Gene-environment interactions at the foraging locus of Drosophila

Bachelor Thesis

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Abstract

Environmental variability during the development of an organism has known impacts on the expression of certain behavioural patterns. The fruit fly *Drosophila melanogaster* was used to investigate on 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. In this study, larval density and nutrient availability could be characterised as environmental key 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, so that rovers and sitters showed no more significant differences regarding their behaviour. Similar results were obtained when starving the larvae. Furthermore, nutrient and yeast deficiency were found to affect the larval foraging behaviour and locomotion.

Zusammenfassung

Eine Variabilität der Umwelt während der Entwicklung eines Organismus hat bekannte Auswirkungen auf die Expression von bestimmten Verhaltensmustern. Die Fruchtfliege *Drosophila melanogaster* wurde benutzt, um zu untersuchen, wie unterschiedliche Umweltbedingungen mit den Allelvarianten von Rovern (for^R) und Sittern (for^s) am *foraging* Genlocus interagieren, um so das Verhalten von Larven in Bezug auf Futter zu beeinflussen. In dieser Studie konnten die larvale Dichte und die Nährstoffverfügbarkeit als bedeutende Umweltfaktoren charakterisiert werden, die das Larvenverhalten während der frühen Entwicklung beeinflussen. Eine hohe larvale Dichte verringerte die Tendenz der Rover eine Nahrungsstelle zu verlassen und verringerte die zurückgelegten Weglängen, so dass Rover und Sitter nicht mehr signifikante Unterschiede bezüglich ihres Verhaltens zeigten. Ähnliche Ergebnisse konnten erzielt werden, nachdem die Larven hungern mussten. Darüber hinaus wurde festgestellt, dass Nährstoff – und Hefemangel das larvale Futtersuchverhalten und die Fortbewegung beeinflussen.

1   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 of an individuum during the development resulting in different behavioural phenotypes (Hoke et al., 2019). The concept of genetic variation as a response to a changing environment is known as gene-environment interaction. Phenotypic plasticity can be described as the extent of influence to which the phenotype can be affected or modified by the environment (Via and Lande, 1985). Many different genes are involved in establishing a certain behavioural phenotype (Mackay et al., 2009), with some genes acting as master regulators (e.g. transcription factors) within this gene pathways (Zayed and Robinson, 2012). 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 regarding the foraging behaviour in *Drosophila melanogaster*. Being conserved in many species with different functions (Anreiter and Sokolowski, 2019) 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) (Osborne et al., 1997) and natural variation of two alleles in *for* affects larval foraging behaviour. Larvae having 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*) alleles (Pereira and Sokolowski, 1993; Sokolowski, 1980). Furthermore, rovers are characterized by a higher PKG enzyme activity compared to sitters in an environment rich of 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 (Kaun et al., 2007a). This plasticity regarding larval foraging behaviour (travelled distances, food intake) and fat levels is regulated by the gene dosage of *for* (Allen et al., 2017).

This work uses the rover/sitter polymorphism of the *for* gene in *Drosophila* to investigate, which environmental changes possibly affect food-related larval behaviour. The aim of

this bachelor thesis is to study how these both genotypes (for^R and for^s) would respond to changes in the environment. An environmental variation was achieved by raising the larvae under different conditions in terms of larval density or nutrient availability.

2   Materials and methods

2.1   Fly stocks and maintenance

The rover and sitter strains used for the experiments are homozygous for the for^R and for^s alleles and isogenic for the second and third chromosome (Belle and Sokolowski, 1987; Sokolowski, 1980). Flies were maintained in plastic vials with food at 25°C and 60% humidity on a 12h/12h light and dark cycle. To start the experiments, about 15 female flies were allowed to lay eggs for one day and then transferred into new vials. Next, the newly hatched offspring was 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 one day. 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 (**Figure 1**).
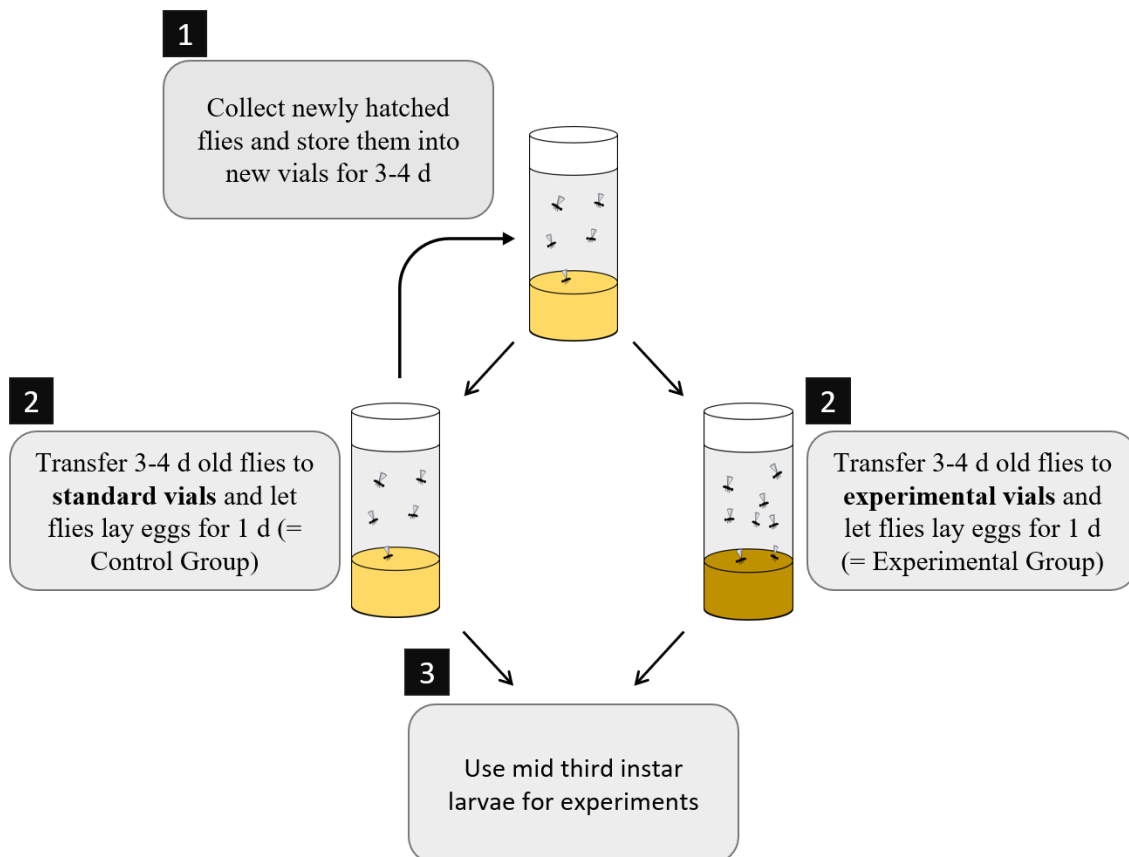
Figure 1: Experimental design: 3-4 days old flies which were collected after they were just hatched were allowed to lay eggs for one day and then removed. Standard vials contained a normal density of flies (about 15 females) and standard fly food. Experimental vials included different experimental treatments (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.

