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ORIGINAL PAPER

Food selection in larval fruit flies: dynamics and effects on larval development

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Abstract Selecting food items and attaining a nutritionally balanced diet is an important challenge for all animals including humans. We aimed to establish fruit fly larvae (*Drosophila melanogaster*) as a simple yet powerful model system for examining the mechanisms of specific hunger and diet selection. In two lab experiments with artificial diets, we found that larvae deprived of either sucrose or protein later selectively fed on a diet providing the missing nutrient. When allowed to freely move between two adjacent food patches, larvae surprisingly preferred to settle on one patch containing yeast and ignored the patch providing sucrose. Moreover, when allowed to move freely between three patches, which provided either yeast only, sucrose only or a balanced mixture of yeast and sucrose, the majority of larvae settled on the yeast-plus-sucrose patch and about one third chose to feed on the yeast only food. While protein (yeast) is essential for development, we also quantified larval success on diets with or without sucrose and show that larvae develop faster on diets containing sucrose. Our data suggest that fruit fly larvae can quickly assess major nutrients in food and seek a diet providing a missing nutrient. The larvae, however, probably prefer to quickly dig into a single food substrate for enhanced protection over achieving an optimal diet.

Keywords *Drosophila melanogaster* · Foraging · Fruit fly larvae · Nutrition · Specific hunger

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Introduction

The nutritional composition of animals' food strongly affect their well-being, survival and reproduction, and accordingly most species appear to seek the mixture of foods that maximises fitness (reviewed in Simpson and Raubenheimer 2012). The behavioural, physiological and neurobiological mechanisms that underlie food selection have been studied for a long time in a wide variety of animal species including rats (*Rattus norvegicus*), mink (*Mustela vison*), locusts (*Locusta migratoria* and *Schistocerca gregaria*), moth caterpillars (e.g. *Heliothis zea*), predatory beetles (*Agonum dorsale*) and spiders (*Pardosa prativaga*) (Waldbauer and Friedmann 1991; Simpson and Raubenheimer 2000; Koehnle et al. 2003; Mayntz et al. 2005, 2009). There are still, however, fundamental questions about the abilities of animals, including humans, to actively seek food types that optimise the proportion and amounts of nutrients in their diet (Power and Schulkin 2009; Galef 1991). These uncertainties probably reflect the enormous complexity involved in nutritional selection and regulation.

Among the variety of species used for research on mechanisms of nutrient selection, one of the most promising animal models is the fruit fly (*Drosophila melanogaster*) owing to the abundance of protocols available for studying its neurogenetics, behaviour, ecology and evolution. Indeed, a recent research effort has been devoted to examining mechanisms of nutritional regulation in adult fruit flies (Amrein and Thorne 2005; Burke and Waddell 2011; Fujita and Tanimura 2011; Ja et al. 2007; Lee et al. 2008; Stafford et al. 2012).

Although the majority of recent research has been devoted to adult fruit flies, the larval stage could potentially be a more powerful model system for a long-term study of the mechanisms and ecological factors that determine nutritional regulation (Scherer et al. 2003; Heisenberg et al. 1985). First, larvae show dramatic, rapid increase in body mass, which strongly depends on the nutritional composition of their food (Sang 1956, 1978). Second, larvae possess only a few thousands functional neurons, which make them a much simpler model system than the adults with approximately 100000 neurons (Iyengar et al. 2006; Huser et al. 2012). Third, in spite of their relative simplicity, the larvae are sensitive to the concentrations of major food components including simple sugars, yeast and sodium (Dukas 1999; Niewalda et al. 2008; Neuser et al. 2005; Durisko and Dukas 2013).

