Gene-environment interactions at the foraging locus of Drosophila

Simon Benmaamar[1], Björn Brembs[1]

[1] Institute of Zoology - Neurogenetics, University of Regensburg, Regensburg, Germany

Correspondence: simon-benmaamar@gmx.de

QUESTION

How forR and forS genotypes in Drosophila respond to changes in the environment?

ABSTRACT

Environmental variability during the development of an organism has known impacts on the expression of certain behavioural patterns. We used the fruit fly Drosophila melanogaster to investigate how different environmental conditions interact with the allelic variants of rover (for^R) and sitter (for^S) at the foraging locus to affect food-related behaviour of larvae. We discovered that larval density and nutrient availability were key environmental factors affecting the larval behaviour during early development. High larval density decreased the tendency of rovers to leave a food patch and reduced their travelled path lengths, such that rovers and sitters showed no more significant differences regarding their behaviour. Similar results were obtained when starving the larvae. Furthermore, cutting the availability only of specific nutrients such as sugar, fat or protein during development all affected larval foraging behaviour and locomotion.

Keywords: animal behaviour, animal tracking, food, gene environment interaction, animal density

INTRODUCTION

For a deeper understanding of the relationship between genes and behaviour, it is essential to be aware of the fact that genetic mechanisms controlling behaviour can be modified by environmental experiences [1]. The concept of genotype-specific phenotypic variation as a response to a changing environment is known as gene-environment interaction [2]. Phenotypic plasticity can be described as the extent of influence to which the phenotype can be affected or modified by the environment [3]. Many different genes are involved in establishing a certain behavioural phenotype [4], with some genes acting as master regulators (e.g. transcription factors) within these gene pathways [5]. An example for one of these master regulators of behaviour is the *foraging* (*for*) gene, as it is known for having an important role as modifier of behaviour. For example, allelic variants of the *for* gene modulate foraging behaviour in *Drosophila melanogaster*. Being conserved in different species with similar functions [6] makes the gene interesting for further research.

The *for* gene in *Drosophila* is an example of an allelic variation in a single gene contributing to behavioural plasticity. The fly gene encodes a cGMP-dependent protein kinase (PKG) [7] and natural variation of two alleles in *for* affects larval foraging behaviour. Larvae with the wild-type rover (for^R) allele cover longer distances while foraging and do not stay as long on the same food patch compared to larvae homozygous for the sitter (for^s) allele [8,9]. Furthermore, rovers are characterized by a higher PKG enzyme activity compared to sitters in an environment rich in nutrients which results in a lower food intake and fat storage than sitter larvae. However, in nutrient-poor environments, PKG levels in both variants drop to a common level. In addition, rover and sitter differ in terms of glucose absorption with rovers having a higher glucose absorption in comparison to sitters [10]. This plasticity regarding larval foraging behaviour (travelled distances, food intake) and fat levels is regulated by the gene dosage of *for* [11].

This work uses the rover/sitter polymorphism of the *for* gene in *Drosophila* to investigate which environmental changes can affect food-related larval behaviour. The aim of this work is to study how these both genotypes (for^R and for^s) would respond to changes in their environment. Environmental variation was achieved by raising the larvae under different conditions in terms of larval density or nutrient availability.

MATERIALS AND METHODS

Fly stocks and maintenance

The rover and sitter strains used for the experiments are homozygous for the for^R and for^s alleles, respectively, and isogenic for the second and third chromosomes [9,12]. Flies were maintained in plastic vials with food at 25°C and 60% humidity on a 12h/12h light/dark cycle. To start the experiments, about 15 female flies were allowed to lay eggs for one day and then transferred into new vials. Newly hatched offspring were transferred into new vials and after 3-4 days these flies were moved to the experimental and standard vials and allowed to lay eggs for 24h. All newly hatched flies from the control groups were always transferred into new vials and again stored for 3-4 days before they were transferred for further experiments in order to have a continuous cycle (Fig. 1).

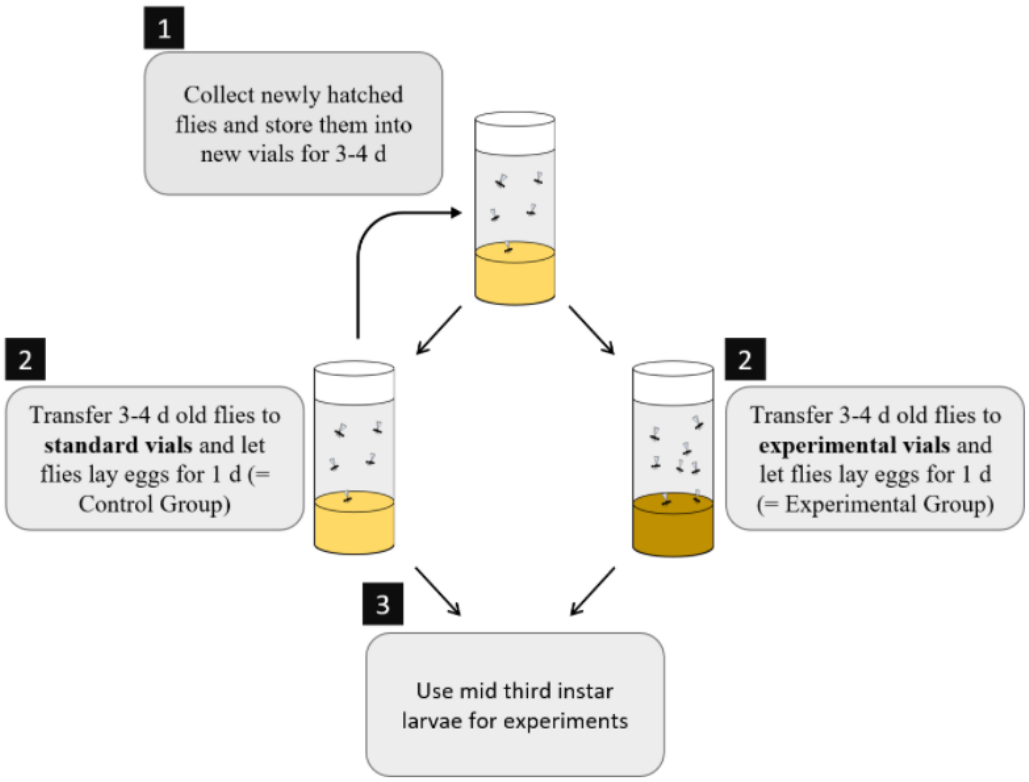

Figure 1: Experimental design - 3-4 days old flies were allowed to lay eggs for one day and then removed. Standard vials contained a normal density of flies (about 15 females) and standard fly food. Experimental vials included different experimental treatments (high/low density, food manipulations). Mid third instar larvae were tested in the experiments. Newly hatched flies of the control groups were transferred into new vials for further experiments.

Fly food and agar plates

Food preparation

Standard fly food and food modified in nutritional composition

Using an induction cooker, 1 l of water was brought to a boil. Then, 17 g of agar-agar dissolved in 350 ml of water was added and also brought to boil. Diastatic malt extract and sugar beet syrup were added to the agar-water solution. Next, cornmeal, brewer's yeast and soy meal dissolved in 350 ml water were added. The mash was boiled for about 10 minutes while stirring continuously. Finally, depending on the mash consistency up to 100 ml of water were added and after a short cooling period, 6 g Nipagin were dissolved in the mixture. Vials were then filled with 40ml of fly food and stored at 4°C. A dab of living yeast paste and a filter paper were added to every vial

except to the food without yeast. About 20 vials could be obtained following each fly food recipe (Table 1).

Table 1: Ingredient list for standard fly food and nutrient manipulation food.

