	**0.0141**	-0.00226	0.69
	t value	**4.03*****	**-8.73*****	-1.38	**9.49*****	-1.80	
Female Weight	β	**-0.0017**	**0.0091**	1.50e-6	**-5.55e-5**	4.2e-6	0.15
	t value	**-2.65****	**7.22*****	0.855	**-7.27*****	0.68	
Male Weight	β	-9.72e-4	**0.0060**	1.50e-6	**-3.19e-5**	-1.30e-6	0.028
	t value	-1.93	**5.76 *** **	0.99	**-5.02 *** **	-0-24	
Ovariole Number	β	-0.020	**0.22**	0.00007	**-0.0012**	-0.00034	0.1
	t value	-0.87	**4.01*****	0.97	**-3.37****	-1.37	

Table 1: Effects of carbohydrate (C), protein (P) and their squares and products in the larval diet on five life history characters: survival from egg to pharate adults, development time, male/female pharate weight and ovariole number. For all traits except survival, the models were linear mixed-effects models fit by maximum likelihood. Survival data was analysed with a generalized linear model, assuming a quasi-binomial distribution of survival probabilities and a logit link. For ovariole number, we used female weight as a covariate. Significant coefficients are in bold: *p<0.05, **p<0.01, ***p<0.001.

Life History Trait A	Life History Trait B	Degrees of Freedom	L ratio	Adjusted p-value
Survival	Ovariole Number	5	7.59	0.720
	Female Weight	5	9.95	0.383
	Male Weight	5	14.4	0.0794
	Inverse Development Time	5	**19.6**	**0.0136***
Ovariole Number	Female Weight	5	3.70	1
	Male Weight	5	2.38	1
	Inverse Development Time	5	**18.1**	**0.0225***
Female Weight	Male Weight	5	7.00	0.720
	Inverse Development Time	5	**17.42**	**0.0263***
Male Weight	Inverse Development Time	5	**38.58**	**<0.001*****

Table 2: Comparisons between the response surfaces of the five life history traits. Using partial F tests, we compared the response surfaces generated from linear mixed effects models on the scaled parameter values. For development time, we inverted the data for comparison. Response surfaces that show significant differences are highlighted in bold. The p values were adjusted using the Holm method. *p<0.05, **p<0.01, ***p<0.001

Experiment 1: Proportion of higher P:C food in the guts over time		
Choice	V Statistic	P value
1:2/1:16	**469**	**0.0010****
1:1/1:8	**1933**	**<0.0001*****
1.5:1/1:4	**634**	**0.039***
1.5:1/1:8	**265**	**<0.0001*****
Experiment 2: Proportion of higher P:C food in the guts for larvae reared on different P:C ratios		
1:2/1:16	**317**	**<0.0001*****
1:1/1:8	**117**	**<0.0001*****
1.5:1/1:4	**68**	**<0.0001*****

Table 3: The percentage of higher P:C foods in the guts deviates significantly from 50% for all choice pairs. Larvae were allowed to feed for 1.5, 3 and 6 hours (Experiment 1) or for 1.5 hours (Experiment 2) on one of the choice pairs. The table shows the V statistic and p values for Wilcoxon tests against $\mu = 50$ (random choice). Significant differences are highlighted in bold. *$p<0.05$, **$p<0.01$, ***$p<0.001$

Protein Eaten (mg)			
Choice Offered	1:2 / 1:16	1:1 / 1:8	1.5:1/1:4
1:1 / 1:8	0.78	-	-
1.5:1/ 1:4	0.78	0.54	-
1.5:1/1:8	**0.0011**	**0.00076**	**0.013**
Carbohydrate Eaten (mg)			
Choice Offered	1:2 / 1:16	1:1 / 1:8	1.5:1/1:4
1:1 / 1:8	**<0.0001**	-	-
1.5:1/ 1:4	**<0.0001**	**<0.0001**	-
1.5:1/1:8	**<0.0001**	**<0.0001**	**<0.0001**

Table 4: The amount of protein and carbohydrate eaten over time depended on the choice offered. A Kruskal-Wallis rank sum test on amount of protein eaten shows a significant difference between choice conditions (χ^2=19.68, df=3, p-value=0.00020). Similarly, the amount of carbohydrate eaten differs between choice tests (χ^2=146.50, df=3, p-value<0.0001). The table above shows the p-values for Wilcoxon rank sum pair-wise comparisons between choices using the Holm p-value adjustment method, with significant differences in bold.