We initiated our work on behavioural mechanisms of nutritional regulation in fruit fly larvae by addressing a few fundamental questions. First, we asked whether larvae fed on diets deficient in a given macronutrient would later seek food rich in that macronutrient, a phenomenon known as 'self-selection' or 'specific hunger' (Waldbauer and Friedmann 1991). Second, having found such selective larval preference for a macronutrient previously deficient in their diet, we quantified the natural tendency of larvae to alternate between distinct food patches that together provided a balanced diet. Third, because, contrary to our expectation, the larvae showed little tendency to alternate between the food patches, we quantified the dynamics of larval choice among three food patches, one providing a balanced diet and the other two being deficient in either sucrose or protein. Finally, to verify that larvae indeed require simple carbohydrates for optimal development, we quantified three standard measures of larval success: survival rate, developmental rate and adult body mass when reared on either a balanced diet or a diet lacking sucrose.

Material and methods

General methods

In all experiments we used *D. melanogaster Canton S* maintained on standard food (20 g/L agar, 75 g/L cornmeal, 32 g/L yeast, 90 g/L sucrose and 2 g/L methylparaben) at 25 °C, 60 % relative humidity and on a 12:12 light/dark cycle with lights on at 1 AM to simplify egg collection. Peak egg-laying thus occurred midday so that we could collect experimental eggs within a very short time window of about 1 h. We collected eggs from 85 mm Petri dishes filled with standard food and covered with 0.7 mL of live-yeast suspension (30 g/L of warm water) to stimulate egg-laying (Sarin and Dukas 2009). Immediately following egg-laying, we transferred these dishes into an incubation chamber maintained at 25 °C and high humidity. All further manipulations were conducted under red light to minimise disturbance.

Experiment 1: larval response to nutrient deficiency

In Experiment 1A, we tested for larval specific hunger for either sucrose or protein. Because protein is essential for larval growth and simple carbohydrates such as fructose or sucrose enhance larval growth rate (Sang 1956), we predicted that larvae deprived of either protein or sucrose would later prefer a diet providing the previously missing nutrient. We reared larvae from eggs until early third instar (3 days after egg laying) on abundant standard food and then, transferred them either to a protein diet or sucrose diet. The sucrose diet consisted of agar mixed with 1 % sucrose (10 g/L) content. The protein diet consisted of an agar mixed with 5 % pure casein (50 g/L), which is a sufficient protein source used in artificial fruit fly diet (Sang 1956). The sucrose and protein concentrations were based on the defined diet developed by Sang (1956), which maximised larval growth rate. With a fine moist paintbrush we placed larvae in small Petri dishes containing either a protein or sucrose diet and left them to eat freely for 6 h, after which we immediately tested their food preference. We rinsed any remaining food from the larvae in a droplet of clean water and then transferred groups of 25 larvae each, previously fed on protein (N=20) or sucrose (N=19), to the edge of an agar Petri dish 3 cm away from two 2.5 mL discs of food (1.0 cm diameter, 0.6 cm thick, 0.3 cm apart). One disc consisted of casein food and the other of sucrose food. We alternated the sides of food discs between replicates to control for any bias. We left the larvae to forage freely for 15 min, after which we counted the number of larvae on each food disc. Larvae that did not reach either of the food discs were excluded from the analysis. We excluded 36±15 % larvae previously fed on casein and 44±19 % previously fed on sucrose. We compared the arcsine square root transformed proportion of larvae on sucrose food (chosen arbitrarily) with a two-sample t-test. We also used a one-sample t-test for each group to test for differences from chance for the arcsine square root transformed proportion of larvae's diet choice.

In nature, the typical source of protein for fruit fly larvae is yeast, which naturally contains carbohydrates (Lee et al. 2008). Thus in Experiment 1B, we repeated the latter experiment to test whether larvae feeding on yeast show specific hunger for sucrose. For each replica, we placed 25 larvae on diets of agar mixed with either 5 % yeast-only (N=19) or 1 % sucrose-only food (N=18) and later tested their food preference as detailed above.