	Corn-meal	Brewer's yeast	Soy meal	Diastatic barley malt extract	Sugar beet syrup
Standard	160 g	36 g	20 g	160 g	44 g
Yeast (0)	160 g	-	20 g	160 g	44 g
Sugar (0,25)	160 g	52,3 g	20 g	97,5 g	-
Carbohydrates (0,5)	50 g	68,8 g	20 g	110,3 g	52,4 g
Fat (0,5)	160 g	45 g	-	103 g	52,5 g

Starch food

On the induction cooker, 560 ml water was brought to boil. Then, 15 g agar-agar dissolved in 200 ml cold water were added to the boiling water while stirring continuously. After that, yeast and cornflour (Mondamin) dissolved in 200 ml cold water were put into the agar-water solution and boiled for about 10 minutes. After a cooling down (food temperature of 75°C), Nipagin dissolved in 40 ml water was added. Vials were then filled with 40ml of fly food and stored in the cooling chamber. A dab of living yeast paste and a filter paper were added to every vial. About 10 vials with starch food could be obtained following this recipe (Table 2).

Table 2: Starch food ingredient list.

	Cornflour	Brewer's yeast	Agar - Agar	Nipagin
Starch food	30 g	50 g	15 g	5 g

Nutrient distribution

The carbohydrate food manipulation contained about half the amount of non-sugar carbohydrates compared to the standard food. Yeast manipulation food contained no yeast. In the fat manipulation food the amount of fats was reduced to about half. Sugar food manipulation contained about a quarter of the amount of sugar in the standard

food. Starch food contained nearly no sugar and a low amount of nutrients in general. We used the nutrient information of the fly food ingredients (Table 3) to calculate the nutrient distribution in our various fly food manipulations (Fig. 2).

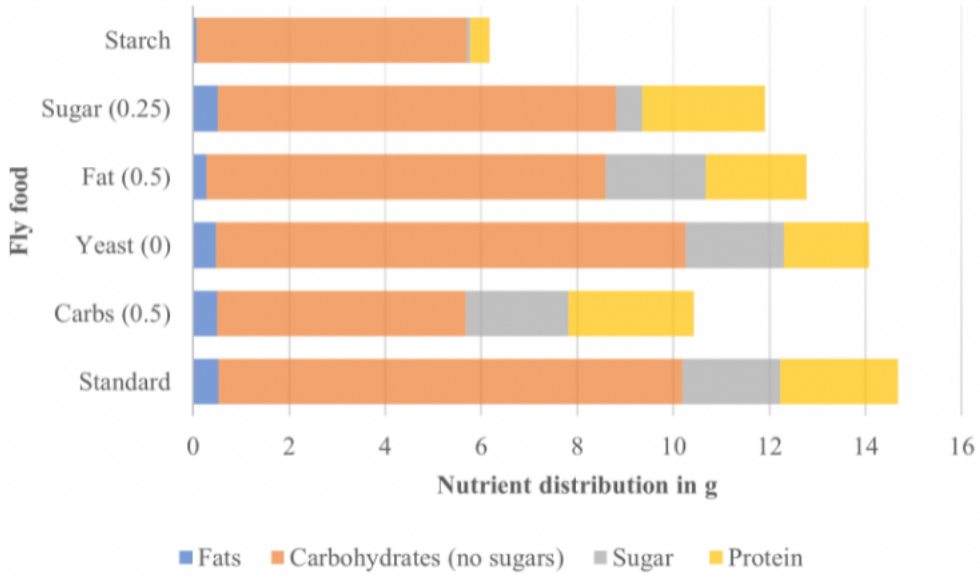

Figure 2: Nutrient distribution in 100 g of each fly food - Number in brackets after the nutritional information of the fly food indicates the ratio in comparison to the standard food.

Table 3: Nutrient distribution in 100 g of the products used for the fly food: Nutritional information were taken directly from the products used for cooking the fly food.

	Corn meal	Brewer's yeast	Soy-meal	Diastatic barley malt extract	Sugar beet syrup	Cornflour (Mondamin)
Fats	1,1g	3,7 g	24,5 g	2,5 g	-	0,5 g
Carbohydrates (no sugars)	72,3 g	5,9 g	10,2 g	59,4 g	4 g	86 g
Sugar	1,5 g	0,2 g	5,3 g	8 g	66 g	-
Protein	8,8 g	44,8 g	39,6 g	10,1 g	2,3 g	0,5 g

Agar plates

Table 4: Ingredient list for about 20 standard or black dyed agar plates.

	Agar-Agar	Water	Activated charcoal powder
standard	24,5 g	700 ml	-
black	24,5 g	700 ml	1,4 g

Starvation protocol

The method for the starvation of the larvae was taken from [13]. A maximum of 30 mid third instar larvae that have previously been removed from the standard food and washed, were placed in a petri dish (Ø 6 cm) containing either a piece of Kim wipe with 350 µl dH20 (starved) or a piece of Kim wipe with 350 µl of 0.2 M sucrose (sucrose). The larvae were allowed to move freely within the petri dishes for 2 hours at room temperature before they were tested.

Control of larval density

In order to have a standard density of larvae in the food, about 15 female flies, together with a few males in one vial, were allowed to lay eggs for 24h. To have a low larval density about 5 female and a few male flies were placed in a vial for one day. A high larval density was achieved by keeping more than 30 females together with the males in one vial for a day. After one day, the adult flies were removed from the vials and the number of laid eggs was inspected.

Larval experiments

Food patch

To assess rover or sitter-like larval behaviour, a small amount of living yeast paste was added to standard agar plates (92 x 16 mm), creating two food patches (A and B) on the agar, each about 1.5 cm in diameter (Fig. 3). The distance between the yeast patches was about 3 cm. About 10 mid third instar larvae, which had been previously removed from the food and carefully washed using small containers with a mesh on the bottom, were placed on food patch A at the beginning of each experiment. After 20 minutes, the number of larvae that were still in food patch A, the number of larvae in food patch B and the number of larvae not in any food patch (elsewhere) were counted to calculate the percentage of larvae that were still in food patch A and the percentage

of larvae having left the first food patch (= larvae in food patch B or elsewhere). All experiments were carried out at 25°C and 60% relative humidity.

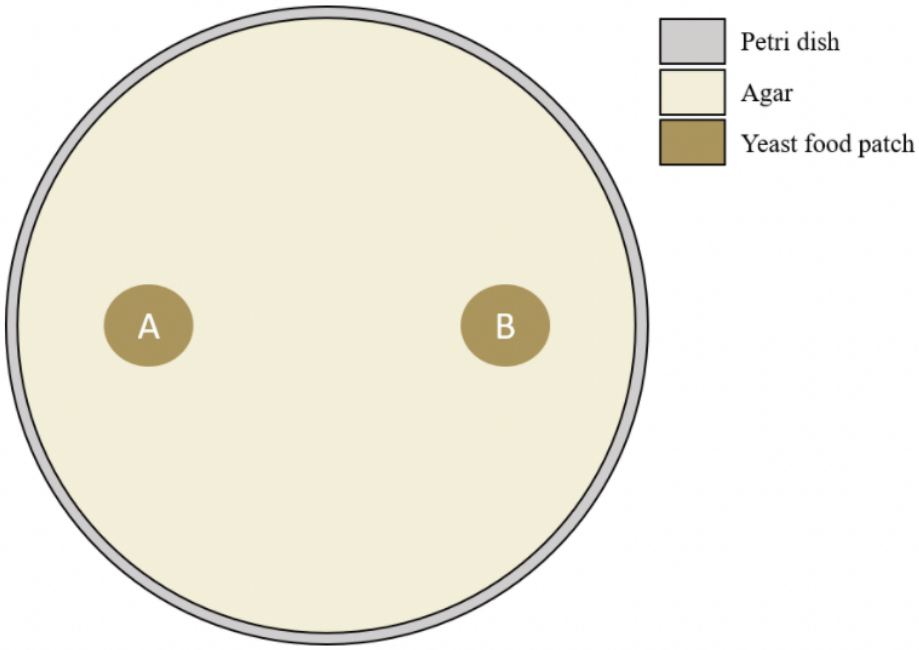

Figure 3: Food patch experiment - Prepared agar plate.