Protein Eaten (mg)		
Choice Offered	1.5:1 / 1:4	1:1 / 1:8
1:1 / 1:8	**0.022**	-
1:2 / 1:16	0.65	**0.00077**
Carbohydrate Eaten (mg)		
Choice Offered	1.5:1 / 1:4	1:1 / 1:8
1:1 / 1:8	**<0.0001**	-
1:2 / 1:16	**<0.0001**	0.31

Table 5: The amount of protein and carbohydrate eaten within 1.5 hours depended on the choice offered. A Kruskal-Wallis rank sum test on amount of protein eaten shows a significant difference between choice conditions (χ^2=13.33, df=2, p-value=0.0013). Similarly, the amount of carbohydrate eaten differs between choice tests (χ^2=40.11, df=2, p-value<0.0001). The table above shows the p-values for Wilcoxon rank sum pair-wise comparisons between choices using the Holm p-value adjustment method, with significant differences in bold.

Protein Eaten (mg)		
Rearing Food	1:8	1:4
1:4	**<0.0001**	-
1:1	**<0.0001**	**0.047**
Carbohydrate Eaten (mg)		
Rearing Food	1:8	1:4
1:4	**<0.0001**	-
1:1	**<0.0001**	0.48

Table 6: Larvae reared on different P:C ratios ate different amounts of protein and carbohydrates. A Kruskal-Wallis rank sum test on amount of protein eaten across rearing treatments shows a significant difference between rearing conditions (χ^2=103.31, df=2, p-value<0.0001). Similarly, the amount of carbohydrate eaten differs between rearing conditions (χ^2=63.90, df=2, p-value<0.0001). The table above shows the p-values for Wilcoxon rank sum pair-wise comparisons between rearing foods, adjusting the p-values using the Holm method, with significant differences in bold.

Development time (hours)		
Third Instar Rearing Food	1:8	1:2
1:2	**<0.0001**	**-**
1.5:1	**<0.0001**	0.13

Table 7: Third instar larvae (L3) reared in different P:C ratios differ in their development time from L3 moult to pupariation. A Kruskal-Wallis rank sum test on development time across rearing treatments shows a significant difference between rearing conditions (χ^2= 52.75, df=2, p-value<0.0001). The table above shows the p-values for Wilcoxon rank sum pair-wise comparisons between rearing foods, adjusting the p-values using the Holm method, with significant differences in bold.

Oviposition Preference for P:C Ratio			
Food P:C	lsmean	st error	group
1:1	-1.24	0.0789	1
1:4	-0.71	0.0700	2
1:8	-0.22	0.0662	3

Table 8: Adult females laid their eggs in food with lower P:C ratios. Generalized linear models using a quasibinomial distribution to account for the overdispersion of the data showed significant differences in the proportion of eggs laid in each P:C ratio (χ^2= 101.72, df=2, p-value<0.0001). The table above shows the least squared means (lsmean), standard errors (st error), and groups for each food type, with significant differences denoted by different numbers in the group column (adjusting p-values using the Bonferroni method for a significance level of 0.05).