Experiment 2: dynamics of feeding on complementary diets

In well-studied animal models such as locusts (*L. migratoria*) and adult fruit flies, individuals presented with two

complementary diets are adept at consuming the optimal proportions of each diet so that they maximise relevant fitness measures such as growth and reproduction (Simpson and Raubenheimer 2000, 2012; Lee et al. 2008; Fanson et al. 2012). Because the fruit fly larvae in Experiment 1 preferentially fed on the diet providing the nutrient missing in their previous food, we examined their ability to optimally alternate between two foods that together provided protein and simple sugars.

First, in Experiment 2A, we monitored the foraging choices and rate of switching between a 5 % yeast-only and a 1 % sucrose-only (10 g/L) food disc. We transferred a single third instar larva from standard food to an agar dish containing the two different food discs. The gap between discs was 0.3 cm, and one disc contained only yeast and the other only sucrose. In half of the replicates, we placed the larva on the yeast disc and in the other half on the sucrose disc. Afterwards, we recorded the location of each larva every 5 min for two 90-min sessions, separated by 2 h. That is, we recorded larval foraging 0–90 min following placement on the food discs and 210–300 min following placement. We did this in order to compare the rate of larval movement between discs in the time immediately following and a few hours following placement on an unbalanced food. We omitted six larvae that were not on either food disc for one whole observation session, and this left us with 19 larvae initially placed on yeast and 15 larvae initially placed on sucrose. We used a generalized estimating equation (GEE) with Poisson distribution and log link function to assess the frequency of switches between the yeast and sucrose food discs in the first and second observation sessions.

Experiment 2A suggested that fruit fly larvae prefer to settle at one patch rather than alternate between patches that together would provide a more balanced diet. To further illuminate the larval diet choices, in Experiment 2B, we let larvae forage among three food discs, one offering sucrose-only, another yeast-only and one with a rather balanced diet of both sucrose and yeast. As in Experiment 2A, we transferred an individual early third instar larva from standard food to an agar dish with three different food discs containing 5 % yeast-only, 1 % sucrose-only and yeast+sucrose together. In all 40 replicates, we placed each larva equidistant from the three food discs, which formed a triangle with 0.3 cm gaps between discs. We omitted one larva that was not on any food disc for one whole hour of observation, and we randomly chose the order of the three food types within the triangle. As before, we recorded the location of each larva every 5 min for a 90-min session. After 90 min, we recorded the location of each larva every 30 min for another 150 min. We analysed the number of larvae on the agar and at each food patch over time using a GEE with a multinomial distribution and cumulative logit link function. The analysis of switches was based on three observations per every 4 h. For this analysis, we used a GEE with a Poisson distribution and log link function.

Experiment 3: effects of sucrose on larval success

In Experiment 1, we documented specific hunger for sucrose. Experiments 2A and 2B, however, suggested that larvae are willing to settle on food with no sucrose even though they can either readily switch to a nearby food containing sucrose (Experiment 2A) or settle on food containing both yeast+sucrose (Experiment 2B). While Experiment 3 was consistent with Sang's (1956) report that simple carbohydrates increase larval growth rate, Experiments 2A and 2B suggested that perhaps larvae are unwilling to leave their current patch for additional carbohydrates. We thus intended to investigate the adaptive value of sucrose to larvae by comparing larval success when fed on either standard food (General methods) with or without 1 % sucrose. We assessed three measures: larval developmental rate from egg to pupation, survival rate from egg to adult and adult dry body mass. We placed 20 freshly laid eggs each into vials containing either 10 mL of standard food (N=13) or 10 mL standard food without sucrose (N=13). To quantify larval development rate, we counted the number of pupae twice per day (11 a.m. and 5 p.m.) starting 90 h after egg laying until all larvae began pupation. We analysed differences in larval developmental rates with Kaplan–Meier survival curves with Mantel–Cox log-rank Chi-square tests. We assessed differences in egg-to-adult survival rate with an independent samples t-test. After eclosion, we sexed the adult flies and dried them for 3 days at 70 °C. We measured the dry adult body mass in groups of five flies on a microbalance and compared the weights from flies of the different nutrition treatments with an ANOVA.