2.2 Fly food and agar plates

2.2.1 Cooking protocols

Standard fly food and food modified in nutritional composition

Using an induction cooker, 1 l of water was brought to a boil in a cooking pot. 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 to the pot. The mash was boiled for about 10 minutes while stirring continuously. Finally, some water was added and after a short cooling down, 6 g Nipagin was put into the mixture. The fly food was then filled into vials and stored in the cooling chamber. A small amount 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

In a cooking pot, 560 ml water was brought to boil. Then, 15 g agar-agar dissolved in 200 ml cold water was 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. Fly food was filled into vials and stored in the cooling chamber. A small amount 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

2.2.2 Nutrient distribution

The carbohydrate food manipulation contained about half the amount of non-sugar carbohydrates as in the standard food. Yeast manipulation food contained no yeast. In the fat manipulation food the amount of fats was reduced by 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. For the nutritional information of the various products used to calculate the nutrient distribution (**Figure 2**) see *Attachment A*.
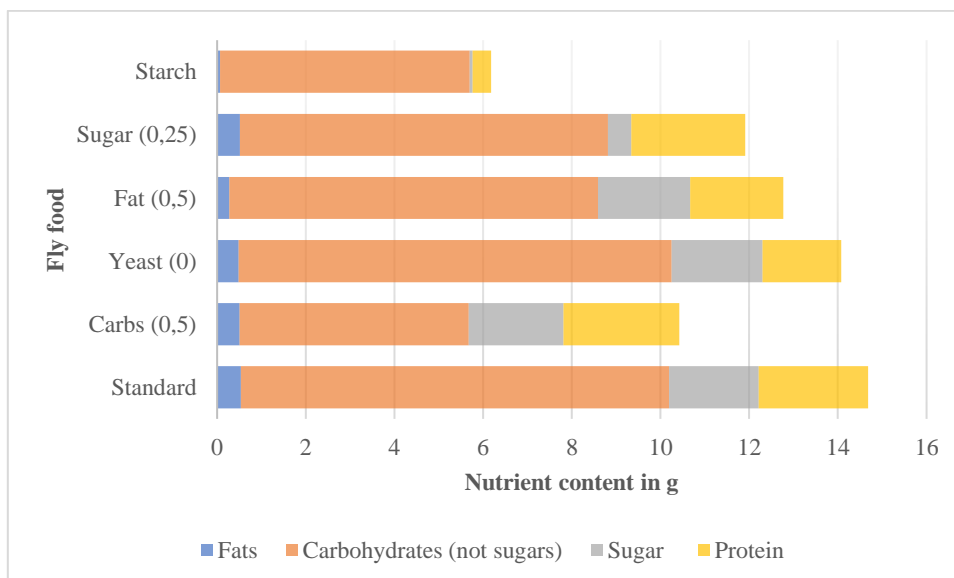
Figure 2: Nutrient distribution in 100 g of each fly food: Number in brackets after the nutritional information of the fly food indicates the ratio in comparison to the standard food.

2.2.3 Agar plates

Table 3: 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

2.3   Starvation protocol

The method for the starvation of the larvae was taken from Slankster et al. (Slankster et al., 2020). 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.

2.4   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 one day. To have a low larval density about 5 female and a few male flies were together 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. The adult flies were then removed from the vials the next day and the number of laid eggs was checked.

2.5   Larval experiments

2.5.1   Food patch

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 (**Figure 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 closed-meshed grid 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 in the 25°C room with 60% humidity.

Figure 3: Food patch experiment: Prepared agar plate.