Figure 1

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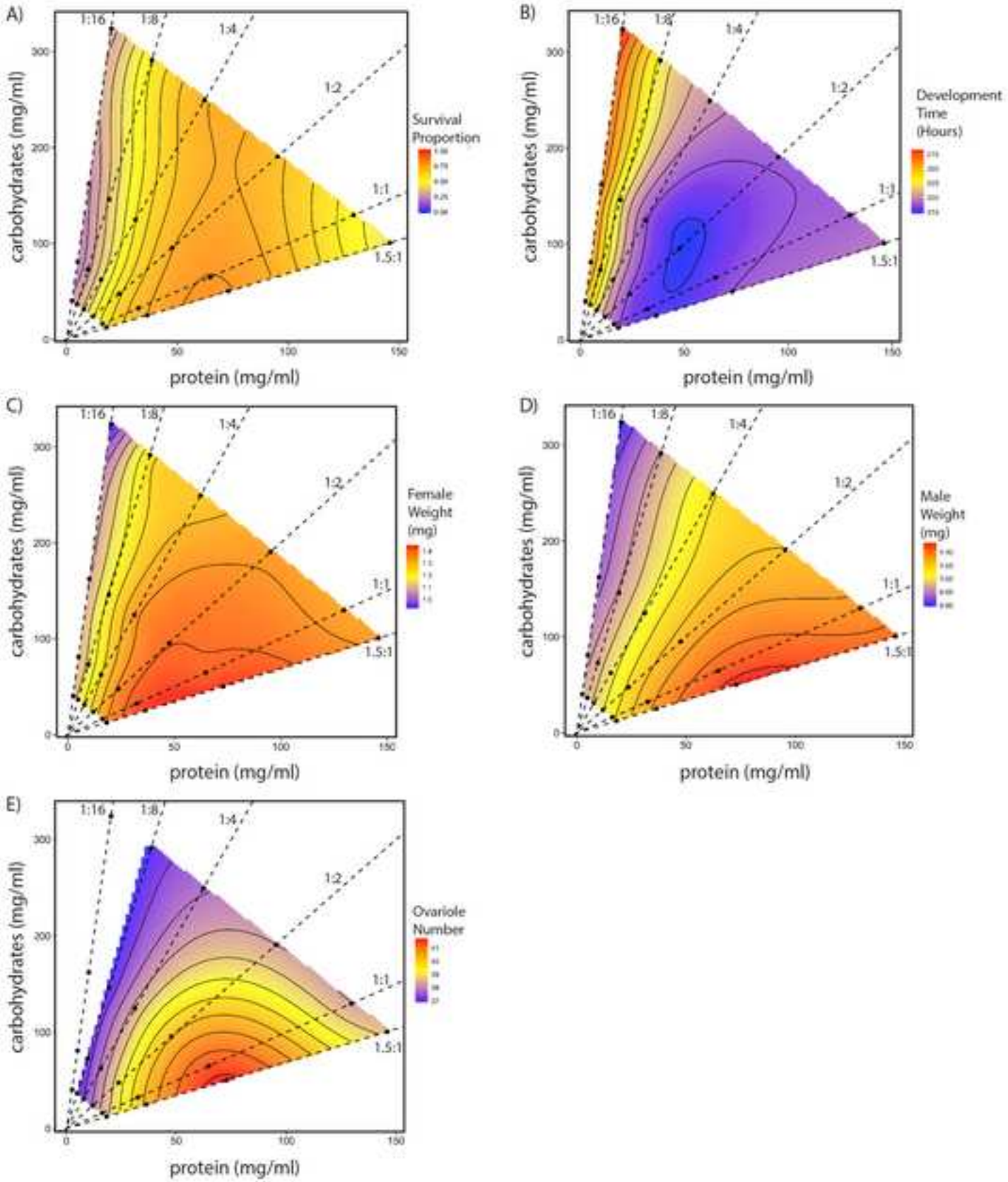

Figure 2

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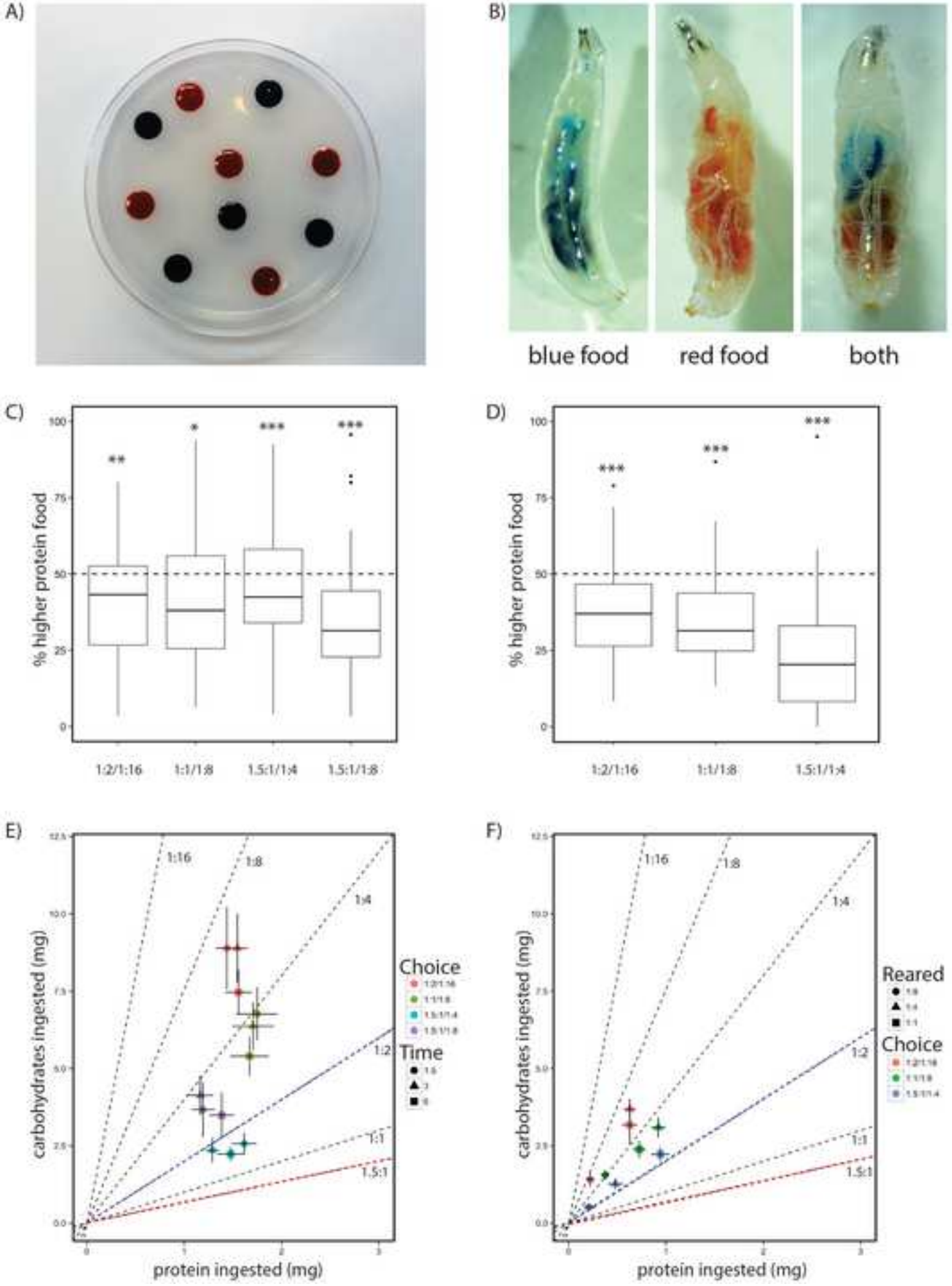