Results

Experiment 1: larval response to nutrient deficiency

In both Experiments 1A and 1B, larvae showed specific hunger as indicated by their preference for the diet containing the nutrient of which they had previously been deprived. In Experiment 1A, a larger percentage of larvae that had fed on casein chose the sucrose (mean (M)=68, standard deviation (SD)=0.18) diet than larvae that had fed on sucrose (M=14, SD=0.11; t_{37}=11.48, P<0.001; Fig. 1a). Furthermore the choice of diet was significantly different from chance when larvae previously fed on either casein (t_{19}=4.166, P=0.001; Fig. 1a) or sucrose (t_{18}=9.911, P<0.001; Fig. 1a). Similarly, in Experiment 1B, a larger percentage of larvae that had fed on yeast diet later preferred the sucrose diet (M=67, SD=0.23) than larvae that had fed on sucrose (M=32, SD=0.24; t_{35}=3.37, P=0.002; Fig. 1b) and the choice of diet was significantly different from chance when larvae previously fed on either yeast (t_{18}=2.994, P=0.008; Fig. 1b) or sucrose (t_{17}=3.054, P=0.007; Fig. 1b).

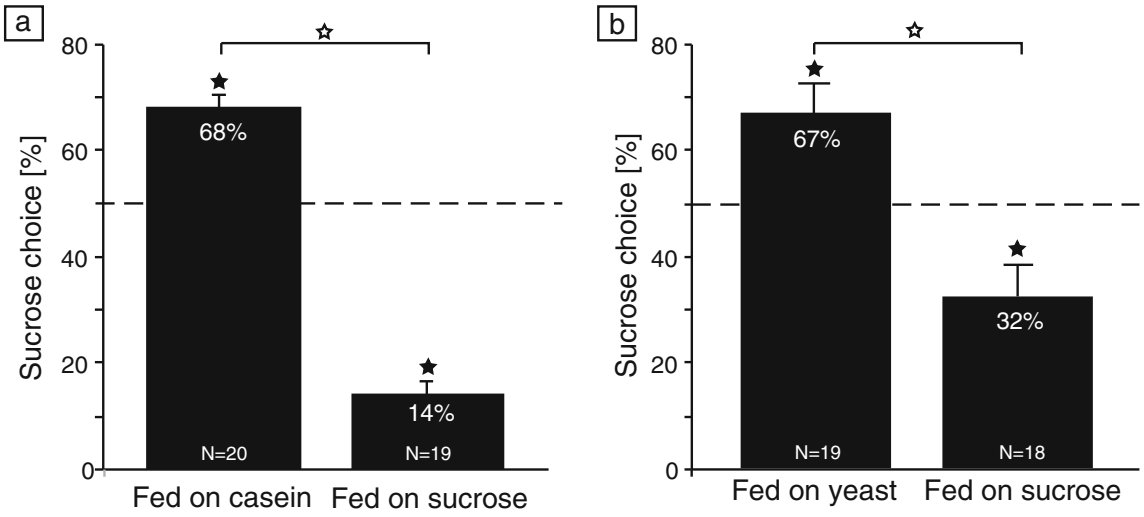

Fig. 1 Experiment 1: larval (*Drosophila melanogaster*) response to nutrient deficiency. After a 6-h deprivation on **a** either casein (sucrose-deprived) or sucrose (protein-deprived) and **b** either yeast (sucrose-deprived) or sucrose (protein-deprived), larvae showed specific hunger and a significant preference for the nutrient they had been deprived of. *Black stars* indicate significant difference between conditions of deprivation (two-sample *t*-tests, $Ps<0.002$), *white stars* indicate significant differences of each group against random choice (one-sample *t*-tests, $Ps<0.008$), *N* sample size and *dotted line* indicates the 50 % threshold

