SOCIAL ATTRACTION MEDIATED BY BACTERIAL VOLATILES

SOCIAL ATTRACTION MEDIATED BY BACTERIAL VOLATILES IN FRUIT FLIES

By
ISVARYA VENU, H. BSc.

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AUTHOR: Isvarya Venu, H. B.Sc. (McMaster University)

SUPERVISOR: Reuven Dukas, Ph.D.

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Abstract

Recent observations illustrate fruit fly larval attraction to the distinct odour emanating from food occupied by other larvae. Growing evidence of bacteria as influential microorganisms of hosts suggested the closer examination of host-microbial interactions. We investigated the origin of the volatiles that are attractive to flies. Focal larvae showed no difference in attraction to axenic used food with axenic larvae and axenic fresh food. Additionally, mated females showed no difference in attraction to axenic used food with axenic larvae and axenic fresh food. When we supplemented the axenic disks with *L. brevis*, larvae showed a significant preference for the axenic used food with axenic larvae and *L. brevis* over axenic fresh food. Also, the supplementation of *L. plantarum* to axenic disks also resulted in larvae showing a significant preference for the axenic used food with axenic larvae and *L. plantarum* over axenic fresh food. Focal larvae showed a significant preference for *L. brevis* on scratched MRS agar and axenic used food with axenic larvae, but did not show a significant preference for *L. brevis* on scratched axenic food. In a learning experiment, focal larvae showed no preference for novel odours previously paired with standard used food over novel odours previously paired with axenic used food. In order to test whether *L. brevis* improves food quality, the three fitness parameters observed, larval development rate, egg-to-adult survival, and adult body mass, revealed inexplicable findings. These results provide evidence for the role of bacterial volatiles in mediating the social attraction observed in fruit flies.

Acknowledgments

I would like to thank, first and foremost, Reuven Dukas for his utmost encouragement and guidance. I would also like to thank Jianping Xu for his continuous insight and assistance. I am grateful for the suggestions and support of my supervisor committee, Ana Campos and Paul Andrews. I would like to express my deepest gratitude to my fellow lab members, Zachary Durisko, Shane Golden, Carling Baxter, and Aaron Vogan of Jianping's lab for tremendous assistance and advice. I would also like to thank my fellow lab members and graduate students of the department for making graduate school an enjoyable and memorable experience.

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INTRODUCTION

Fruit flies: a model system for social behaviour

Fruit flies are a prime model system widely used to study evolution and neurogenetic mechanisms. Their simple nervous system, learning abilities, and ease of culturing and reproduction make them an ideal candidate for studying social behaviour. While many socially influenced behaviours have been primarily associated with vertebrates and social hymenoptera, the phenomenon has only recently been examined in insects (von Frisch 1967; Heyes & Galef 1996; Leadbeater & Chittka 2007; Kendal et al. 2009). Inherent conditions such as parental care and overlapping generations have been identified to enhance the utility of social learning behaviour (Dukas, 2010). Reducing the risk of incurring costs in time, energy, and predation has also greatly contributed to the evolution of this beneficial behaviour (Galef & Laland, 2005). Engaging in social behaviours ultimately promotes the passage of information from experienced to inexperienced individuals. We have established novel protocols examining fruit fly social behaviour in an attempt to understand the evolution and neurogenetics underlying social behaviour.

Social information use in larvae

The use of social information by *Drosophila melanogaster* larvae in foraging decisions has been documented in our lab. In an experiment testing simple social information use, fruit fly larvae exhibit social attraction to other larvae and to a substrate frequented by others (Durisko & Dukas, in press). To eliminate the possibility that focal larvae were simply attracted to other larvae out of familiarity, since they were reared in groups, another experiment reared focal larvae in isolation prior to testing with no experience of other larvae. Despite being reared in isolation or in groups, larvae significantly preferred the food

occupied and consumed by other larvae over the unoccupied food. This work was then extended to a wild-caught population of flies. Consistent with larvae from the previous two experiments, focal larvae preferred the banana slice occupied and consumed by other larvae over the unoccupied banana slice. Food consumed by fruit fly larvae has a distinctive odour and Durisko & Dukas (in press) tested which component of the used food was attractive to larvae, whether it was the used food or the presence of larvae. Work by Durisko & Dukas (in press) suggests that larvae are attracted to the smell of used food as larvae showed significant attraction to used food from which larvae are removed but not to fresh food supplemented with larvae.

Following the observation of robust attraction by focal larvae to other larvae, another experiment tested whether larvae were capable of learning novel cues associated with other larvae (Durisko & Dukas, in press). Work by Durisko & Dukas (in press) demonstrated the ability of focal larvae to prefer cues previously paired with food occupied by other larvae over cues previously paired with unoccupied food. In a learning experiment, focal larvae exhibited a preference for novel odours previously paired with food occupied by other larvae over novel odours previously paired with unoccupied food.

Social information use in adults

Social information use in adults fruit lies have been reported in several studies. One study examining the preference of females to lay eggs, found a greater number of females laying eggs on novel food present with mated models than on novel food alone (Sarin & Dukas, 2009). Fruit fly social learning has also been observed in Mery et al's (2009) study of mate choice. Females exhibited a preference to males of a certain colour following exposure to those males copulating, over males of colour who were not seen copulating. Durisko et al.'s (submitted) study of egg laying preference, found a preference of focal females to

lay eggs on novel food present with larvae over novel food unoccupied by larvae. Recent work by Durisko et al. (submitted) extended their larval work and examined social attraction and social learning in adult male and female fruit flies. Adult male and female fruit flies showed a significant attraction to odours emanating from foraging larvae, as they preferred vials containing larvae and used food over vials containing unused food (Durisko et al., submitted). Additionally, adults learned to prefer odours from food substrates occupied by larvae over odours from unoccupied substrates of similar quality (Durisko et al., submitted.) in a similar learning experiment.