Distance tracking

Experimental setup

To record the locomotion of individual larvae, the setup of the Buridan apparatus was used to maintain an environment with constant temperature and lighting, using four circular fluorescent tubes to illuminate the larvae homogeneously [14]. Black dyed agar plates (see above) were used to increase the contrast to the white larvae. Larvae, removed from the food and washed with water, were carefully placed in the centre of the agar plate. A camera, connected to a computer running the recording software, was positioned above the agar plate in order to record the position of each larva during the experiment (Fig. 4). A one-minute video was recorded immediately after the larva was placed on the agar using OBS Studio (https://obsproject.com) as video recording

software. Larvae that crawled under the agar before one minute had passed were discarded.

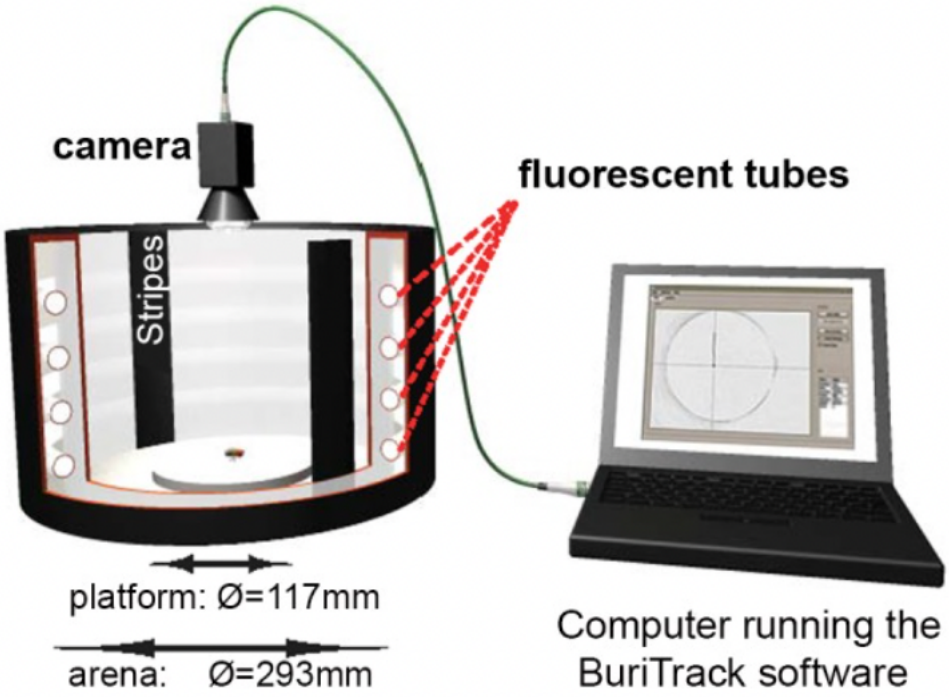

Figure 4: Burridan's paradigm - Setup used to record videos of the crawling larvae. We used OBS Studio software to record the videos.

Kinovea tracking software

The program Kinovea (https://www.kinovea.org) was used to analyze the video files recorded by OBS studio. Kinovea is a video player for sport analysis, but provides different features like the ability of tracking objects [15]. After successfully tracking a larva (Fig. 5), the program generates a text file containing the x and y coordinates (in cm) at each point in time (in ms). For further instructions on how to use the program see DOI: 10.17504/protocols.io.bz3ip8ke.

Figure 5: Path tracking using the Kinovea software - Example of a finished trajectory of a larva moving on black dyed agar for one minute.

Trajectory analysis

A script based on R (version 4.0.5) was used to evaluate the Kinovea data in order to determine the individual travelled distances of each larva. The travelled distance D (in cm) was calculated with the following equation [16]:

Equation 1: Distance equation - x = x-coordinates, y = y-coordinates and z = total number of coordinate pairs.

$$D = \sum_{t=1}^{z-1} \sqrt{(x_t - x_{t+1})^2 + (y_t - y_{t+1})^2}$$

The R-Script shortens the raw data to the desired duration (60 s) before the distance is calculated. The R-script also detects the time at which the larva starts moving (in ms) by determining the point in time after which the first distance is different from zero. The mean distance (in cm) and the mean latency to first movement (in ms) were

calculated for all files within one group. For further information on how to use the script see DOI: 10.17504/protocols.io.bz3ip8ke.

Statistical analysis

Sample size estimation

A two-sample t-test power analysis was carried out prior to the experiments with data from the food patch and the distance tracking experiment of larvae that grew up in standard food with a normal density in order to determine the sample size n (Table 5). Effect sizes were obtained by recording *rover* and *sitter* larvae under standard conditions.

Table 5:Two-sample power analysis.

Experiment	n	delta	sd	sig.level	power	alternative
Food patch	13,23	0,17	0,17	0,05	0,8	one.sided
Distance tracking	18,17	1,38	1,64	0,05	0,8	one.sided

Hence, for the food patch experiments, we used a target sample size of 14 experiments (with about 10 larvae in each experiment), while for experiments where the distance travelled by individual larvae was recorded, the target sample size was 19 individual larvae.

The fairly high [17] alpha value of 5% was chosen both for practical reasons and because the difference between *rover* and *sitter* phenotypes is so highly reproducible such that the sample sizes calculated here are sufficient to detect the effect if it is present. Finally, food patch and trajectory length experiments were chosen assuming they replicate each other, providing additional statistical power.

Testing for statistical significance

Food patch experiment

As the data were not normally distributed (Shapiro-Wilk-Test, $p < 0.05$), we performed R's Wilcoxon tests. To compare the respective variant (rover/sitter) of the control group to the equal variant of the experimental groups, a Kruskal-Wallis-test with post hoc Dunn's test was used in order to look for significant effects of the larval environment.

Distance tracking experiment

A t-test was used to compare the travelled distances of rovers and sitters within one experimental group as the data was normally distributed (Shapiro-Wilk-Test, $p > 0.05$). To look for significant differences in path lengths between control group and experimental groups of each variant (rover/sitter), an Anova with a post hoc Dunnett-test was carried out.

Data availability

All raw data is available at DOI: 10.6084/m9.figshare.17032442.

RESULTS

Larval density manipulation

Previous studies had shown an impact of larval density on the phenotypic expression in *Drosophila*, for example regarding body size [18]. To determine whether larval density could affect the foraging behaviour or the locomotion of *rovers* and *sitters*, mid third instar larvae were tested after growing up under high-density, low-density or standard-density conditions (*Methods*) (Fig. 6). Larvae that grew up under a high larval density reached mid third instar stage about one day later than larvae that grew up under a low or standard density.

Rover and *sitter* larvae showed a significant difference regarding their tendency of staying in the first food patch in the control group with a standard density

(wilcox.exact, W = 32.5, p = 0.0014) as well as in the experimental group with a low density (wilcox.exact, W = 30.5, p = 0.0012) (Fig. 6*A*). In contrast, *rovers* and *sitters* that grew up under a high larval density had a significant higher tendency to stay in their first food patch compared to control larvae (*rovers*: Kruskal-Wallis-test, $F_{(2)} = 21.3$, $p < 0.001$ with post hoc Dunn's test, $p = 0.001$; *sitters*: Kruskal-Wallis-test, $F_{(2)} = 12.8$, $p = 0.002$ with post hoc Dunn's test, $p = 0.041$). As a result, no more difference between *rover* and *sitter* foraging behaviour could be observed in the high density treatment (wilcox.exact, W = 84, p = 0.596).