Experiment 2: dynamics of feeding on complementary diets

In Experiment 2A, most larvae (94 % placed on sucrose and 88 % placed on yeast) left their initial food disc and explored, during which 68 % of larvae switched between food discs at an average rate of about 1 per 90 min regardless of the larval initial placement, most larvae settled on the yeast patch (Fig. 2a). In the second 90 min observation period (minutes 210–300), only 9 % of the larvae switched between patches and the average rate of switching declined to only about 0.25 per 90 min (GEE, Wald $\chi^2_1=5$, $P=0.026$ for the effect of time period on switching rate; $\chi^2_1=0.04$, $P=0.842$ for the effect of initial placement; Fig. 2b).

In Experiment 2B, while most larvae started by exploring their new settings, almost half (44 %) settled on one patch, which they did not leave for the remainder of the observation period. The number of larvae occupying the yeast+sucrose and yeast-only disc increased over time, whereas the number of larvae occupying the agar and sucrose-only food patch decreased (GEE, Wald $\chi^2_{19}=3653$, $P<0.001$; Fig. 3a). Although no larva settled on the sucrose patch, a sizable proportion (about 30 %) remained permanently on the yeast patch. Interestingly, 78 % of the larvae that settled on the yeast disc had previously been on the yeast+sucrose patch. Overall, the rate of larval switching between patches showed a nearly significant decrease over time (GEE, Wald $\chi^2_3=7.1$, $P=0.068$; Fig. 3b). After the first hour, most larvae did not switch patches. Only 5 % of the larvae alternated between patches in the second and third hour, and only 2.5 % did so in the fourth hour.

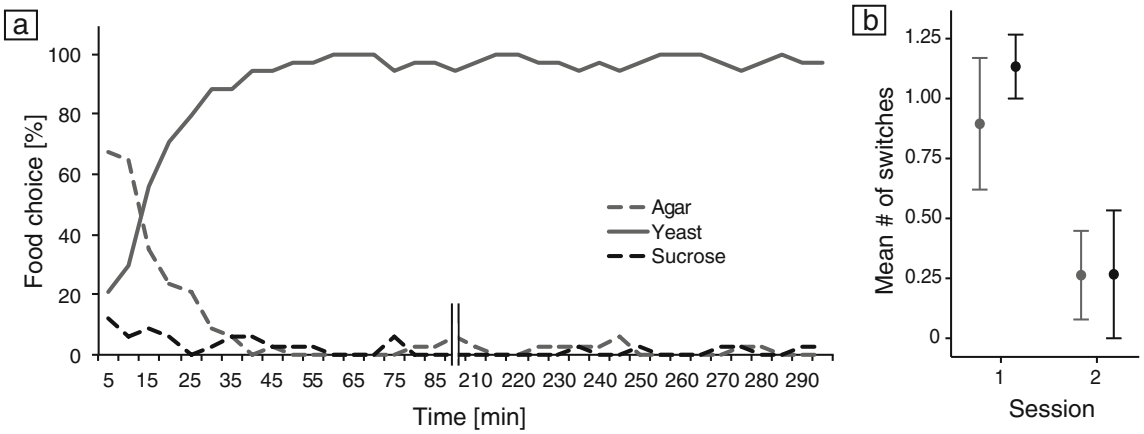

Fig. 2 Experiment 2: dynamics of feeding on complementary diets. **a** The proportion of larvae (*Drosophila melanogaster*) occupying each food patch. *Double slash* indicates 2-h observation break. **b** Larval average number of switches between the sucrose and yeast patches during the initial 90 min (session 1) and during the final 90 min (session 2) as a function of their initial placement on either the sucrose (*grey bars*) or yeast (*black bars*) patches