Host-microbial interactions

In an attempt to determine the origin of the volatiles that are attractive to the flies, Durisko & Dukas (in prep) hypothesized that the prominent compound of larval fecal matter, ammonia (Borash et al. 1998), served as the attractant. However, odour preference tests with varying concentrations of ammonia solutions ruled out ammonia as the attractant (Durisko & Dukas, in prep). Growing evidence of bacteria as influential microorganisms of hosts suggested the closer examination of host-microbial interactions. Interactions between bacteria and their animal hosts are plentiful in nature and occur in many different hosts. *Buchnera aphidicola*, a bacterial endosymbiont of the pea aphid (*Acyrthosiphon pisum*) confers resistance to *Aphidius erv*i, a natural enemy of to the aphid host (Oliver et al., 2003). While infection of the aphids by the ovipositing parasitoid is equal in aphids with or without *B. aphidicola*, infected aphids reduced parasitoid larval development resulting in their mortality (Oliver et al., 2003). Work by Bouskra et al. (2008) revealed developmental defects in germ-free mice. Development and maturation of isolated lymphoid follicles were compromised in germ-free mice (Bouskra et al., 2008). Infection of the germ-free mice with their gut bacteria resulted in the normal development of these structures

(Bouskra et al., 2008). The gut bacteria of rats have also proven to be an important factor in stimulating and maintaining the mucosal barrier (Szentkuti et al., 1990). The mucus layer overlaying the GI-tract epithelium of conventional rats was twice as thick as that of germ-free rats (Szentkuti et al., 1990).

While host-microbial interactions can encompass various relationships such as colonization, defined as "a state in which the microbe may be present in the host for a variable duration of time" (Casadevall & Pirofski, 2000) and infection, described as the "acquisition of a microbe by a host" (Casadevall & Pirofski, 2000), our focus will remain on the commensal relationship between hosts and microbes. Commensalism comes from a latin term meaning ''eating at the same table'' and refers to "a host-microbial interaction that does not result in perceptible, ongoing, and/or persistent host damage" (Casadevall and Pirofski, 2000). "Commensal bacteria colonize their host generally at birth, through vertical transfer, and are acquired constantly during the host life from the environment through ingestion" (Storelli et al., 2000).

Fruit fly-microbial interactions

Fruit flies are excellent model system to study host-microbial interactions owing to their simple nature with less diverse microbiota than mammals. Storelli et al. (2011) studied the contribution of *Drosophila* microbiota by comparing the timing of adult emergence in germ free and conventionally reared siblings. A significant delay in growth was observed between fruit flies experimentally deprived of bacteria compared to conventional flies when larvae were reared on poor-nutrient conditions but not when raised on rich-nutrient conditions (Storelli et al., 2011). Poor-nutrient conditions refer to a diet containing only 10% of the regular yeast extract and resulted in a 2.5 day delay in adult emergence for conventionally reared individuals and an additional 2.9 day delay for germ free adults (Storelli et al., 2011). Ridley et al. (2012) also assessed the impact of

eliminating the microbiota by examining indices such as survival to adulthood, adult weight, fecundity, nutritional status, and metabolic rate. While indices such as survival to adulthood, adult weight, fecundity did not differ between the conventional and axenic treatments, development time to adulthood differed significantly (Ridley et al. (2012). Slower development of larvae was noted for adults of the axenic treatment as a median value of 1-day delay was observed the axenic treatment (Ridley et al. (2012). Work by Sharon et al. (2010) revealed a role of the commensal bacteria of *D. melanogaster* in mating preferences. Antibiotic supplementation to fly media abolished positive assortative mating preference to random. An infection experiment with the supplementation of *Lactobacillus plantarum* revealed a similar mating preference to flies prior to antibiotic treatment and suggested a role of the symbiotic bacteria in mating preference. The impact of the elimination of microbiota in the above studies collectively makes bacterial odour a strong candidate as the attractant. Although we suspected microbes, we limited our analysis to bacteria as we supplemented the lab diet with antifungals. Larvae may be less attracted to food consumed by conspecifics if it is experimentally deprived of microbiota.

Gut bacteria of the fruit fly

The diversity of microbiota in animal guts reveals the presence of two ecologically distinct forms: the resident (autochthonous) and non-resident (allochthonous) taxa (Wong et al., 2011). "Autochthonous strains have a long-term association with a particular host, and they form stable populations of a characteristic size in a particular region of the gut" (Walter, 2008). Allochthonous taxa on the other hand "are ingested with, and pass through, the gut with the food" (Wong et al., 2011). Work by Wong et al. (2011) overcomes 3 major limitations of previous studies attempting to characterize the bacteria of fruit flies. Wong et al. (2011) attempted to identify low-abundance taxa that may have been missed in

previous studies, bacteria specific to the gut, and minimized the incidence of non-resident allochthonous groups which may have inflated the microbial diversity reported previously. Although the abundance of taxa varied as a function of developmental age, the taxa most common among samples included *Acetobacter pomorum*, *Acetobacter tropicalis*, *Lactobacillus brevis*, *Lactobacillus fructivorans* and *Lactobacillus plantarum* (Wong et al., 2011). *A. pomorum*, *A. tropicalis*, *L. brevis*, *L. fructivorans* and *L. plantarum* accounted for 1.75%, 3.56%, 22.42%, 4.3%, 60.9%, respectively, of the reads in third-instar larvae. *L. brevis* and *L. plantarum* were strongly represented in third-instar larvae (Wong et al., 2011), the focal individuals of most of our experiments.