Figure 3

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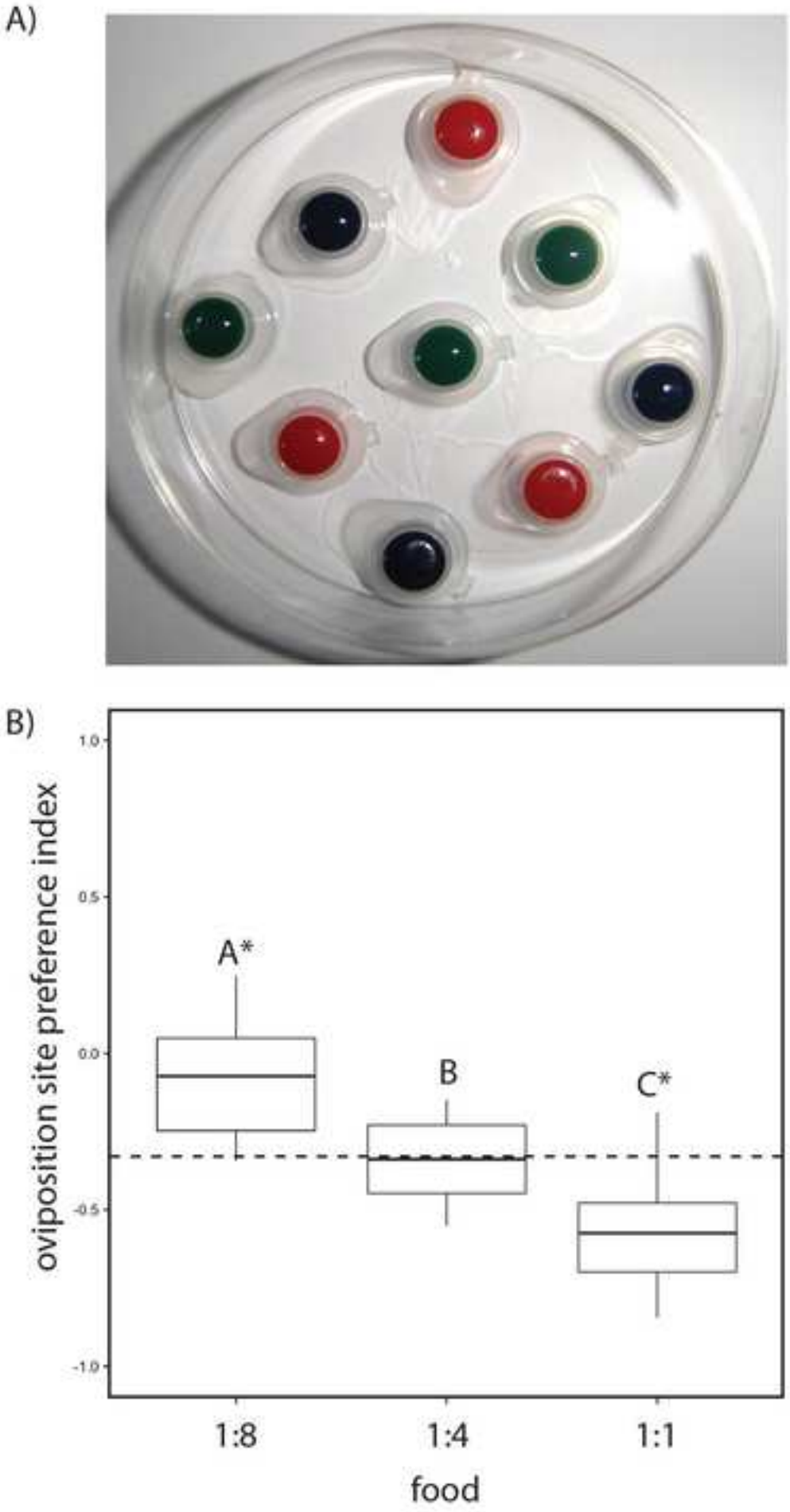