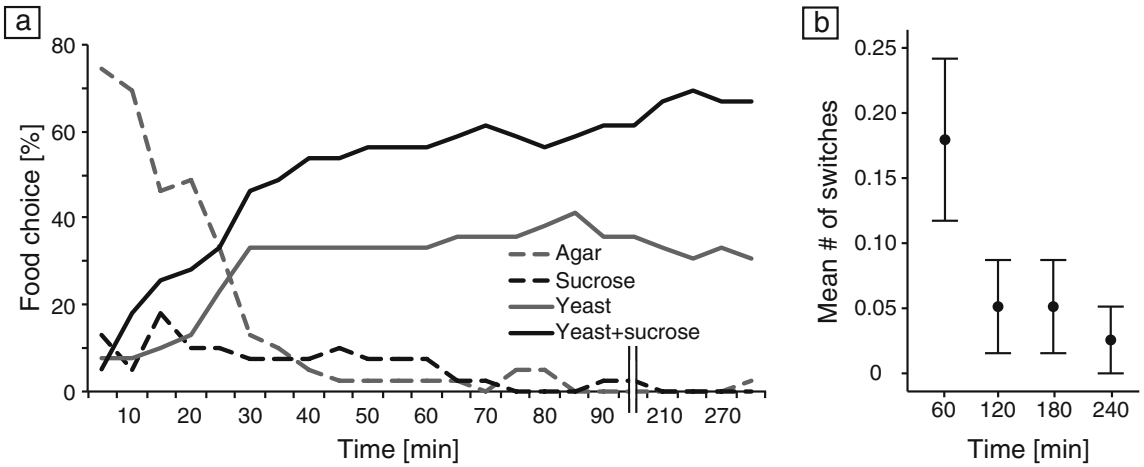

Fig. 3 Experiment 2: dynamics of feeding on complementary diets. **a** The proportion of larvae (*Drosophila melanogaster*) observed away from food (agar) and at food discs containing sucrose, yeast or yeast+sucrose. *Double slash* indicates switch from 5- to 30-min observation intervals. **b** Larval average number of switches between patches in each of four successive hours

Experiment 3: effects of sucrose on larval success

Larvae that fed on standard food with sucrose developed faster than larvae that fed on standard food with no sucrose (Mantel–Cox log-ranked chi square, χ^2=13.1, P<0.001; Fig. 4a). After the third counting (148 h after egg laying), most larvae on standard food reached pupation (54±6 %), whereas only 14±4 % of larvae without sucrose reached this stage (Fig. 4a). Survival rate was similar between the diet treatments (t_{24}=0.179, P=0.859) with a mean mortality of 29±20 % in the standard food group and 27±24 % in the standard food with no sucrose. Surprisingly, larvae that fed on standard food without sucrose had higher average adult body mass than those that fed on standard food with sucrose ($F_{1,69}$=442.16, P<0.001; Fig. 4b). Females weighed significantly more than males ($F_{1,69}$=32.22, P<0.001; Fig. 4b), and there was no significant sex by diet interaction ($F_{1,69}$=1.69, P=0.198).

Discussion

In two experiments, we found that fruit fly larvae deprived of either simple carbohydrates or protein later preferentially fed on the food providing the missing macronutrient (Fig. 1). Experimental protocols to study nutritional intake are already well established for the adult stage (e.g. the CAFE assay; Ja et al. 2007; Fanson et al. 2012); however, the larvae possess a nervous system comprising only a few thousands functional neurons. Given this relative simplicity of the larval brain coupled with the amenability of larvae for neurogenetic