Lactic acid bacteria

"Bacteria belonging to the genus *Lactobacillus* are members of the lactic acid bacteria (LAB), a broadly defined group characterized by the formation of lactic acid as the sole or main end product of carbohydrate metabolism" (Walter, 2008). Storelli et al. (2011) examined the ability of *Lactobacillus plantarum* to accelerate larval growth upon nutrient scarcity and found that the addition of *Lactobacillus plantarum* to poor medium conditions was sufficient to accelerate larval growth and resulted in earlier emergence of adults (Storelli et al. 2011). Sharon et al. (2010) conducted infection experiments to determine whether *Lactobacillus plantarum* could restore the abolished mating presence of *Drosophila melanogaster* following antibiotic treatment. *Lactobacillus plantarum* isolated from the flies was partially responsible for the mating preference. Work by Tannock (1992) in rodents, chickens and pigs describes an "adherence of lactobacilli to the surface of the nonsecretory epithelium lining of these sites, which enables the bacteria to form a biofilm-like structure that provides a bacterial inoculum of the digesta". More recent work by Storelli et al. (2011) illustrates *Lactobacillus plantarum*'s ability to reside in the midgut and "resist the

passage through the digestive tract of its host" (Storelli et al., 2011). This suggests the closer examination of bacteria residing in the gut of *Drosophila* larvae as the attractant.

GENERAL METHODS

We conducted population maintenance of *Drosophila melanogaster* Canton S in a population cage kept at 25°C, 60% relative humidity (RH), on a 12:12 hour light/dark cycle with lights on at 1 am. The Canton S population cage is supplied with 2 bottles of the standard lab diet and 1 bottle of the standard lab diet sprinkled with live yeast for population maintenance egg collection. 1 L of the standard lab diet contains 75 g cornmeal, 20 g agar, 60 g dextrose, 30 g sucrose, 32 g yeast and 2 g methyl paraben. We collected eggs for experiments using 100 mm Petri dishes of standard food media. We collected eggs for the axenic conditions approximately 24 hours prior to egg laying for the standard conditions to control for size disparities that occur between the standard and axenic larvae when eggs are collected for both conditions at the same time.

We generated axenic cultures using the protocol used by Brummel et al (2004). Under a laminar flow cabinet, we created axenic cultures by sterilizing 12 hour embryos with 2 minutes of immersion in 2.5% sodium hypochlorite, followed by 2 washes each with 70% ethanol and sterile distilled water. We transferred sterilized embryos to autoclaved axenic food dishes, which consisted of autoclaved standard food supplemented with ampicillin (50 mg/l food) and chloramphenicol (20 mg/l) and methyl paraben (2 g/l) and fluconazole (10 mg/l). We verified the achievement of axenic cultures by plating homogenates on LB agar plates. For standard flies, we washed 12-hour embryos 4 times with sterile distilled water before transferring to standard food dishes supplemented only with methyl paraben. We transferred Petri dishes containing standard or axenic embryos to a Tupperware chamber maintained at 25°C, 90% RH and kept in total

darkness. For all experiments, an observer blind to the experimental treatments recorded the data.

CHAPTER 1: ORIGIN OF VOLATILES ATTRACTIVE TO FLIES

1a. Are larvae attracted to bacterial volatiles?

Rationale

The purpose of this experiment was to test whether bacterial volatiles serve as the attractant to larvae displaying attraction to a substrate frequented by others. We predicted that focal larvae would show no difference in attraction to axenic used food with axenic larvae versus axenic fresh food. As in previous research, we expected that focal larvae would be more attracted to standard used food with larvae than to standard fresh food.

Protocol

The experimental setup and design is a modified version of the social attraction protocol described by Durisko and Dukas (in press). We randomly selected axenic mid third-instar larvae (approximately 120 hours after egg-laying) as characterized by morphological traits such as large mouth parts and orange ringed posterior spiracles and behavioural traits such as voracious foraging as focals individuals. We stayed away from late third-instar larvae characterized by wandering behaviour in which larvae cease to forage and search a site for metamorphosis instead. Focal larvae are maintained on low-density dishes (~65 focals/ 100mm dish) prior to testing. We tested one focal individual at a time in a trial alternating between 1 of 2 food type conditions (standard or axenic). We randomly used 128 axenic third-instar larvae as focal individuals for this experiment. One focal individual at a time was placed (head pointing up) in the center of a 100 mm agar Petri dish through a 1 cm opening in the lid.

Depending on the trial (standard or axenic), focal larvae had a choice between 1) standard used food occupied and consumed by 30 standard larvae for 24 hours and standard fresh food or 2) axenic used food occupied and consumed by 30 axenic larvae for 24 hours and axenic fresh food. The 2.5 ml food disks were located 0.5 cm equidistantly from the midline and alternated sides between trials. We recycled pairs of used and fresh food disks for 6 trials but used a new agar dish for each trial. We recorded the choice of focal larvae, as indicated by physical contact with a food disk, within a maximum time window of 5 minutes. We excluded from the analyses the focal larvae that did not make a choice within 5 mins. We analyzed larval choices using generalized linear models with a binomial distribution, logit link function, and included side chosen as a factor. We also compared the frequency of choices between treatments using generalized linear models with a binomial distribution, logit link function, and side as a factor.

Results

While larvae showed significant preference for the standard used food with standard larvae over standard fresh food (66.1%, N = 62, GzLM: $\chi^2_{1} = 6.2$, p = 0.01), they showed no preference between the axenic used food with axenic larvae and axenic fresh food (47.5%, N = 59, GzLM: $\chi^2_{1} = 0.138$, p = 0.8). Larval preference for the used food was significantly higher when it was standard than axenic (GzLM: $\chi^2_{1} = 4.1$, p = 0.04), but they showed no significant side preference (GzLM: $\chi^2_{1} = 0.001$, p = 1.0).