The evaluation of the larval path lengths revealed similar results as in the food patch experiments (Fig. 6*B*). *Rover* and *sitter* larvae showed a significant difference regarding their trajectory lengths when grown at standard density (t-test, $t_{(34.6)} = 3.14$, 95% CI = [0.45, 2.08], $p = 0.003$) or at low larval density (t-test, $t_{(33.6)} = 5.31$, 95% CI = [1.3, 2.91], $p < 0.0001$). Low-density *rovers* covered a longer distance on the agar within 1 minute compared to the standard density *rovers*, but the difference was not significant (Anova, $F_{(2)} = 8.5$, $p < 0.001$ with post hoc Dunnett test, $p = 0.17$). However, *rover* larvae grown at high density showed significantly lower path lengths when compared to the *rovers* grown at standard density (Anova, $F_{(2)} = 8.5$, $p < 0.001$ with post hoc Dunnett test, $p = 0.04$) resulting in no more significant difference in path lengths between high-density for^R and for^s larvae (t-test, $t_{(35.9)} = 0.76$, 95% CI = [-0.47, 1.03], $p = 0.45$).

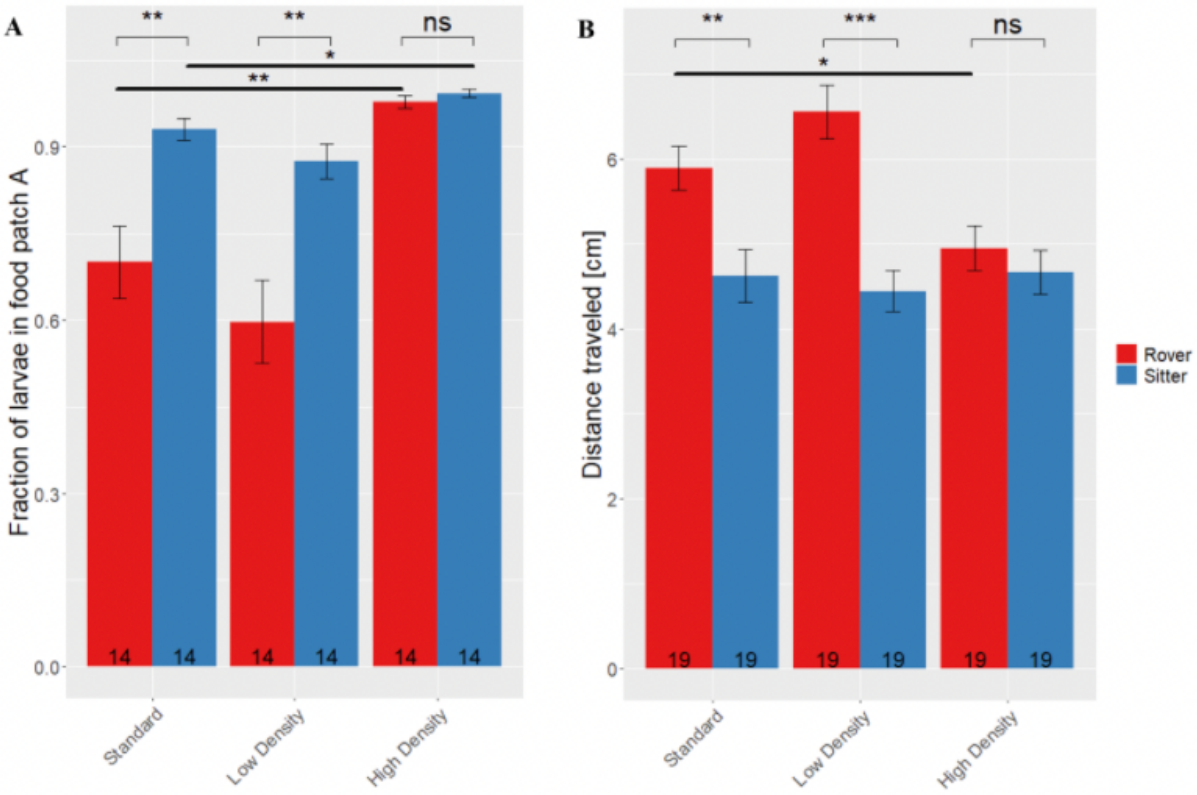

*Figure 6: Behaviour analysis of rover and sitter larvae grown up under different larval densities - (A) Mean percentage of larvae still in food patch A after 20 minutes. Brackets above each experimental group with asterisks indicate whether there is a significant difference between rover and sitter behaviour (Wilcoxon test). Thick lines with asterisks denote significant differences between the rovers/sitters grown at high/low larval density, respectively, in comparison to the rovers/sitters grown at standard density (non-parametric Kruskal-Wallis-test with post hoc Dunn's test for multiple comparisons). (B) Mean distance travelled of larvae moving for one minute on an agar plate. Brackets above each experimental group with asterisks denote a significant difference between rover and sitter path lengths (t-test). Thick lines with asterisks denote significant differences in path lengths between the rovers/sitters grown at high/low larval density, respectively, in comparison to the rovers/sitters grown up under standard density (Anova with post hoc Dunnett test was performed). Error bars denote standard error of the mean (SEM). Number of asterisks denotes p-value (*p < 0.05, **p < 0.01, ***p < 0.001, ns p > 0.05). Numbers within bars denote the number of experiments (n).*

Larval starvation

Increasing larval density may affect nutrient uptake either positively via cooperative feeding or negatively via food competition. To compare nutrient deprivation to standard food conditions, mid third instar *rover* and *sitter* larvae were exposed to an environment either without any nutrients (dH20, starved) or with only sucrose as the nutrient (sucrose) for 2 hours (*Methods*). After that, their tendency to stay in the food patch as well as their trajectory lengths were analysed (Fig. 7). As a control group, larvae that were taken directly out of the food were also tested.

Analysing the foraging behaviour of *rover* and *sitter* larvae (Fig. 7*A*) revealed that both starved and sucrose-only *rovers* had a significantly higher tendency to stay in the first food patch compared to the controls taken directly out of regular fly food (Kruskal-Wallis-test, $F_{(2)} = 26.11$, $p < 0.001$ with post hoc Dunn's test, $p < 0.001$ for both cases). As a result, the significant difference between *rover* and *sitter* behaviour as it was observed in the control group (wilcox.exact, $W = 6$, $p < 0.0001$) could no longer be seen in the starved experimental group (wilcox.exact, $W = 79$, $p = 0.35$), as well as in the experimental group with only sucrose as nutrient (wilcox.exact, $W = 66$, $p = 0.12$). *Sitter* larvae that were restricted in their nutrient availability (starved group and sucrose group) did not differ significantly from *sitter* larvae of the control group.