2.5.2 Distance tracking

Experimental setup

To record the larval locomotion, the setup of the Burridan apparatus was used to maintain an environment with constant temperature and light using four circular fluorescent tubes to illuminate the arena homogenously (Colomb et al., 2012). Black dyed agar plates where used to increase the contrast to the white larvae. The agar plates were placed in an elevated position within the arena. Larvae, which had previously been removed from the food and washed with water, were carefully placed in the centre of the agar plate. The camera, connected to a computer running the recording software, was positioned above the arena in order to film each larva during the experiment (**Figure 4**). A one-minute video was recorded immediately after the larva was placed on the agar using OBS Studio as video recording software. Larvae that crawled under the agar before one minute were discarded.
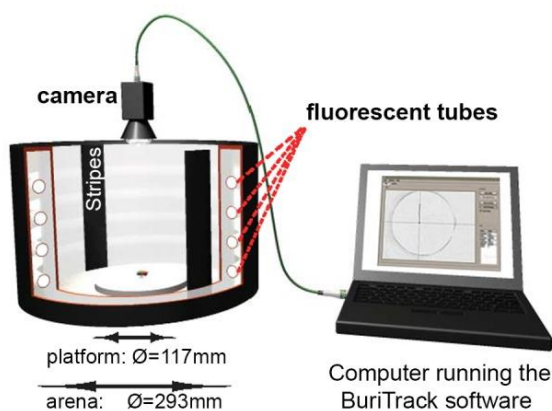

Figure 4: Burridan's paradigm (Colomb et al., 2012): Setup was used to record videos of the crawling larvae. Computer used OBS Studio software (not BuriTrack) to record the videos.

Kinovea tracking software

The program Kinovea was used to track the movement of each larva. Kinovea is a video player for sport analysis, but provides different features like the ability of tracking objects (Kinovea 0.8.15, 2009). After successfully tracking a larva (**Figure 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 *Attachment B*.

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

R script

A script based on R studio was used to evaluate the Kinovea data in order to determine the individual travelled distances of the larvae. The following formula shows how the travelled distance D (in cm) was calculated, with x = x-coordinates, y = y-coordinates and z = total number of coordinate pairs (Hemmerich, 2011-2020):

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

Using the script, the data will be shortened to the desired end time (60 s) before the distance is calculated. The script is also able to detect the time at which the larva starts moving (in ms) by determining the point in time after which the first distance is not equal to zero. The mean distance (in cm) and the mean time of first movement (in ms) can be calculated for all text files within one group. For further information on how to use the script see *Attachment C*.

2.6 Statistical analysis

2.6.1 Sample size calculation

Using R Studio, a two-sample t test power calculation 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 4).

Table 4: 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

2.6.2 Testing for statistical significance

Food patch experiment

To look for a significant difference in the comparison of rovers and sitters regarding the food patch experiment, a wilcox.exact test was carried out, using R studio due to the fact that the data was not normally distributed (Shaipiro-Wilk-Test, $p < 0,05$). To compare the respective variant (rover/sitter) of the control group to the equal variant of the experimental groups, a Kruskal-Wallis-test with posthoc 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 (Shaipiro-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 posthoc Dunnet-test was carried out.

3   Results

3.1   Food manipulation

As previous studies had shown, that the expression of phenotypes related to the foraging behaviour regarding the *for* gene is affected by the food quality in the environment (Anreiter et al., 2016; Kaun et al., 2007a), I wanted to determine whether deficiency of a certain nutrient in the fly food could have an impact on food-related larval behaviour. To do this, standard fly food was analysed for the nutritional composition and the main nutritional components were reduced as much as possible in the respective fly food (**Figure 2**). Due to the fact, that it was difficult to reduce 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 (**Figure 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 (**Figure 3,** *Methods*) and their travelled distances on an agar plate within one minute (**Figure 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) (**Figure 6**). Larvae were delayed in their development by about one day when growing up in the carbohydrate, fat and sugar manipulation food. Yeast deficiency in the food resulted in a larval development delayed by about 4 days.

Looking at the behavioural differences between rovers and sitters regarding the foraging behaviour (**Figure 6** *A*) revealed, that rovers had a significant higher tendency to leave the first food patch compared to sitters when the larvae were raised in standard food (wilcox.exact, $p = 0,009$), carbohydrates (0,5) food (wilcox.exact, $p < 0,0001$), starch food (wilcox.exact, $p = 0,03$), and food with no yeast (wilcox.exact, $p < 0,001$). No significant differences were found between rovers and sitters when they were exposed to the food with a reduced amount of fat (wilcox.exact, $p = 0,27$) or sugar (wilcox.exact, $p = 0,31$). Next, a non-parametric Kruskal-Wallis-test with a posthoc Dunn's test for

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multiple comparison 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 (Kruskal-Wallis-test with posthoc Dunn's test, sitters, p = 0,002; Kruskal-Wallis-test with posthoc 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 with posthoc Dunn's test, p = 0,003).