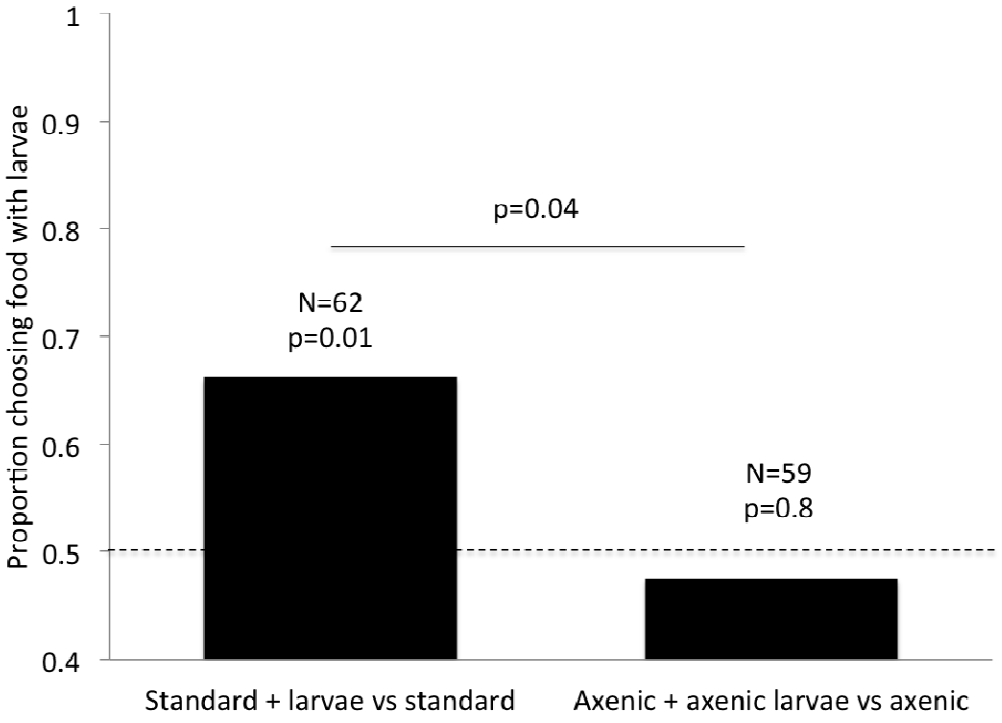

Figure 1. Focal larvae were observed in a binary choice task. Focals were given either a choice between standard used food with standard larvae and standard fresh food (left) or a choice between axenic used food with axenic larvae and axenic fresh food (right).

1b. Are adults attracted to bacterial volatiles?

Rationale

The purpose of this experiment was to test whether bacterial volatiles also serve as the attractant to mated females displaying social attraction to a substrate frequented by others. We predicted that focals would show no difference in attraction to axenic used food with axenic larvae versus axenic fresh food. As in previous research, we expected that focals would be more attracted to standard used food with larvae than to standard fresh food.

Protocol

The experimental setup and design will be a modified version of the adult male attraction to larvae protocol described by Durisko et al. (submitted) Sexing of adult flies took place post-clearing within eight hours of eclosion, three days prior to testing. We randomly selected 180 3-day-old mated females as focal individuals for this experiment. We tested one focal individual at a time in a trial alternating between 1 of 2 food type conditions (standard or axenic). One focal individual at a time was gently aspirated into a cage containing two vials located in the posterior corners of each cage. Stimuli for the adult social attraction experiment consisted of vials containing approximately 5 ml of food.

Depending on the trial (standard or axenic), focals had a choice between 1) standard used food occupied and consumed by 30 standard larvae for 24 hours and standard fresh food or 2) axenic used food occupied and consumed by 30 axenic larvae for 24 hours and axenic fresh food. Funnels were placed atop the two vials to trap the focal adults after making a choice. Sixteen hours later, the choice of focal adults, indicated by their presence inside vials, was recorded. We conducted three replicates of this experiment during which 60 females were tested per replicate. We excluded from the analyses the focals that did not enter a vial.

We analyzed adult female choices using generalized linear models with a binomial distribution, logit link function, and included side chosen as a factor. We also compared the frequency of choices between treatments using generalized linear models with a binomial distribution, logit link function, and side as a factor.

Results

We replicated the social attraction results using mated females. While flies showed significant preference for the standard used food with standard larvae over standard fresh food (69.3%, N = 88, GzLM: $\chi^2_{1} = 12.5$, p = 0.000), they showed no preference between the axenic used food with axenic larvae and axenic fresh food (53.4%, N = 88, GzLM: $\chi^2_{1} = 0.455$, p = 0.5). Larval preference for the used food was significantly higher when it was standard than axenic (GzLM: $\chi^2_{1} = 4.5$, p = 0.03), but they showed no significant side preference (GzLM: $\chi^2_{1} = 0.000$, p = 1.0).