Supplementary Tables

Experiment 1: Proportion of larvae choosing blue over time		
Choice	V Statistic	P value
1:2/1:16	**1185**	**0.047 ***
1:1/1:8	2530	0.052
1.5:1/1:4	697.5	0.11
1.5:1/1:8	1044	0.34
Experiment 2: Proportion of larvae choosing blue for larvae reared on different P:C ratios		
1:2/1:16	**1126.5**	**0.036***
1:1/1:8	820	0.49
1.5:1/1:4	**1190.5**	**0.043***

Supplementary Table S1: The percentage of blue food in the guts deviates significantly from 50% for some choice pairs. Larvae were allowed to feed for 1.5, 3 and 6 hours (Experiment 1) or for 1.5 hours (Experiment 2) on one of the choice pairs. The table shows the V statistic and p values for Wilcox tests against $\mu = 50$ (random choice). Significant differences from 50% are highlighted in bold. *p<0.05, **p<0.01, ***p<0.001

P:C ratio Eaten			
Choice Offered	1:2 / 1:16	1:1 / 1:8	1.5:1/1:4
1:1 / 1:8	**<0.0001**	-	-
1.5:1/ 1:4	**<0.0001**	**<0.0001**	-
1.5:1/1:8	**<0.0001**	**0.00031**	**<0.0001**

Supplementary Table S2: The P:C ratio eaten over time depended on the choice offered. A Kruskal-Wallis rank sum test on the P:C ratio eaten shows a significant difference between choice conditions (χ^2= 151.0067, df=3, p-value= < 2.2e-16). The table above shows the p-values for Wilcoxon rank sum pair-wise comparisons between choices using the Holm method for p-value adjustment. Significant differences are in bold.

P:C ratio Eaten		
Choice Offered	1.5:1 / 1:4	1:1 / 1:8
1:1 / 1:8	**<0.0001**	-
1:2 / 1:16	**<0.0001**	**<0.0001**

Supplementary Table S3: The P:C ratio eaten within 1.5 hours depended on the choice offered. A Kruskal-Wallis rank sum test on the P:C ratio eaten shows a significant difference between choice conditions (χ^2= 93.51, df=2, p-value= < 2.2e-16). The table above shows the p-values for Wilcoxon rank sum pair-wise comparisons between choices using the Holm method for p-value adjustment. Significant differences are in bold.

P:C ratio Eaten		
Rearing Food	1:8	1:4
1:4	0.2580	-
1:1	**0.0096**	0.1249

Supplementary Table S4: The P:C ratio eaten depended on the larval rearing conditions. A Kruskal-Wallis rank sum test on the P:C ratio eaten shows a significant difference between rearing conditions (χ^2=8.99, df=2, p-value= 0.01119). The table above shows the p-values for Wilcoxon rank sum pair-wise comparisons between rearing conditions using the Holm method for adjusting p-values, with significant difference in bold.