Analysing the travelled distances of *for^R* and *for^s* larvae (**Figure 6 *B***) revealed significant differences between rover and sitter larval path lengths after they grew up in the standard food (t-test, p =0,048) the carbs (0,5) food (t-test, p =0,016), the fat (0,5) food (t-test, p =0,002), and the yeast (0) food (t-test, p =0,002). There was no significant difference between rover and sitter distances when larvae had to grow up in the starch food treatment (t-test, p =0,067). Sitter larvae that grew up in food with no yeast covered a significantly shorter distance compared to sitters growing up in standard food (Anova with posthoc Dunnet-test, p = 0,043).
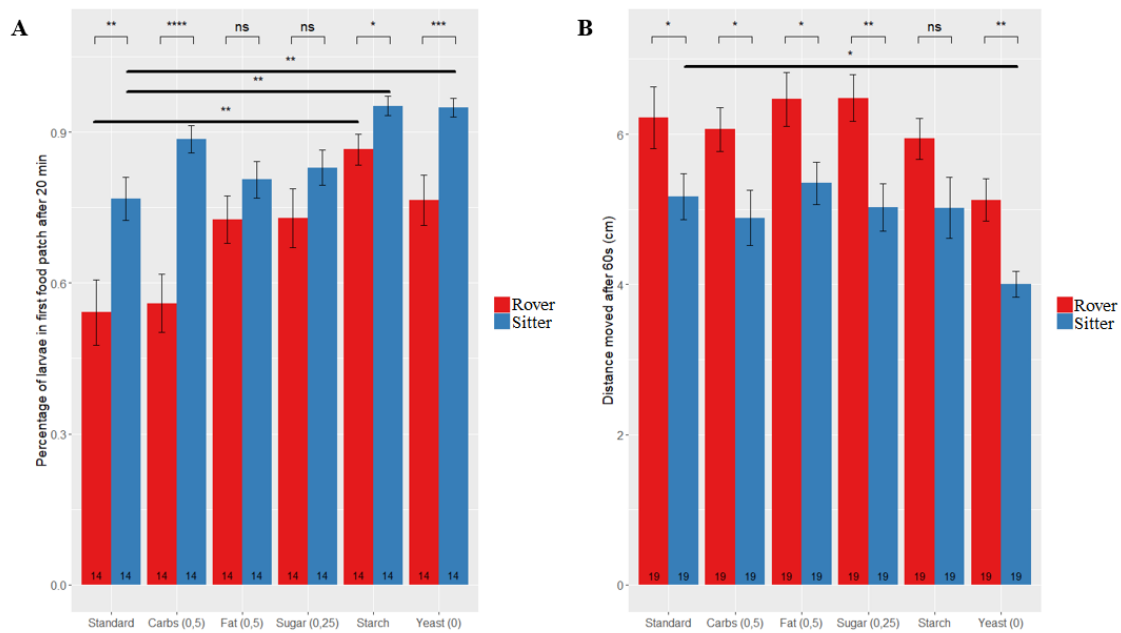
Figure 6: Behaviour analysis of rover and sitter larvae exposed to different nutritional conditions throughout their larval life: *(A)* Mean percentage of larvae still in food patch A after 20 minutes. Brackets above each experimental group with stars indicate whether there is a significant difference between rover and sitter behaviour (non-parametric wilcox.exact test). Thick lines with significance stars above 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 posthoc Dunn's test for multiple comparison). *(B)* Mean distance of larvae moving for one minute on an agar plate. Brackets above each experimental group with stars 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 path lengths between the rovers/sitters of a food manipulation treatment in comparison to the rovers/sitters of the standard food treatment (Anova with posthoc Dunnet test). Error bars represent standard error of the mean (SEM). Number of stars represent p-value (*p < 0,05, **p < 0,01, ***p < 0,001, ****p < 0,0001, ns p > 0,05). Number within the bar charts represents the number of experiments (n).

3.2 Larval starvation

To understand how larvae would react to starvation, 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 path lengths were analysed (**Figure 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 (**Figure 7 A**) revealed, that rovers had a significant higher tendency to stay in the first food patch compared to the controls taken directly out of the food when they had been starved (Kruskal-Wallis-test with posthoc Dunn's test, p < 0,001) or when they had sucrose as only nutrient (Kruskal-Wallis-test with posthoc Dunn's test, p < 0,001) for 2 hours. As a result, the significant