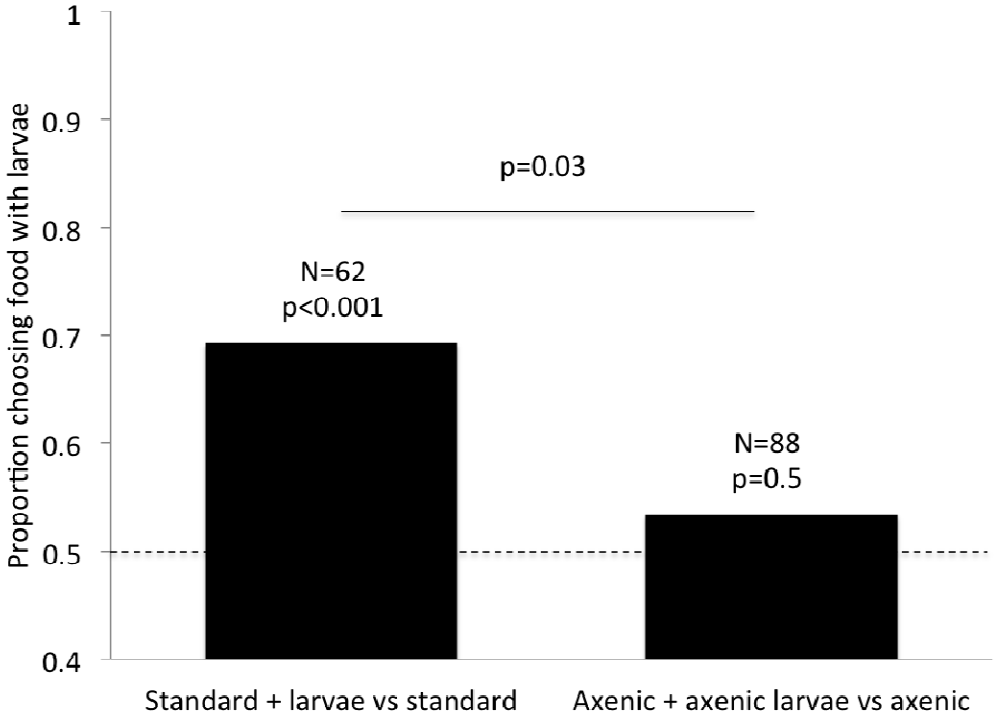

Figure 2. Mated females were observed in a binary choice task. Depending on the treatment, focals were given a choice between standard used food with standard larvae and standard fresh food (left) or axenic used food with axenic larvae and axenic fresh food (right).

1c. Are larvae attracted to volatiles of the internal bacterium we isolated from larvae?

Rationale

The purpose of this experiment was to test whether larvae are attracted to volatiles of the internal bacterium we isolated from larvae. We predicted that focal larvae would be more attracted to axenic used food with axenic larvae and the internal bacterium isolated from larvae than to axenic fresh food. As in previous research, we expected that focal larvae would be more attracted to standard used food with larvae than to standard fresh food.

Protocol

The experimental setup and design is a modified version of the social attraction protocol described by Durisko and Dukas (in press). We maintained focal individuals on low-density dishes (~65 focals/ 100mm dish) prior to testing. We randomly used 120 axenic third-instar larvae as focal individuals and tested one focal individual at a time in a trial alternating between 1 of 2 food type conditions (standard or axenic + *L. brevis*). For the standard condition, approximately 24 hours prior to testing, we transferred 30 standard larvae per 2.5 ml of new standard food disks for consumption. For the axenic + *L. brevis* condition, approximately 24 hours prior to testing, we transferred 30 axenic larvae and 50 µl of *L. brevis* per 2.5 ml of axenic food disks, which consisted of autoclaved standard food supplemented with two antifungals (methyl paraben and fluconazole) and no antibiotics. One focal individual at a time was placed (head pointing up) in the center of a 100 mm agar Petri dish through a 1 cm opening in the lid.

Depending on the trial (standard or axenic + *L. brevis*), focal larvae had a choice between 1) standard used food occupied and consumed by 30 standard

larvae for 24 hours and standard fresh food or 2) axenic used food occupied and consumed by 30 axenic larvae and *L. brevis* for 24 hours and axenic fresh food. The 2.5 ml food disks were located 0.5 cm equidistantly from the midline and alternated sides between trials. We recycled pairs of used and fresh food disks for 6 trials but used a new agar dish for each trial. We recorded the choice of focal larvae, as indicated by physical contact with a food disk, within a maximum time window of 5 minutes. We excluded from the analyses the focal larvae that did not make a choice within 5 min. We analyzed larval choices using generalized linear models with a binomial distribution, logit link function, and included side chosen as a factor. We also compared the frequency of choices between treatments using generalized linear models with a binomial distribution, logit link function, and side as a factor.

In order to isolate the internal bacteria from larvae, we transferred 25 third-instar larvae into a 12 ml two-staged centrifuge tube. We washed these larvae twice with 70% ethanol and twice with sterile distilled water. Using a sterilized loop, we crushed the larvae and streaked the liquid remains on a Lactobacilli MRS plate, a special growth medium that fosters good growth of a number of *Lactobacilli* strains (De Man et al., 1960).

We incubated the Lactobacilli MRS plate in a high humidity chamber at 25°C. Following growth of the bacteria, we streaked out cells from a single colony onto a second plate of Lactobacilli MRS to achieve a pure culture. Using a sterilized loop, we transferred cells from a single colony to an Erlenmeyer flask containing 100 ml of Lactobacilli MRS Broth incubated at 31°C on a rotary shaker. Prior to inoculation, to prevent carryover of medium components, we centrifuged 12 ml of the bacterial culture in each of two two-stage capped test tubes at 4000 rpm for 4 minutes.

We conducted DNA extractions with an alkaline lysis miniprep procedure (Miller 1992). We added cells to a 1.5 ml microfuge tube containing 500 ml of

nuclease free water. We centrifuged the tube at 13000 rpm for 5 minutes. We then discarded the supernatant and resuspended the pellet in 467 µl of TE buffer. We added 30 µl of 20% SDS and 3 µl of proteinase K before incubating for an hour at 37°C. We repeatedly added phenol-chloroform and followed-up with vortexing and centrifugation. We precipitated the DNA by adding 100% ethanol and 50 µl sodium acetate. Following subsequent vortexing, centrifugation and washing with 70% ethanol, we dried the pellet by incubating the tube for 30 minutes at 37°C. The primers we used for the 16S PCR are 5'-GTGCCAGGMGCCGCGGTAA and 5'-CCGTCAATTCMTTTRAGTTT. We used gel electrophoresis and UV light exposure for PCR product visualization. The PCR products were sequenced by fluorescence-based DNA sequencing (Mobix Laboratory, McMaster University, Hamilton). The sequences were then used to search for homologs using the Blast program at the National Center for Biotechnology Information.Identification of the bacteria using 16S PCR yielded *L. brevis*.