Oviposition Preference for Colour			
Food Colour	lsmean	st error	group
Blue	-0.6580559	0.1037053	1
Green	-0.7101727	0.1045991	1
Red	-0.7115210	0.1046231	1

Supplementary Table S5: *D. melanogaster* females do not show significant preferences for food colour for oviposition. Generalized linear models using a quasibinomial distribution to account for the overdispersion of the data showed no significant differences in the proportion of eggs laid in each colour (χ^2= 0.17, df=2, p-value=0.92). The table above shows the least squared means (lsmean), standard errors (st error), and groups for each food type, with significant differences denoted by different numbers in the group column (adjusting p-values using the Bonferroni method for a significance level of 0.05).

P:C Ratio for Ovipostion	**1:1**			**1:4**			**1:8**		
Rearing Food	lsmean	st error	group	lsmean	st error	group	lsmean	st error	group
1:1	-1.2	0.110	12	-0.69	0.098	1	-0.26	0.093	1
1:4	-1.01	0.122	2	-0.74	0.116	1	-0.36	0.110	1
1:8	-1.71	0.184	1	-0.69	0.141	1	0.05	0.133	1

Supplementary Table S6: Females reared in different P:C ratios lay different proportions of their eggs in 1:1 food. We fit the data with generalized linear models, using a quasibinomial distribution to account for the overdispersion of the data. Our models showed significant differences in the proportion of eggs laid in the 1:1 food for females reared in different P:C ratios (Food: χ^2=114.40, df=2, p-value<0.0001, Rearing Food: χ^2=0.00, df=2, p-value=1.00, Food x Rearing Food: χ^2=16.58, df=4, p-value=0.0023). The table above shows the least squared means (lsmean), standard errors (st error), and groups for each species in each food type, with significant differences denoted by different numbers in the group column (adjusting p-values using the Bonferroni method for a significance level of 0.05).

Supplmentary Figure S1

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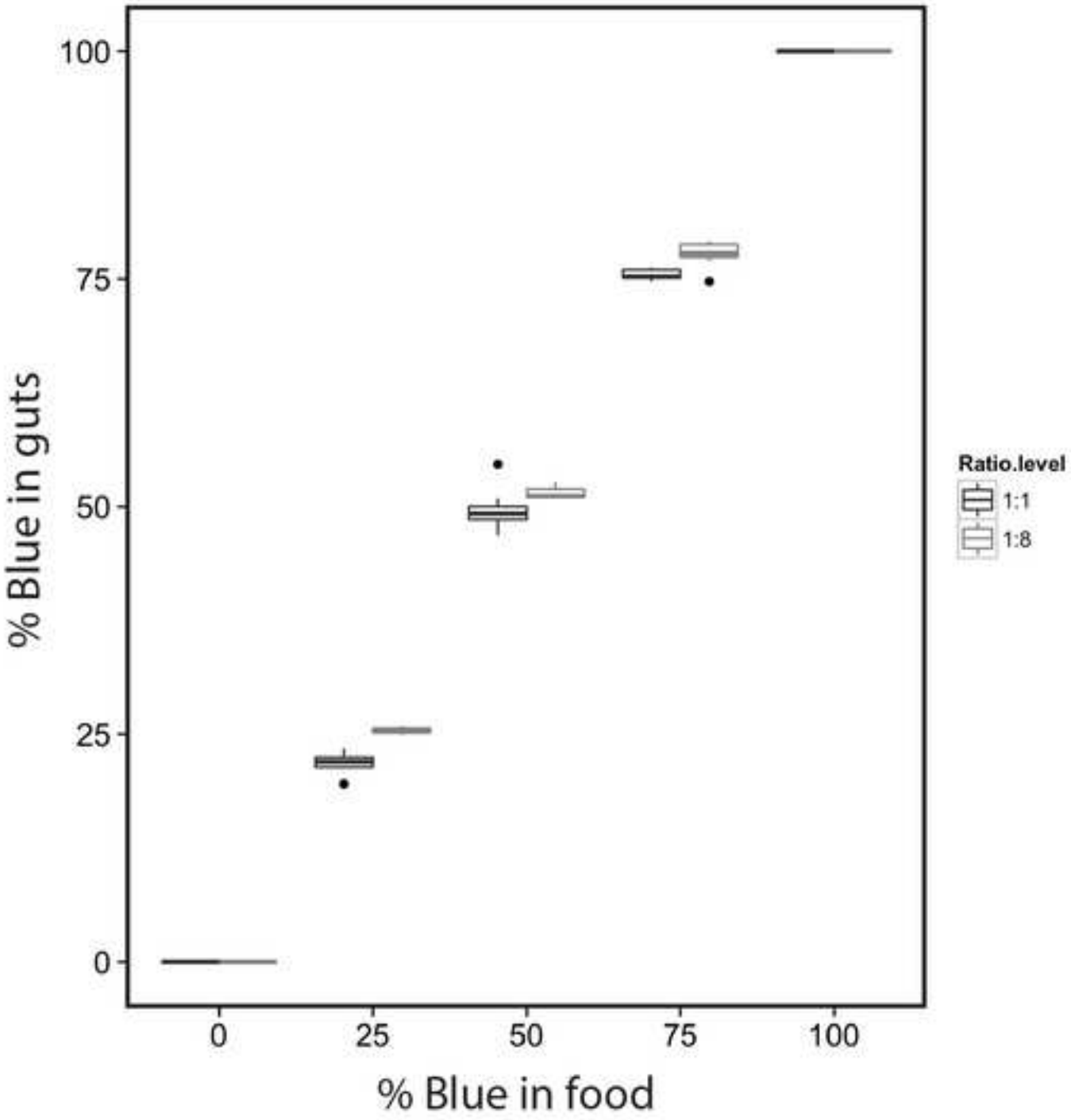