difference between rover and sitter behaviour as it was observed in the control group (wilcox.exact, p < 0,0001) could no longer be seen in the starved experimental group (wilcox.exact, p = 0,35), as well as in the experimental group with only sucrose as nutrient (wilcox.exact, 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 path lengths (**Figure 7 *B***). Rover larvae that had been under starved conditions showed significant shorter path lengths when compared to the control rover larvae (Anova with posthoc Dunnet-test, p < 0,001). Also rovers that were exposed to sucrose as only nutrient covered a significant shorter distance compared to the controls (Anova with posthoc Dunnet-test, p = 0,009). Furthermore, sitters showed significant lower path lengths compared to the control sitters when they had been starved for 2 hours (Anova with posthoc Dunnet-test, p = 0,04). The significant difference between covered distances of rovers and sitters that could be seen in the control group (t-test, p = 0,017) could no longer be observed when the larvae had been starved (t-test, p = 0,67) or had sucrose as only nutrient (t-test, 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 (**Figure 7 *C***).
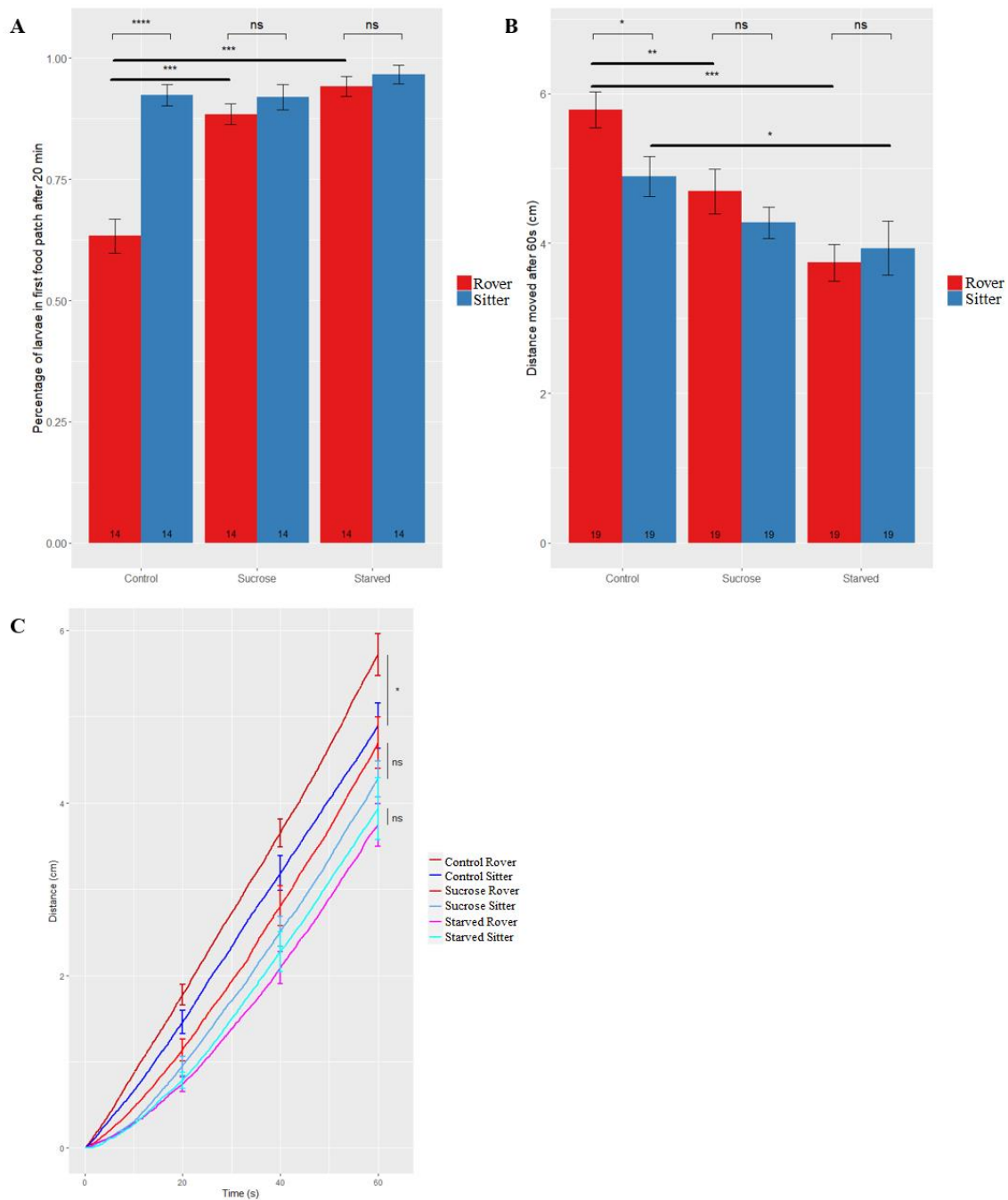
Figure 7: Behaviour analysis of rover and sitter larvae under starvation conditions: *(A)* Mean percentage of larvae still in food patch A after 20 minutes. Brackets above each experimental group with stars indicate whether there is a significant difference between rover and sitter behaviour (non-parametric wilcox.exact test). Thick lines with significance stars above indicate that there is a significant difference between nutrient-limited rovers/sitters (sucrose/starved) in comparison to non-starved control rovers/sitters (non-parametric Kruskal-Wallis-test with posthoc Dunn's test for multiple comparison). *(B)* Mean distance of larvae moving for one minute on an agar plate. Brackets above each experimental group with stars 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 path lengths between nutrient-limited rovers/sitters (sucrose/starved) in comparison to the non-starved control rovers/sitters (Anova with posthoc Dunnet). *(C)* Mean distance (in cm) as a function of time (in s). Lines with significance stars show if there is a significant difference between rover and sitter path lengths after 60 s (t-test). Error bars represent standard error of the mean (SEM). Number of stars represent p-value ($*p < 0,05$, $**p < 0,01$, $***p < 0,001$, $****p < 0,0001$, ns $p > 0,05$). Number within the bar charts represents the number of experiments (n).

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3.3 Larval density manipulation

Previous studies had shown an impact of larval density on the phenotypic expression in *Drosophila*, for example regarding the body size (Santos et al., 1994). To determine whether larval density could affect the foraging behaviour or the locomotion of rovers and sitters, mid third instar larvae where tested after growing up under high-density, low-density and standard-density conditions (*Methods*) (**Figure 7**). 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, p = 0,0014) as well as in the experimental group with a low density (wilcox.exact, p = 0,0012) (**Figure 8 *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 (Kruskal-Wallis-test with posthoc Dunn's test, rovers, p = 0,001; Kruskal-Wallis-test with posthoc Dunn's test, sitters, 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, p = 0,596).