Results

While larvae showed significant preference for the standard used food with standard larvae over standard fresh food (67.6%, N = 37, GzLM: $\chi^2_{1} = 7.7$, p = 0.005), they also showed a significant preference for the axenic used food with axenic larvae and *L. brevis* over axenic fresh food (67.1%, N = 70, GzLM: $\chi^2_{1} = 5.3$, p = 0.02). Larval preference for the used food was not significantly higher when it was standard than axenic with *L. brevis* (GzLM: $\chi^2_{1} = 1.5$, p = 0.2), and they showed no significant side preference (GzLM: $\chi^2_{1} = 0.08$, p = 0.8).

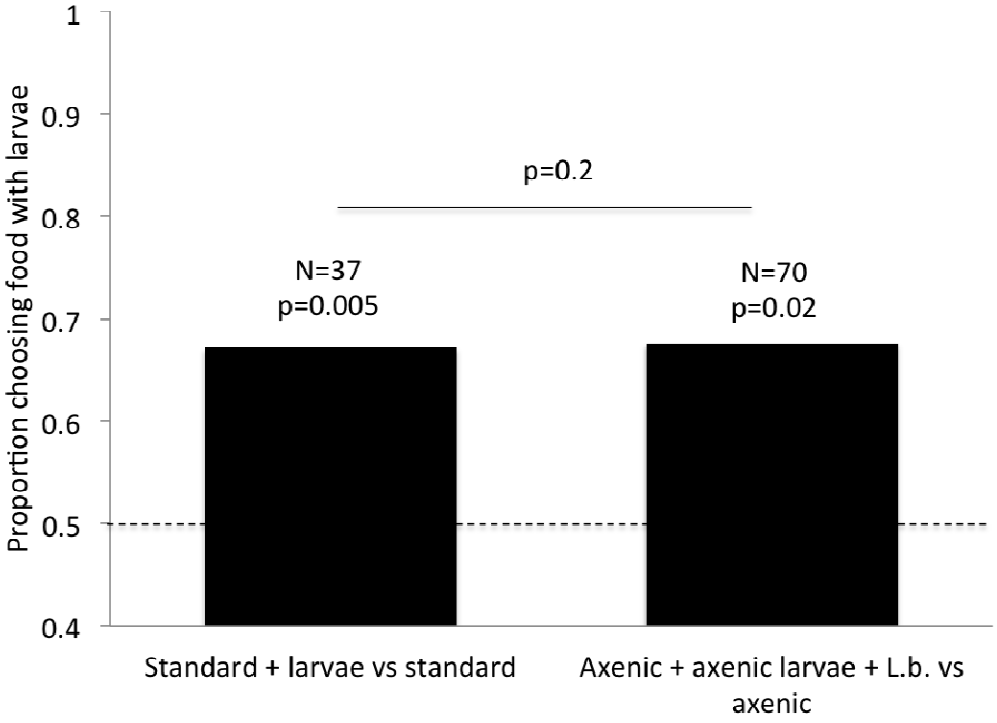

Figure 3. Focal larvae were observed in a binary choice task. Depending on the treatment, focals were given a choice between standard used food with standard larvae and standard fresh food (left) or axenic used food with axenic larvae and *L. brevis* and axenic fresh food (right).

1d. Are larvae attracted to volatiles of a common internal bacterium isolated from larvae in other labs?

Rationale

The purpose of this experiment was to test whether larvae are attracted to volatiles of a common internal bacterium of larvae as indicated in other laboratories. We predicted that focal larvae would be more attracted to axenic used food with axenic larvae and the common internal bacterium isolated from larvae in other labs than to axenic fresh food. As in previous research, we expected that focal larvae would be more attracted to standard used food with larvae than to standard fresh food.

Protocol

The experimental setup and design is a modified version of the social attraction protocol described by Durisko and Dukas (in press). We maintained focal individuals on low-density dishes (~65 focals/ 100mm dish) prior to testing. We randomly used 199 axenic third-instar larvae as focal individuals and tested one focal individual at a time in a trial alternating between 1 of 2 food type conditions (standard or axenic + *L. plantarum*). One focal individual at a time was placed (head pointing up) in the center of a 100 mm agar Petri dish through a 1 cm opening in the lid.

Depending on the trial (standard or axenic + *L. plantarum*), focal larvae had a choice between 1) standard used food occupied and consumed by 30 standard larvae for 26 hours and standard fresh food or 2) axenic used food occupied and consumed by 30 axenic larvae for 26 hours and 50 µl of *L. plantarum* for 2.5 hours and axenic fresh food. The 2.5 ml food disks were located 0.5 cm equidistantly from the midline and alternated sides between trials. We recycled pairs of used and fresh food disks for 6 trials but used a new agar dish for

each trial. We recorded the choice of focal larvae, as indicated by physical contact with a food disk, within a maximum time window of 5 minutes. We excluded from the analyses the focal larvae that did not make a choice within 5 mins. We analyzed larval choices using generalized linear models with a binomial distribution, logit link function, and included side chosen as a factor. We also compared the frequency of choices between treatments using generalized linear models with a binomial distribution, logit link function, and side as a factor.

We cultured *L. plantarum*, obtained from the American Type Culture Collection (ATCC strain 14917), in an Erlenmeyer flask containing 100 ml of Lactobacilli MRS Broth incubated at 37°C on a rotary shaker. Prior to inoculation, to prevent carryover of medium components, we centrifuged 10 ml of the bacterial culture in each of two 12 ml two-stage capped test tubes at 4000 rpm for 4 minutes.