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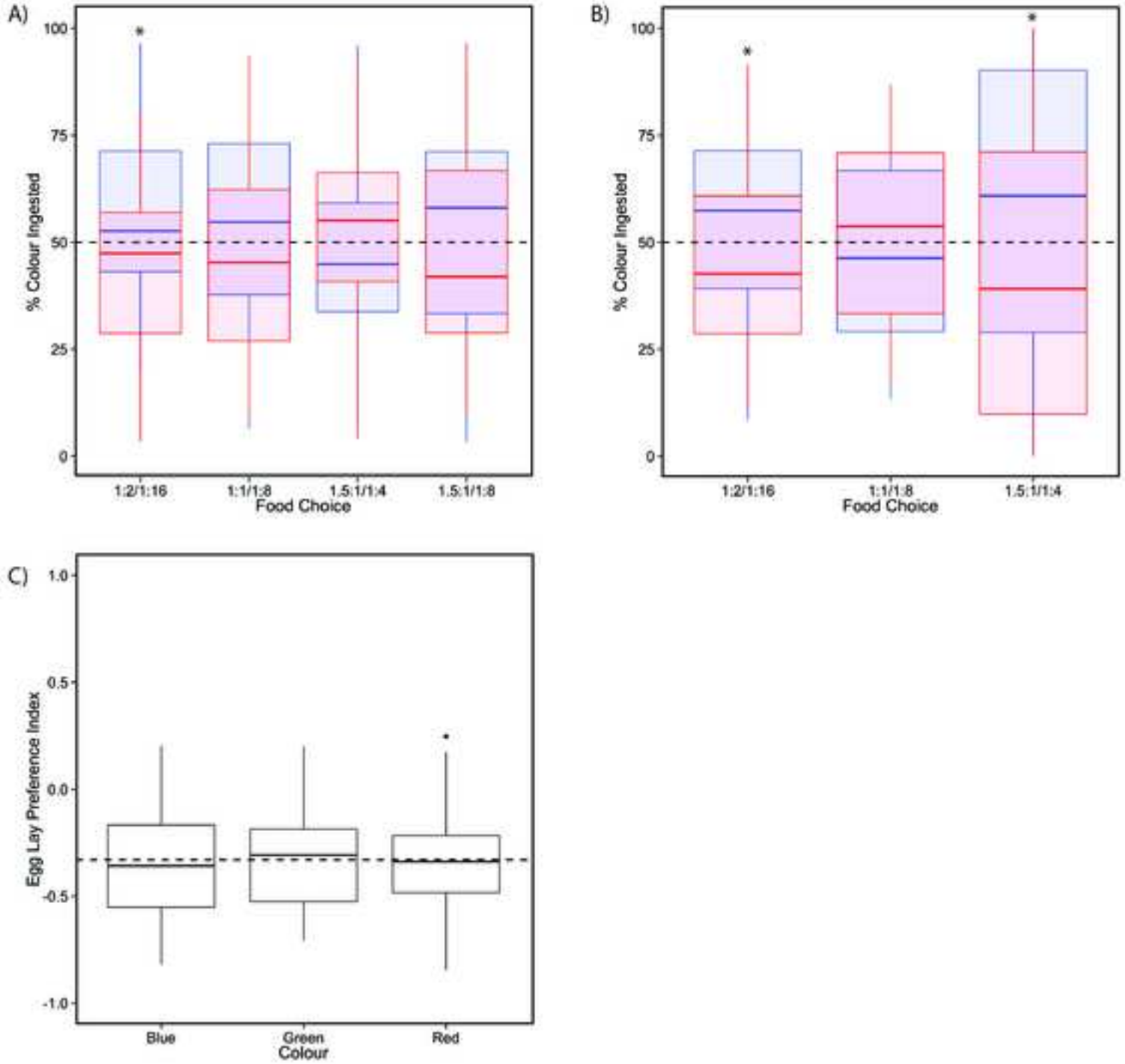