Similar results were found when looking at the differences in trajectory lengths (Fig. 7 *B*). *Rover* larvae that had been under starved conditions showed significantly shorter trajectory lengths when compared to the control *rover* larvae (Anova, $F_{(2)} = 15.28$, $p < 0.001$ with post hoc Dunnett-test, $p < 0.0001$). Also, *rovers* that were exposed to sucrose as only nutrient covered a significantly shorter distance compared to the controls (Anova, $F_{(2)} = 15.28$, $p < 0.001$ with post hoc Dunnett-test, $p = 0.009$). *Sitters* did not significantly alter their behavior when they had been starved for 2 hours (Anova, $F_{(2)} = 2.93$, $p = 0.06$). The significant difference between covered distances of *rovers* and *sitters* that could be seen in the control group (t-test, $t_{(35.6)} = 2.5$, 95% CI = [0.16, 1.61], $p = 0.017$) could no longer be observed when the larvae had been starved (t-test, $t_{(31.7)} = -0.44$, 95% CI = [-1.1, 0.7], $p = 0.67$) or had sucrose as only nutrient (t-test, $t_{(32.3)} = 1.14$, 95% CI = [-0.36, 1.15], $p = 0.26$). The differences between *rovers* and *sitters* regarding their travelled distances could already be seen at an earlier point in time in the control group (Fig. 7 *C*).

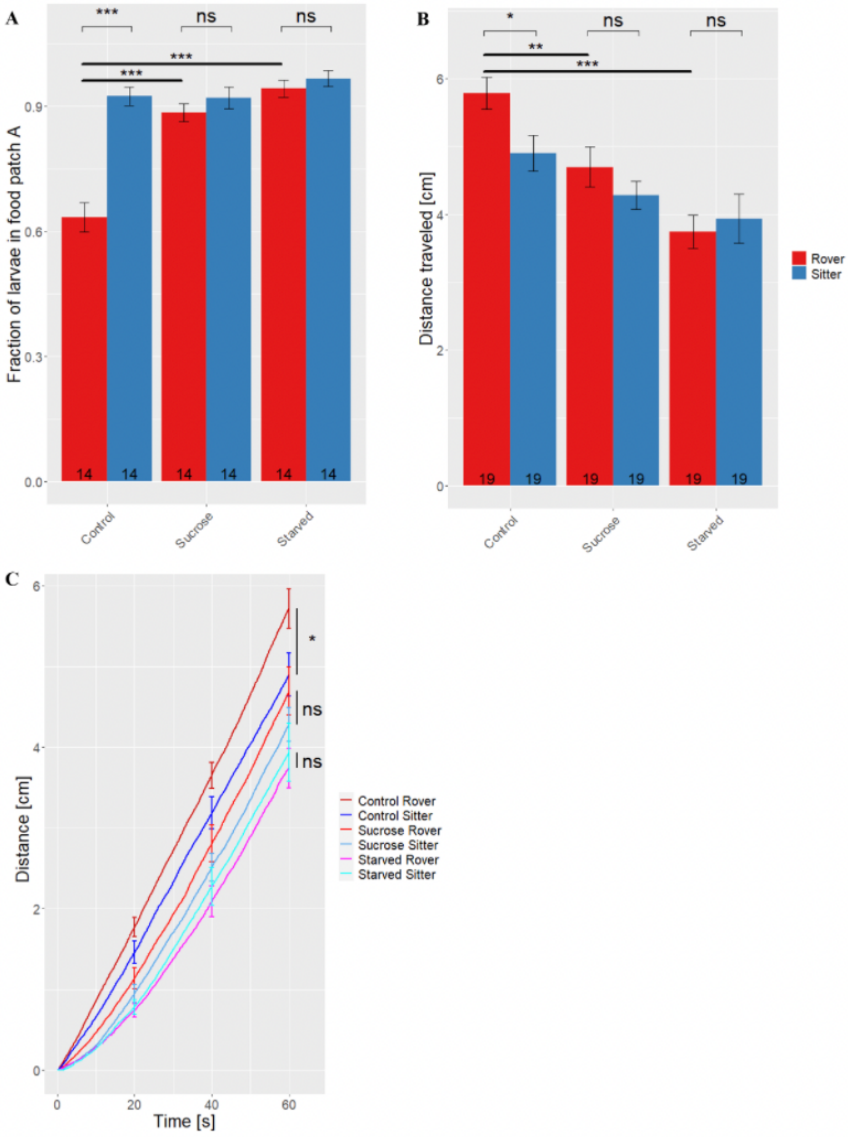

*Figure 7: Behaviour analysis of rover and sitter larvae under starvation conditions - (A) Mean percentage of larvae still in food patch A after 20 minutes. Brackets above each experimental group with asterisks indicate whether there is a significant difference between rover and sitter behaviour (Wilcoxon test). Thick lines with asterisks denote that there is a significant difference between nutrient-limited rovers/sitters (sucrose/starved) in comparison to non-starved control animals (non-parametric Kruskal-Wallis-test with post hoc Dunn's test for multiple comparisons). (B) Mean distance of larvae moving for one minute on an agar plate. Brackets above each experimental group with asterisks indicate whether there is a significant difference between rover and sitter path lengths (t-test). Thick lines with asterisks indicate that there is a significant difference in path lengths between nutrient-limited rovers/sitters (sucrose/starved) in comparison to the non-starved control rovers/sitters (Anova with post hoc Dunnett test). (C) Mean distance (in cm) as a function of time (in s). Lines with asterisks denote a significant difference between rover and sitter travelled distances after 60 s (t-test). Error bars represent standard error of the mean (SEM). Number of asterisks denotes p-value (*p < 0.05, **p < 0.01, ***p < 0.001, ns p > 0.05). Numbers within bars denote the number of experiments (n).*

Food manipulation

Similar to previous studies that had shown that the expression of phenotypes related to foraging behaviour associated with the *for* gene is affected by the food quality in the environment, we also found (Fig. 7) that starvation for 2h affected larval foraging behaviour in ways analogous to low food quality [10,19]. To understand which nutrients are specifically involved in these changes, we analysed standard fly food for its nutritional composition and reduced the main nutritional components as much as possible in the respective fly food variants (Fig. 2). Due to the difficulty of reducing the amount of protein as a single nutritional component, fly food containing no yeast was used as a low-protein manipulation. A nutrient-poor starch fly food containing nearly no sugar was used as a further treatment. The experimental design (Fig. 1) allowed *rover* and *sitter* larvae to grow up in an environment that differed only in terms of the nutritional composition of the food. Mid third instar larvae were tested for their tendency to leave the first food patch (food patch A) after 20 minutes (Fig. 3**,** *Methods*) and their travelled distances on an agar plate within one minute (Fig. 5, *Methods*) were measured after growing up under six different food conditions: standard food (standard), food containing half the amount of non-sugar carbohydrates (carbs 0.5) or half the amount of fats (fat 0.5) compared to the standard food, food containing a quarter of the amount of sugar (sugar 0.25) in the standard food, as well as food containing no yeast (yeast 0) and starch food (starch) (Fig. 8). Larvae were delayed in their development by about one day when growing up in the carbohydrate, fat and sugar manipulated food. Yeast deficiency in the food resulted in a larval development that was delayed by about 4 days.

Looking at the behavioural differences between *rovers* and *sitters* regarding the foraging behaviour (Fig. 8*A*) revealed that *rovers* had a significantly higher tendency to leave the first food patch compared to *sitters* when the larvae were raised in standard food (wilcox.exact, $W = 42$, $p = 0.009$), carbohydrates (0.5) food (wilcox.exact, $W = 18$, $p < 0.0001$), starch food (wilcox.exact, $W = 54$, $p = 0.03$), and food with no yeast

(wilcox.exact, W = 30, p < 0.001). No significant differences were found between *rovers* and *sitters* when they were exposed to the food with a reduced amount of fat (wilcox.exact, W = 73.5, p = 0.27) or sugar (wilcox.exact, W = 75.5, p = 0.31). Next, a non-parametric Kruskal-Wallis-test with a post hoc Dunn's test for multiple comparisons was performed. Both mid third instar *sitter* and *rover* larvae had a significant higher tendency to stay in the first food patch when they were exposed to the starch food compared to *sitter* and *rover* larvae growing up in standard food (*sitters*: Kruskal-Wallis-test, $F_{(5)}$ = 26.5, p < 0.001 with post hoc Dunn's test, sitters, p = 0.002; *rovers*: Kruskal-Wallis-test, $F_{(5)}$ = 21.8, p < 0.001 with post hoc Dunn's test, rovers, p = 0.002). There was also a significant difference between *sitters* growing up in standard food and *sitters* growing up in the no-yeast food (Kruskal-Wallis-test, $F_{(5)}$ = 26.5, p < 0.001 with post hoc Dunn's test, p = 0.003).