Results

While larvae showed significant preference for the standard used food with standard larvae over standard fresh food (73.1%, N = 93, GzLM: $\chi^2_{1} = 18.4$, p = 0.000), they also showed a significant preference for the axenic used food with axenic larvae and *L. plantarum* over axenic fresh food (62.5%, N = 80, GzLM: $\chi^2_{1} = 4.6$, p = 0.03). Larval preference for the used food was not significantly higher when it was standard than axenic with *L. plantarum* (GzLM: $\chi^2_{1} = 2.4$, p = 0.1), and they showed no significant side preference (GzLM: $\chi^2_{1} = 0.728$, p = 0.4).

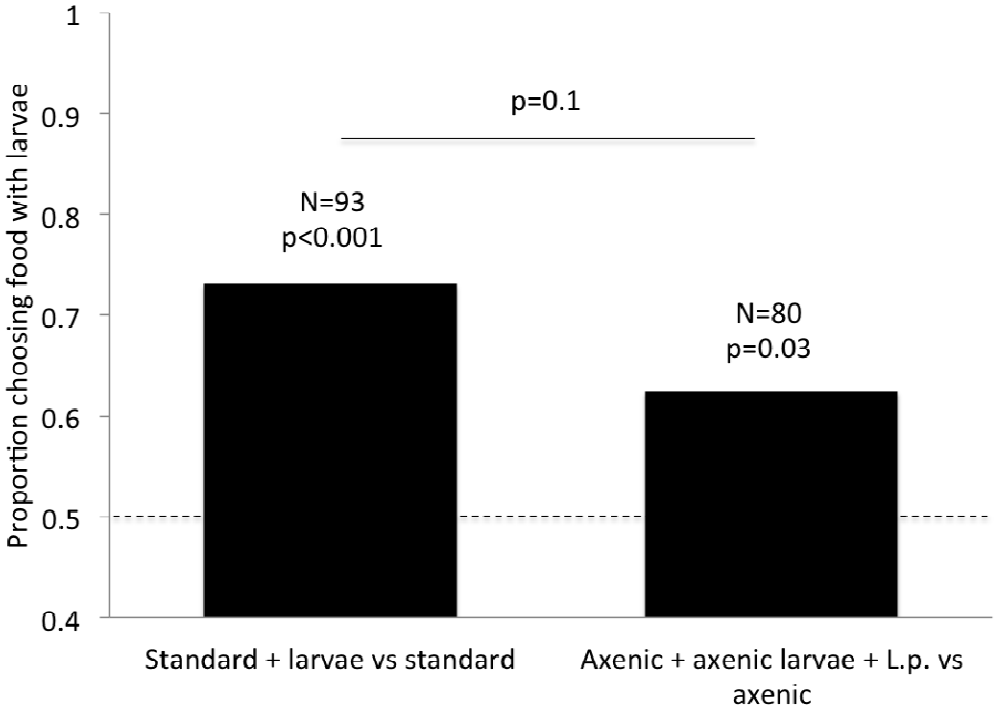

Figure 4. Focal larvae were observed in a binary choice task. Depending on the treatment, focals were given a choice between standard used food with standard larvae and standard fresh food (left) or axenic used food with axenic larvae and *L. plantarum* and axenic fresh food (right).

CHAPTER 2: ATTRACTIVENESS OF *L. BREVIS* TO LARVAE

2.1 Are *L. brevis* attractive on their own, or only in the presence of other larvae?

Rationale

The purpose of this experiment was to test whether *L. brevis* are attractive to larvae on their own or only in the presence of larvae. We predicted that focal larvae would be more attracted to a few types of media when supplemented with L. brevis than when they are bacteria-free.

Protocol

The experimental setup and design is a modified version of the social attraction protocol described by Durisko and Dukas (in press). We maintained focal individuals on low-density dishes (~65 focals/ 100mm dish) prior to testing. We randomly used 200 axenic third-instar larvae as focal individuals and tested one focal individual at a time in a trial alternating between 1 of 3 conditions (*Lactobacilli* MRS, axenic food, and axenic food with axenic larvae). For the scratched *Lactobacilli* MRS + *L. brevis* disks, approximately 24 hours prior to testing, we transferred 50 µl of *L. brevis* per 2.5 ml of axenic *Lactobacilli* MRS agar scratched on the surface and bottom with a sterilized needle to create grooves. For the scratched *Lactobacilli* MRS disk, we only scratched the *Lactobacilli* MRS agar with a sterilized needle. For the scratched axenic food + *Lactobacilli brevis* disks, approximately 24 hours prior to testing, we transferred 50 µl of *L. brevis* per 2.5 ml of axenic food disks scratched with a sterilized needle. The axenic food consisted of autoclaved standard food supplemented with 1 ml and 5 ml of fluconazole and methyl paraben, respectively. For the scratched axenic food, we only scratched the axenic food disks, which consisted of autoclaved standard food supplemented with 1 ml and 5 ml of fluconazole and

methyl paraben, respectively. For the axenic food with axenic larvae and *Lactobacilli brevis* disks, approximately 24 hours prior to testing, we transferred 30 axenic larvae and 50 µl of *L. brevis* per 2.5 ml of axenic food disks, which consisted of autoclaved standard food supplemented with 1 ml and 5 ml of fluconazole and methyl paraben, respectively. For the axenic food with axenic larvae, we transferred only 30 axenic larvae per 2.5 ml of axenic food disks, which consisted of autoclaved standard food supplemented with 1 ml and 5 ml of fluconazole and methyl paraben, respectively. One focal individual at a time was placed (head pointing up) in the center of a 100 mm agar Petri dish through a 1 cm opening in the lid.