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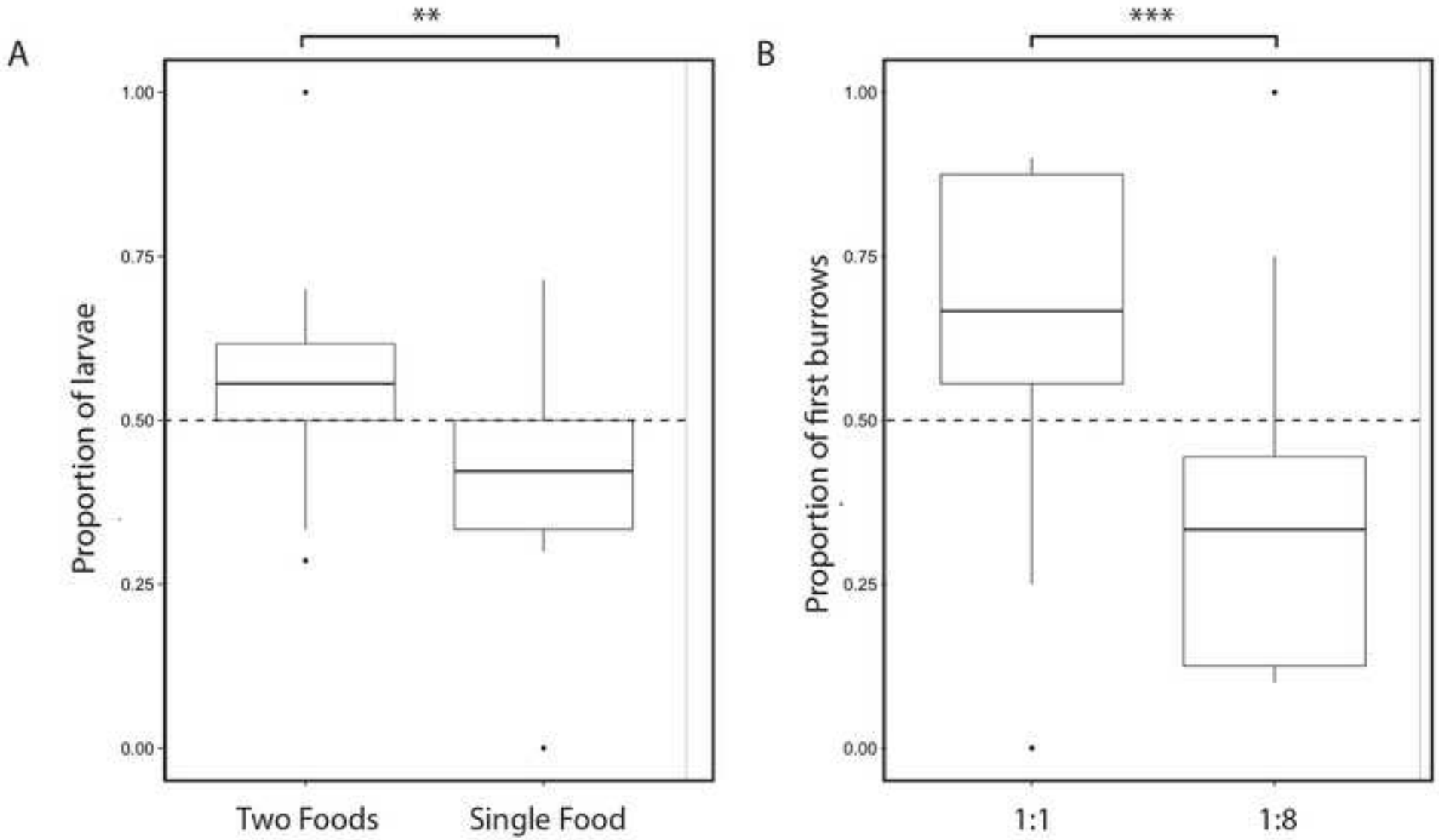

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