Analysing the travelled distances of for^R and for^s larvae (Fig. 8*B*) revealed significant differences between rover and sitter larval path lengths after they grew up in the standard food (t-test, $t_{(33.4)}$ = 2.05, 95% CI = [0.009, 2.09], p =0.048) the carbs (0.5) food (t-test, $t_{(34.1)}$ = 2.53, 95% CI = [0.23, 2.13], p =0.016), the fat (0.5) food (t-test, $t_{(34)}$ = 2.45, 95% CI = [0.19, 2.05], p =0.02), the sugar (0.25) food (t-test, $t_{(36)}$ = 3.29, 95% CI = [0.56, 2.36], p =0.002) and the yeast (0) food (t-test, $t_{(30)}$ = 3.4, 95% CI = [0.45, 1.8], p =0.002). There was no significant difference between rover and sitter distances when larvae had grown in the starch food (t-test, $t_{(31.3)}$ = 1.9, 95% CI = [-0.07, 1.91], p =0.067). *Sitter* larvae that grew in food with no yeast did not show significantly shorter distances compared to *sitters* growing up in standard food (Anova, $F_{(5)}$ = 2.21, p = 0.058).

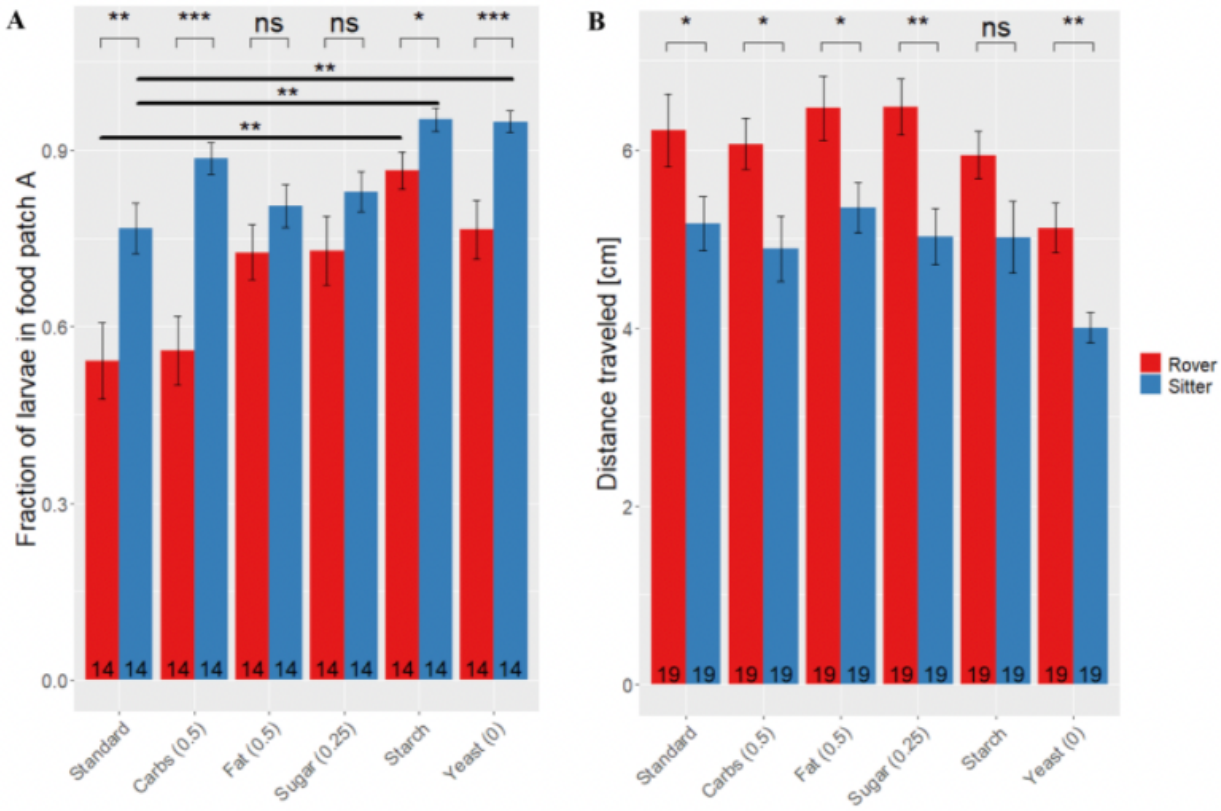

*Figure 8: Behaviour analysis of rover and sitter larvae exposed to different nutritional conditions throughout their larval life - (A) Mean percentage of larvae still in food patch A after 20 minutes. Brackets above each experimental group with asterisks indicate whether there is a significant difference between rover and sitter behaviour (non-parametric Wilcoxon test). Thick lines with asterisks indicate that there is a significant difference between the rovers/sitters of a food manipulation treatment in comparison to the rovers/sitters of the standard food treatment (non-parametric Kruskal-Wallis-test with post hoc Dunn's test for multiple comparisons). (B) Mean trajectory length of larvae moving for one minute on an agar plate. Brackets above each experimental group with asterisks indicate whether there is a significant difference between rover and sitter path lengths (t-test). Thick lines with significance stars above indicate that there is a significant difference in distance travelled between the rovers/sitters of a food manipulation treatment in comparison to the rovers/sitters of the standard food treatment (Anova with post hoc Dunnett test). Error bars represent standard error of the mean (SEM). Number of asterisks denotes p-value (*p < 0.05, **p < 0.01, ***p < 0.001, ns p > 0.05). Numbers within the bars denote the number of experiments (n).*

Time to first movement

In general, the differences between the times to first movement of *rover* and *sitter* larvae we observed within our experimental treatments were less pronounced than the differences reported above. Larvae started to move after about 1.5 s on average (Fig. 9). Confirming previous results, the time of the first movement depended to some extent on how much the larvae were restricted in their nutrient availability, with a high restriction (starvation) leading to a later first movement (Fig. 9*B*). However, large variability in these data prevented strong conclusions to be drawn.