The evaluation of the larval path lengths revealed similar results as in the food patch experiment (**Figure 8 *B***). Rover and sitter larvae showed a significant difference regarding their path lengths when grown up under a normal standard density (t-test, p = 0,003) or under a low larval density (t-test, 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 with posthoc Dunnet test, p = 0,17). However, rover larvae grown up under a high density showed significant lower path lengths when compared to the rovers grown up under standard density (Anova with posthoc Dunnet test, p = 0,04) resulting in no more significant difference in path lengths between high-density *for^R* and *for^s* larvae (t-test, p = 0,45).
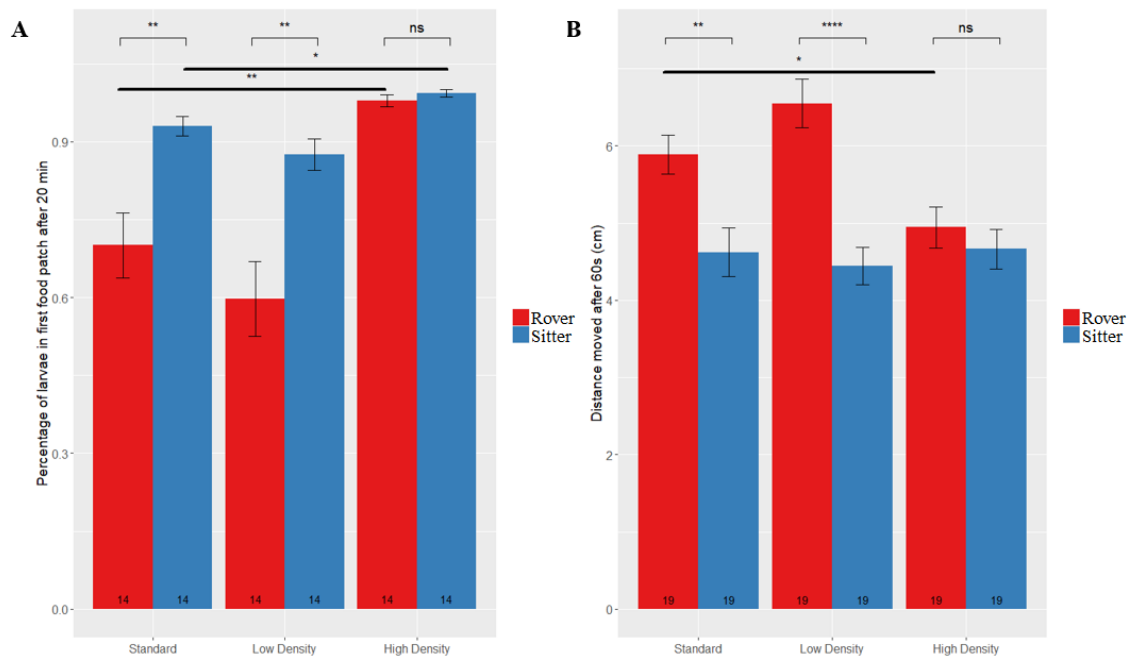
Figure 8: Behaviour analysis of rover and sitter larvae grown up under different larval densities: *(A)* Mean percentage of larvae still in food patch A after 20 minutes. Brackets above each experimental group with stars indicate whether there is a significant difference between rover and sitter behaviour (non-parametric wilcox.exact test). Thick lines with significance stars above indicate that there is a significant difference between the rovers/sitters grown up under a high/low larval density in comparison to the rovers/sitters grown up under standard density (non-parametric Kruskal-Wallis-test with posthoc Dunn's test for multiple comparison). *(B)* Mean distance of larvae moving for one minute on an agar plate. Brackets above each experimental group with stars 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 path lengths between the rovers/sitters grown up under a high/low larval density in comparison to the rovers/sitters grown up under standard density (Anova with posthoc Dunnet test was performed). Error bars represent standard error of the mean (SEM). Number of stars represent p-value (*p < 0,05, **p < 0,01, ***p < 0,001, ****p < 0,0001, ns p > 0,05). Number within the bar charts represents the number of experiments (n).

3.4 Times of first movement

In general, only slight differences regarding the times of the first movement of rover and sitter larvae were observed within all experimental treatments with larvae starting to move after about 1,5 s on average (**Figure 9**). Not surprisingly, the time of the first movement depended on how much the larvae were restricted in their nutrient availability, with a high restriction (starvation) leading to a later first movement (**Figure 9 *B***).
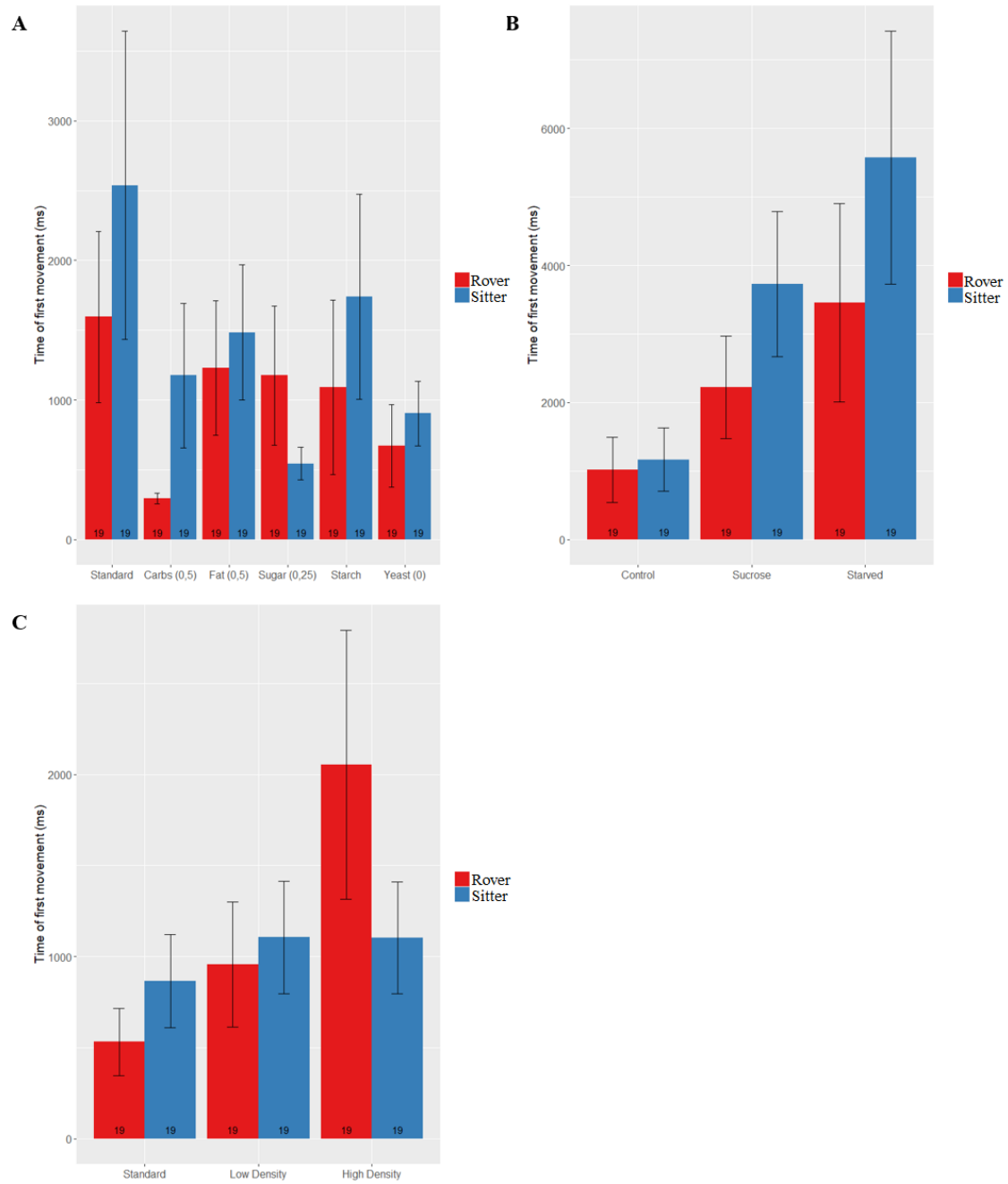
Figure 9: Mean times of first movement (in ms): *(A)* Food manipulation experiment *(B)* Starvation experiment *(C)* Density manipulation experiment. Error bars represent standard error of the mean (SEM). Number within the bar charts represents the number of experiments (n).