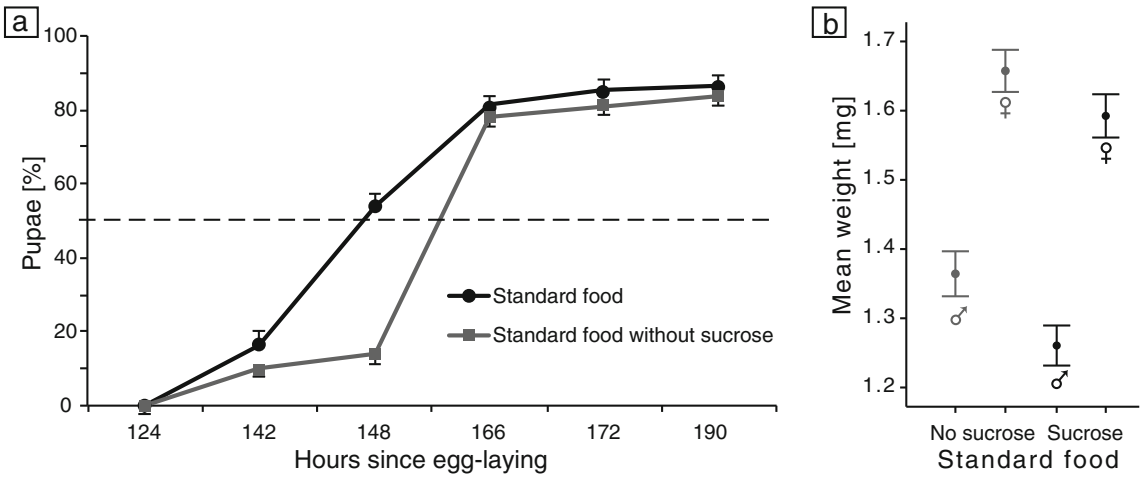

Fig. 4 Experiment 3: effects of sucrose on larval success. **a** Developmental rate of fruit fly larvae (*Drosophila melanogaster*) reared on different diets. *Dotted line* represents the 50 % threshold. Larvae reared on standard food (*black line*) developed faster than larvae reared on standard food without sucrose (*grey line*). **b** Mean adult dry body mass from larvae reared on standard food without sucrose (*grey bars*) and standard food (*black bars*). See text for statistical details

analyses, finding specific hunger at the larval stage positions fruit fly larvae as a promising model system for further research on the mechanisms and evolutionary ecology of nutrient regulation. While assays quantifying precise nutritional intake are probably more difficult in the larvae than in the adults, there do exist established protocols in which the amount of ingested food is estimated by the consumption of coloured dye, the number of bites and mouth hook contractions (Wu et al. 2003, 2005). Furthermore, and similar to adult fruit flies, it is cheap and easy to rear numerous larvae, but larvae possess the obvious advantage of developing faster than adults. Finally, there is detailed information on larval nutritional needs (Sang 1956, 1978), which one can rely on for modern nutritional research.

Based on our results indicating specific hunger in fruit fly larvae and studies in other insects (Chambers et al. 1995; Cohen et al. 1987), we expected that larvae provided with distinct protein and sucrose foods would alternate between them in order to obtain a balanced diet. Contrary to our prediction, however, the larvae preferred to settle on the yeast food in Experiment 2A (Fig. 2a) and a fair percentage of them (about 30 %) settled on a yeast food even when yeast+sucrose food was available within 3 mm in Experiment 2B (Fig. 3a). Because these behavioural data somewhat conflicted with the results of Experiment 1B, we verified in Experiment 3 that larvae achieve a higher growth rate when their diet contains sucrose (Sang 1956) in addition to the complex carbohydrate available in cornmeal and yeast. We replicated Sang's (1956) classic results (Fig. 4a), but our other relevant measure for larval success, adult body mass, which was not quantified by Sang (1956), surprisingly indicated a lower body mass with than without sucrose (Fig. 4b). While growth rate can strongly affect fitness, especially in growing populations (Charlesworth 1994), adult body mass is also important because reproductive success is positively correlated with adult body mass in both male and female fruit flies (Lefranc and Bundgaard 2000; Partridge et al. 1987; Partridge and Farquhar 1983). However, the temporal advantage of the higher growth rate might outweigh the increased body mass when reared on yeast-only food. Furthermore, the total caloric content in the yeast-only food is lower than in the food with additional sucrose. It is possible that the larvae compensated for the lacking calories by ingesting more of the yeast-only food and thus became heavier as adult flies. On the other hand, the increased body mass based on the yeast-only diet and the associated overconsumption of protein and other nutrients in yeast might have other deleterious effects, which may be quantified in future research (Metcalfe and Monaghan 2001; Lee et al. 2008; Waldbauer and Friedmann 1991).