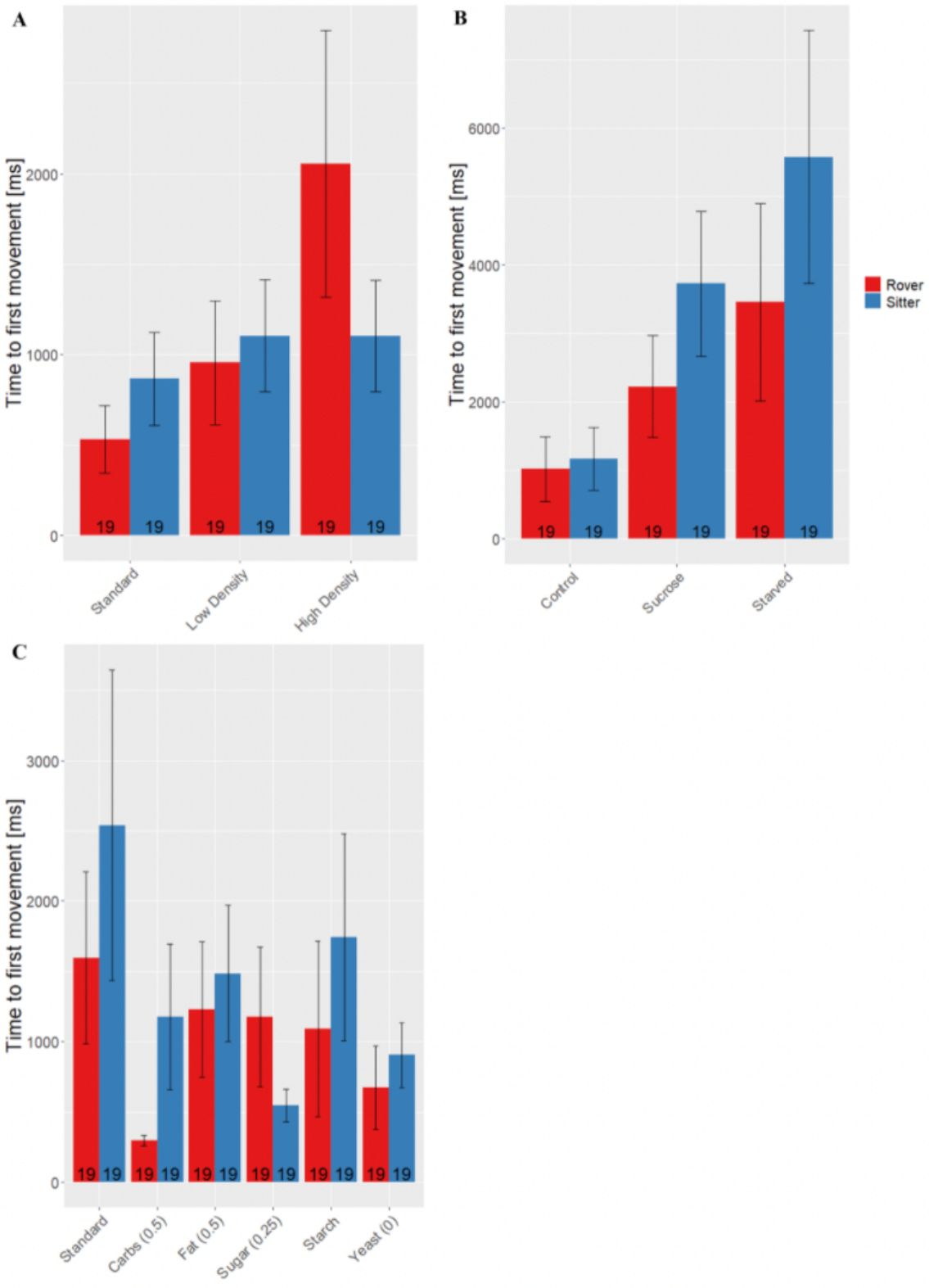

Figure 9: Mean times to first movement (in ms) - (A) Food manipulation experiment (B) Starvation experiment (C) Larval density manipulation experiment. Error bars denote standard error of the mean (SEM). Numbers within bars denote the number of experiments (n).

DISCUSSION

Two of our three quantifications (propensity to leave the food patch and distance travelled) provided reliable behavioural differences between *rovers* and *sitters* grown at standard conditions (Figs 6, 7 and 8). Control *rovers* showed a significantly higher tendency to leave the food patch as well as significantly longer path lengths on the agar plate, when compared to the control *sitters*. Interestingly, Kaun *et al.* found analogous differences in foraging trails between *rovers* and *sitters* during a 15-minute test on an agar plate with a small layer of yeast, but no such differences in path lengths between *rover* and *sitter* during a test on non-nutritive agar [10]. However, due to the fact that in the experiment used for this study, larvae were taken directly out of the food and the covered distance on the agar was determined immediately after a much shorter time of only 60 seconds, larvae might still have been looking for food on the agar and thus show a foraging behaviour, resulting in significantly different path lengths between *rover* and *sitter* larvae.

As the results of this work suggest, larval density seems to be a key factor affecting larval behaviour connected to food (Fig. 6). A high larval density during development produced a gene-environment interaction that led to significantly lower path lengths of *rover* larvae when compared to *rovers* grown at normal density. In contrast, *sitter* path lengths did not differ significantly compared to the control group after developing at a high larval density. As a result, *rover-sitter* differences regarding the distances travelled on the non-nutritive agar vanished.

In addition, both *rover (*and to a much smaller extent *sitter)* larvae raised under a high larval density showed a significantly higher propensity of remaining on the first food patch when compared to the control group, i.e., both groups showed increased sitter-like behaviour. The dramatic shift towards sitter-like behaviour in the rovers raised at high larval densities abolished the *rover-sitter* differences observed at low and standard densities. One possible explanation for the reduced tendency of *rover* and

sitter larvae to leave the food patch after growing up at high larval density could be their cooperative feeding behaviour. Social aggregation can be seen in different species including *Drosophila* larvae [20]. Participation in a cooperative feeding group can lead to different fitness benefits, for example animals can process food more easily in order to get nutrients [21] or reduce the risk of predation [22]. Dombrovski *et al.* showed that *Drosophila* larvae, foraging in liquid food under crowded conditions, form feeding clusters, in order to dig deeper or drag a common air cavity [23]. As a result of growing at high density, larvae could consequently still show a cooperative feeding behaviour by forming feeding clusters on the yeast patch and therefore remain together on the food they are placed on first.

Another study showed a delay in development for *Drosophila* larvae raised under crowded conditions, possibly due to a decrease in nutrients in the food medium [24]. The high-density larvae used in our experiments were also delayed in their development and thus tested one day later compared to the larvae raised under a normal or low larval density. Therefore, the decreased nutritional value of the substrate could be a further explanation for the observed behaviour, as previous studies had also demonstrated an effect of lower food quality on foraging behaviour [10,19].

Our results from the food manipulation experiments may help shed light on the causal relationship between crowded living conditions, the availability of nutrients - or lack thereof - and their effect on larval foraging behaviour. Which specific nutrients, if any, may have a more important effect on the observed behavioural change of the larvae (Figs. 7, 8)?

Starved *rovers* and *rovers* restricted to sucrose as the sole food source showed a significantly lower tendency to leave the food patch, as well as significantly shorter path lengths compared to non-starved *rovers* (Fig. 7). In contrast, *sitters* did not significantly change their behaviour in the restricted situations, even though there was a tendency in the same direction as in *rovers*. Consequently, both complete starvation

or sucrose as a single food source abolished the standard rover-sitter difference (Fig. 7), reproducing earlier results [10].

As the availability of nutrients is essential for any organism to provide the energy needed to survive and maintain metabolic activities and as starvation can lead to drastic consequences such as a rapid suppression of the protein expression or the progression of the cell cycle [25], it may not be overly surprising that especially *rovers* react very sensitively to nutrient limitations in their environment by leaving their food patch less often and covering shorter distances on the agar in order to rapidly replenish energy reserves or to save energy. This makes it very clear that nutrient availability is an important environmental factor and that even short-term restrictions in nutrient availability can cause serious changes in the phenotypic expression of food-related behavioural patterns in larvae.

As sucrose alone was not sufficient to maintain the *rover-sitter* differences, are there other nutrients in the fly food that are especially important for these phenotypes? To address this question, we manipulated the sugar, (non-sugar) carbohydrate, fat and protein content of the food the larvae were grown in (see Materials and Methods). In contrast to the other results, here the congruence between the food patch experiment and the trajectory length experiment was less clear. While nearly all treatments appeared to increase the tendency to stay in the first foodpatch in both *rovers* and *sitters* (Fig. 8A), path length only appears to be affected by complete elimination of yeast or a broad reduction in many nutrients, such as in our starch medium (Fig. 8B). While sugar and fat reductions significantly reduced the rover sitter difference in the food patch experiment, only the starch medium managed to accomplish this for path length.