4 Discussion

With both experiments carried out (food patch, distance tracking) it was possible to determine behavioural differences between rovers and sitters who grew up under standard conditions (**Figures 6, 7 and 8**). Control rovers showed a significant higher tendency of leaving the food patch as well as significant longer path lengths on the agar plate, when compared to the control sitters. Interestingly, Kaun, et al. found significant differences in foraging trails between rover and sitter during a 15 minute test on an agar plate with a small layer of yeast, but no significant differences in path lengths between rover and sitter during a test on non-nutritive agar (Kaun et al., 2007a). 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 significant different path lengths between rover and sitter larvae.

As the results of this study suggest, larval density seems to be a key factor affecting larval behaviour connected to food (**Figure 8**). A high larval density during the development produced a gene environment interaction that led to significant lower path lengths of rover larvae when compared to rovers growing up under a normal density. In contrast, sitter path lengths did not differ significantly compared to the control group after growing up under a high larval density. As a result, rover-sitter differences regarding the distances travelled on the non-nutritive agar could no longer be observed. In addition, both rover and sitter larvae raised under a high larval density showed a significant higher tendency of leaving the food patch when compared to the control group, which led to the result, that no more significant rover-sitter differences regarding this behaviour could be observed. Furthermore, larvae grown up under a low larval density showed a similar behaviour regarding their tendency of leaving their food patch and their path lengths compared to larvae grown up under a standard larval density. One possible explanation for the reduced tendency of rover and sitter larvae to leave the food patch after growing up under a high larval density could be the cooperative feeding behaviour. Social aggregation can be seen in different species including *Drosophila* larvae. A 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 (Valone, 1989) or reduce the risk

of predation (Turchin and Kareiva, 1989). 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 (Dombrovski et al., 2017). As a result of growing up under a 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 of the medium (Dombrovski et al., 2020). The high-density larvae used in these 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 a previous study had already demonstrated an effect of lower food quality on foraging behaviour (Anreiter et al., 2016; Kaun et al., 2007a). Also, intra-group competition could play a role, as the larvae have to compete for the reduced amount of nutrients within the food. Furthermore, larvae growing up under a normal or low density are able to roam freely within the food whereas larvae developing under crowded conditions may be restricted in terms of their ability to move freely within the food, which could also be an explanation for their reduced tendencies to explore their environment resulting in the observed significantly shorter path lengths of rover larvae that grew up under a high larval density.

To be able to further interpret the results of the food manipulation experiment (**Figure 6**), it is important to be aware of the previously described impact of the larval density on the larval behaviour. Due to the fact that larval density within these experiments was only controlled via the number of females (about 15), that were allowed to lay eggs for one day, there may have been differences in the density of larvae for the different nutritional treatments, since food quality can also influence the egg laying rate (Watada et al., 2020). However, the results of this study are still sufficient, to show the effect of food quality on the larval behaviour. As expected, growing up in food, in which only the amount of non-sugar carbohydrates was reduced, but the sugar, protein, fat and yeast amount was similar to the standard food, did not lead to a significant change of the larval behaviour, so that significant rover-sitter differences regarding path lengths and food-leaving tendencies could still be observed. Furthermore, even if there was still a significant difference between rovers and sitters regarding the tendency to stay in the food patch, both rover

and sitter larvae stayed significantly more often in the food patch they were placed on first, when they were exposed to nutrient-poor starch food with a highly reduced amount of sugar, protein and fat. When developed in food with a reduced amount of fat (fat 0,5) or sugar (sugar 0,25), significant rover-sitter differences regarding the larval tendency of leaving the food patch could no longer be observed. However, neither sitters nor rovers that grew up in the low-fat or low-sugar food showed a significant difference in their food-leaving behaviour compared to the controls (standard food), which means, that it cannot be clearly determined, whether one of these nutrients really has an influence on this larval behaviour. Analysing the path lengths revealed, that the treatment with starch food was sufficient to achieve no more significant differences in path lengths between rover and sitter. These results confirm previous studies in which it was shown, that larval path length depends on food quality (Anreiter et al., 2016; Kaun et al., 2007a) and therefore indicate that food quality is an environmental factor modulating the plasticity of food-related behaviour in *Drosophila* larvae.