Why did larvae not show a stronger preference for additional sucrose in Experiment 2A (Fig. 2), and why were they willing to settle on yeast-only instead of yeast+sucrose in Experiment 2B (Fig. 3)? One possibility suggested by the results of Experiment 3 (Fig. 4) is that sucrose has only a limited value for the larvae owing to trade-offs between the rate of development and body mass. It is also possible that larvae chose the yeast-only food due to their particular stage of development. In the Tephritid fruit fly (*Ceratitis capitata*), younger larvae (2 days old) prefer protein food to facilitate development, whereas older larvae (6 days old) tend to choose carbohydrates as energy for the movements during wandering state for pupation (Zucoloto 1987). Another aspect could be due to sex differences in the nutritional intake in larvae. For instance, female larvae might have chosen the yeast-only food in order to gain a higher adult body mass, whereas males might have chosen the yeast+sucrose food to increase their developmental rate (Fig. 4). Different nutritional composition during larval development in Hymenoptera (Gadagkar et al. 2008), Diptera (House 1967) and Lepidoptera (Delisle and Bouchard 1995; Delisle and Hardy 2003) have been shown to be involved in the reproductive success of the subsequent adult life stage for both sexes. The influence of sex differences in food balancing in fruit fly larvae is an interesting topic for future studies. We should note, however, that the closely studied larval locust (*L. migratoria*) shows little difference in nutrient utilisation except that the larger females eat more than males (Simpson 1982). Furthermore, our observations suggest that fruit fly larvae prefer to dig into their food substrate, perhaps in order to reduce exposure to natural enemies such as parasitoids and to optimise abiotic factors including high humidity and darkness. This may explain larval tendencies to settle on the yeast food in Experiment 2A. That is, the larvae may have placed a higher value on digging into the food than on consuming sucrose. This explanation is consistent with a study of grasshopper nymphs (*Schistocerca americana*), which documented decreased tendencies of individuals to balance their diets when the distinct foods were farther away from each other. In both—fruit fly and grasshopper nymphs—there may be a trade-off between the risk of predation and the quality of the current food substrate (Bernays et al. 1997).

The results of Experiment 2B are somewhat more difficult to interpret. While a significant majority of larvae (about 70 %) chose the balanced diet containing yeast+sucrose, a fair proportion (about 30 %) settled on the yeast-only food (Fig. 3a) even though most of the larvae settling on the yeast-only food (78 %) had previously been on the yeast+sucrose food. We suspect that this reflects poor decision making by a minority of the larvae. It is possible that, given the relative short duration of the larval stage and the probable advantage of digging into the substrate, larvae prefer to settle quickly into continuous feeding rather than engage in an extended search for the best food when the present food is above some threshold quality. We expect such behaviour to vary in time and space as function of competition and the perceived danger from desiccation and parasitoids. Another likely source of variation could have a genetic basis. For example, the well

studied, naturally occurring fruit fly morphs, rovers and sitters (e.g. Osborne et al. 1997), might differ in response to moving between nearby food sources.

To summarise, we have documented specific hunger for both protein and sucrose in fruit fly larvae, which are a promising model system for further work on the mechanisms underlying nutrient regulation and hunger. Larvae valued protein in their diets very highly, but a fair proportion of them settled on a patch even when it lacked sucrose and resulted in a lower growth rate. While we focused here on key macronutrients, future work can assess larval abilities to regulate other essential nutrients and the mechanisms underlying such abilities.

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