Yeast elimination seemed to have an outsize effect exclusively on *sitters*. It has been reported previously that yeast deficiency may be underlying effects on life-history traits in *Drosophila* by larval crowding, as a yeast-rich larval diet was able to rescue the consequences of high larval density, for example regarding body size or

developmental time [26]. The observation that *sitter* larvae grown in food without any yeast showed a significantly higher tendency to stay in the food patch compared to *sitter* larvae that were able to develop in the standard food, indicates that yeast availability during larval development may have played a critical role in our experiments with high larval density. Further studies will be needed to investigate the possible relationship between yeast availability and larval density and the associated effects on the behaviour of *rover* and *sitter* larvae.

The only treatment with no effect in either experiment was to reduce non-sugar carbohydrates by 50%. Taking the results together, it appears as if yeast (protein), fats and sugar appear to each have specific effects on foraging behaviour, but better-controlled food manipulations are required to corroborate or correct this conclusion.

In addition to more sophisticated nutrient manipulations, one may also want to consider a more precise control of larval density in the manipulated food medium. In our experiments, there may have been differences in the density of larvae for the different nutritional treatments, because food quality can influence the egg laying rate [27] and we only controlled the number of egg-laying females, but not the number of eggs laid.

In *Drosophila,* the *for* gene is known to affect a wide range of behavioural traits apart of food-related behaviour, for example learning and memory [28,29], olfactory behaviour [14,30] as well as sleep [31], which opens a wide field for further investigations. Given the fact, that the *for* gene is conserved across many different species [6], further studies will show whether similar gene-environment interactions can also be observed at the *for* gene orthologs of other species and for other traits.

REFERENCES

1. Hoke, K. L., Adkins-Regan, E., Bass, A. H., McCune, A. R. & Wolfner, M. F. Co-opting evo-devo concepts for new insights into mechanisms of behavioural diversity. J. Exp. Biol. 222, (2019).

2. Anreiter, I., Sokolowski, H. M. & Sokolowski, M. B. Gene–environment interplay and individual differences in behavior. Mind Brain Educ. 12, 200–211 (2018).

3. Via, S. & Lande, R. Genotype-environment interaction and the evolution of phenotypic plasticity. Evolution 39, 505–522 (1985).

4. Mackay, T. F. C., Stone, E. A. & Ayroles, J. F. The genetics of quantitative traits: challenges and prospects. Nat. Rev. Genet. 10, 565–577 (2009).

5. Zayed, A. & Robinson, G. E. Understanding the relationship between brain gene expression and social behavior: lessons from the honey bee. Annu. Rev. Genet. 46, 591–615 (2012).

6. Anreiter, I. & Sokolowski, M. B. The foraging Gene and Its Behavioral Effects: Pleiotropy and Plasticity. Annu. Rev. Genet. 53, 373–392 (2019).

7. Osborne, K. A. et al. Natural behavior polymorphism due to a cGMP-dependent protein kinase of Drosophila. Science vol. 277 834–836 (1997).

8. Pereira, H. S. & Sokolowski, M. B. Mutations in the larval foraging gene affect adult locomotory behavior after feeding in Drosophila melanogaster. Proc. Natl. Acad. Sci. U. S. A. 90, 5044–5046 (1993).

9. Sokolowski, M. B. Foraging strategies of Drosophila melanogaster: a chromosomal analysis. Behav. Genet. 10, 291–302 (1980).

10. Kaun, K. R. et al. Natural variation in food acquisition mediated via a Drosophila cGMP-dependent protein kinase. J. Exp. Biol. 210, 3547–3558 (2007).

11. Allen, A. M., Anreiter, I., Neville, M. C. & Sokolowski, M. B. Feeding-Related Traits Are Affected by Dosage of the foraging Gene in Drosophila melanogaster. Genetics 205, 761–773 (2017).

12. de Belle, J. S. & Sokolowski, M. B. Heredity of rover/sitter: Alternative foraging strategies of Drosophila melanogaster larvae. Heredity 59, 73–83 (1987).

13. Slankster, E. et al. Mechanism underlying starvation-dependent modulation of olfactory behavior in Drosophila larva. Sci. Rep. 10, 3119 (2020).

14. Colomb, J., Reiter, L., Blaszkiewicz, J., Wessnitzer, J. & Brembs, B. Open source tracking and analysis of adult Drosophila locomotion in Buridan's paradigm with and without visual targets. PLoS One 7, e42247 (2012).

15. Charmant, J. Kinovea. kinovea.org https://www.kinovea.org/.

16. Hemmerich, W. Abstand zwischen zwei Punkten. matheguru.com https://matheguru.com/lineare-algebra/abstand-zwischen-zwei-punkten.html.

17. Benjamin, D. J. et al. Redefine statistical significance. Nat Hum Behav 2, 6–10 (2018).

18. Santos, M., Fowler, K. & Partridge, L. Gene–environment interaction for body size and larval density in Drosophila melanogaster: an investigation of effects on development time, thorax length and adult sex ratio. Heredity 72, 515–521 (1994).

19. Anreiter, I., Vasquez, O. E., Allen, A. M. & Sokolowski, M. B. Foraging Path-length Protocol for Drosophila melanogaster Larvae. J. Vis. Exp. (2016) doi:10.3791/53980 .

20. Allee, W. C. Animal Aggregations. Q. Rev. Biol. 2, 367–398 (1927).

21. Valone, T. J. Group Foraging, Public Information, and Patch Estimation. Oikos 56, 357–363 (1989).

22. Turchin, P. & Kareiva, P. Aggregation in Aphis varians: An effective strategy for reducing predation risk. Ecology 70, 1008–1016 (1989).

23. Dombrovski, M. et al. Cooperative Behavior Emerges among Drosophila Larvae. Curr. Biol. 27, 2821–2826.e2 (2017).

24. Dombrovski, M., Kuhar, R., Mitchell, A., Shelton, H. & Condron, B. Cooperative foraging during larval stage affects fitness in Drosophila. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. 206, 743–755 (2020).

25. Scott, R. C., Schuldiner, O. & Neufeld, T. P. Role and regulation of starvation-induced autophagy in the Drosophila fat body. Dev. Cell 7, 167–178 (2004).

26. Klepsatel, P., Procházka, E. & Gáliková, M. Crowding of Drosophila larvae affects lifespan and other life-history traits via reduced availability of dietary yeast. Exp. Gerontol. 110, 298–308 (2018).

27. Watada, M. et al. Divergence of Drosophila species: Longevity and reproduction under different nutrient balances. Genes Cells 25, 626–636 (2020).

552 28. Kaun, K. R., Hendel, T., Gerber, B. & Sokolowski, M. B. Natural variation in
553 Drosophila larval reward learning and memory due to a cGMP-dependent protein
554 kinase. Learn. Mem. 14, 342–349 (2007).

555 29. Mery, F., Belay, A. T., So, A. K.-C., Sokolowski, M. B. & Kawecki, T. J. Natural
556 polymorphism affecting learning and memory in Drosophila. Proc. Natl. Acad. Sci. U.
557 S. A. 104, 13051–13055 (2007).

558 30. Arya, G. H. et al. The genetic basis for variation in olfactory behavior in Drosophila
559 melanogaster. Chem. Senses 40, 233–243 (2015).

560 31. Donlea, J. et al. Foraging alters resilience/vulnerability to sleep disruption and
561 starvation in Drosophila. Proc. Natl. Acad. Sci. U. S. A. 109, 2613–2618 (2012).