In addition, yeast deficiency seems to affect foraging behaviour and path lengths of sitter larvae. Interestingly, Klepsatel et al. found, that yeast deficiency could be the reason for previous observed life-history traits in *Drosophila* due to larval crowding, as a yeast-rich larval diet was able to rescue the effects of a high larval density, for example regarding body size or developmental time. The fact that sitter larvae, that grew up in food without any yeast showed a significant higher tendency to stay in the food patch, as well as significant lower path lengths compared to sitter larvae, that were able to develop in the standard food containing yeast, indicates, that yeast availability during larval development may play an important role in the expression of the observed behavioural patterns and might consequently be another explanation for the previous described effects of a high larval density. However, as the limited yeast availability only had a significant influence on the larval path lengths of the sitters, whereas a high larval density only significantly affected the larval path lengths of rovers, further studies will be needed to investigate on a possible relationship between yeast availability and larval density and the associated effects on the behaviour of rover and sitter larvae.

Looking at the previous results, it is no surprise that also starvation affects larval behaviour (**Figure 7**). Starved rovers and rovers restricted in terms of nutrients (only sucrose) showed a significantly lower tendency to leave the food patch, as well as

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significantly shorter path lengths compared to non-starved rovers. Also sitter path lengths were significantly shorter after starving the larvae when compared to the control sitters, but the availability of only sucrose was sufficient to prevent the previous reduction in travelled distances due to starvation. Furthermore, there was no significant effect of starvation or nutrient restriction on food leaving tendencies of sitter larvae when compared to the non-starved sitters. 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 (Scott et al., 2004), it is not surprising that that especially rovers react very sensitive 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.

In *Drosophila* the *for* gene is known to effect a wide range of behavioural traits apart of food-related behaviour, for example learning and memory (Kaun et al., 2007b; Mery et al., 2007), olfactory behaviour (Arya et al., 2015; Colomb et al.) as well as sleep (Donlea et al., 2012), which opens a wide field for further investigations. Given the fact, that the *for* gene is conserved across many different species (Anreiter and Sokolowski, 2019), further studies will show whether similar gene-environment interactions can also be observed at the *for* gene orthologs of other species.

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Attachments

A) Nutritional information of the respective products

Table 5: 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

B) Kinovea tracking guide

1. Start Kinovea program.

2. Import video by clicking on *File -> Open Video File.*

3. Set time format: *Options -> Time markers format -> Total milliseconds*.

4. To express distance in real life units add a line with the diagonal length of the black agar, then right click on the line -> *Calibrate measure ->* Change *Real size of the segment* to *8.5* cm (diameter of the agar plate).

5. For tracking the object: Right click in the middle of the larva -> *Track path.*

6. Start tracking by clicking on *play.*

7. If the tracking was misplaced go to the image corresponding to the misplaced point, then right click the path -> *Restart Path Edition* and adjust the point location. After editing the tracking, click on *End Path Edition.*

8. Safe trajectories by clicking on *File -> Export to Spreadsheet -> Trajectories to simple text*

More information about the program can be obtained at the Kinovea website (https://www.kinovea.org/help/en/109.html).

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C) R script guide

Analyse larval movement of a single group (Calculate_Distance.R)

1. Safe your text files from the Kinovea tracking program in folders referring to the Experiment and Fly Group (for example "Starved" -> "ForR").
2. Open the *Calculate_Distance.R* script in R studio.
3. Make sure the required libraries are installed.
4. Set working directory by changing *setwd* to the file path where your folders are stored.
5. Change *Experiment* to the name of the experiment (= name of the Experiment folder, for example "Starved") and *Group* to the fly group (= name of Group folder in the Experiment folder, for example "ForR") you want to evaluate. If you have no subfolders in the Experiment-folder you need to adjust the code.
6. Run the script -> A csv-file is generated containing information about the name of the Experiment and Group, the distances in cm of each larva and the time of the first movement of each larva. To adjust where this file should be stored change *setwd* above the *write.csv* function to your desired file path.
7. The plotted trajectories containing information about the name of the text file and the calculated distance can now be checked for each file.
8. You can also check the bar plots of mean distance and mean time of first movement.

Compare larval movement data of different groups (Compare_Distances.R)

1. Store the csv-files from the previous script in a folder where the same experimental groups are in the same folder (for example "Starved" folder contains the csv files of "Starved_ForR" and "Starved_ForS").
2. Open the *Compare_Distances.R* script in R studio
3. Make sure the required libraries are installed.
4. Set working directory by changing *setwd* to the file path where your folder containing the different experimental groups is stored
5. Run the script.

Both R scripts are available at "https://github.com/SimonBnm/Kinovea-Trajectories-Evaluation".

Declaration of authorship

The submitted printed copies and the submitted electronic version of the thesis are identical. I have written the thesis independently, have not used any sources or tools other than those indicated and have not already submitted the thesis to another university for the purpose of obtaining an academic degree. Furthermore, I confirm that I am aware of the legal consequences provided in § 27 (3) of the current examination regulations.

Die vorgelegten Druckexemplare und die vorgelegte elektronische Version der Arbeit sind identisch. Ich habe die Arbeit selbstständig verfasst, keine anderen als die angegebenen Quellen und Hilfsmittel benutzt und die Arbeit nicht bereits an einer anderen Hochschule zur Erlangung eines akademischen Grades eingereicht. Weiterhin bestätige ich, dass ich von den in § 27 Abs. 3 der geltenden Prüfungsordnung vorgesehenen Rechtsfolgen Kenntnis habe.

_____   _____

Date   Signature