Depending on the treatment, focal larvae had the choice between 1) scratched axenic *Lactobacilli* MRS agar with *Lactobacilli brevis* and scratched axenic *Lactobacilli* MRS agar 2) scratched axenic food with *L. brevis* and scratched axenic fresh food 3) axenic food with axenic larvae and *L. brevis* and axenic food with axenic larvae. The stimuli disks for treatments excluding larvae were scratched in order to mimic an effect of foraging by the larvae. Perhaps the foraging behaviour of the larvae increases the surface area and allows the volatiles of the medium to dissipate better. The food disks were located 0.5 cm equidistantly from the midline and alternated sides between trials. We recycled pairs of used and fresh food disks for 6 trials but used a new agar dish for each trial. We recorded the choice of focal larvae, as indicated by physical contact with a food disk, within a maximum time window of 5 minutes. We excluded from the analyses the focal larvae that did not make a choice within 5 mins. We analyzed larval choices using generalized linear models with a binomial distribution, logit link function, and included side chosen as a factor. We also compared the frequency of choices between treatments using generalized linear models with a binomial distribution, logit link function, and side as a factor. Post-hoc pairwise comparisons were conducted using the sequential Bonferroni method adjusting for

multiple comparisons.

Results

Larvae showed a significant preference for *L. brevis* on scratched MRS agar (66.0%, N = 50, GzLM: $\chi^2_{1} = 4.9$, p = 0.03 and axenic used food with axenic larvae (65.3%, N = 49, GzLM: $\chi^2_{1} = 4.9$ p = 0.03). Larvae did not show a significant preference for *L. brevis* on scratched axenic food (44.9%, N = 49, GzLM: $\chi^2_{1} = 0.568$, p = 0.451) or a significant side preference (GzLM: $\chi^2_{1} = 0.202$, p = 0.653). Adjusting for multiple comparisons revealed no significant differences between the three treatments (all p > 0.07). Using plate counting to estimate the number of viable bacterial cells in both types of media revealed a statistically significant difference (t = 33.98, p<0.001) in the number of colony-forming units (cfu). With a mean ± SE of 5065 cfu/50 ml ± 43.98 of *L. brevis* on MRS agar and a mean of 2275 cfu/50 ml ± 69.36 of *L. brevis* on axenic food.

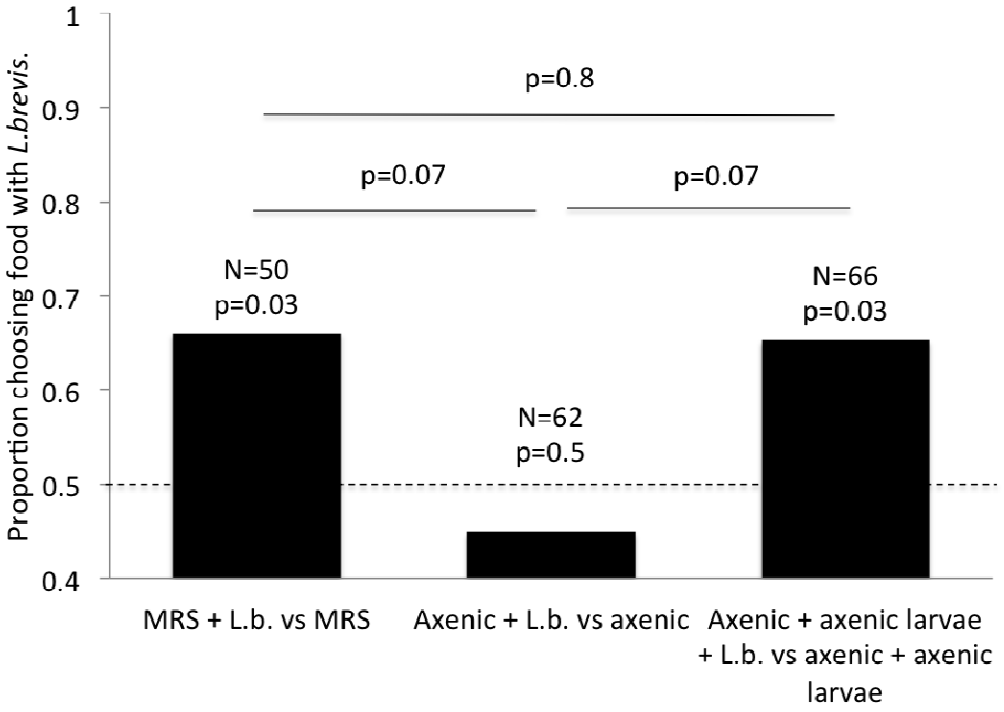

Figure 5. Focal larvae were observed in a binary choice task. Depending on the treatment, focals were given a choice between scratched MRS food and scratched MRS food with *L. brevis* (left) or scratched axenic food and scratched axenic food with *L. brevis* (middle) or axenic food with axenic larvae and axenic food with axenic larvae and *L. brevis* (right).

CHAPTER 3: PERCEPTION OF FOOD WITH *L. BREVIS*

3.1 Do larvae perceive food with *L. brevis* as better than axenic food?

Rationale

The purpose of this experiment was to test whether larvae perceive food with *L. brevis* volatiles as better than axenic food. We predicted that focal larvae would be more attracted to novel odours paired with standard used food over novel odours paired with axenic used food.

Protocol

The experimental setup and design is a modified version of the social learning protocol described by Durisko and Dukas (in press). We randomly selected 96 axenic mid third-instar larvae (approximately 120 hours after egg-laying) as focal individuals and provided them droplets of blue food colouring 24 hours prior to testing. Each focal individual was provided with 6 3-minute trainings. One focal individual at a time was trained by placing the larvae (head pointing up) in the center of a 100 mm agar Petri dish containing food and 2 odour cups (polypropylene NMR tube caps, Sigma).

Depending on the trial (control or treatment), focal larvae were trained with novel odours paired with either 1) standard used food occupied and consumed by 30 standard larvae for 24 hours and standard fresh food (control) or 2) standard used food occupied and consumed by 30 standard larvae for 24 hours and axenic used food occupied and consumed by 30 axenic larvae for 24 hours (treatment). For both treatments, we removed larvae consuming the food prior to testing. The novel odours used in this experiment were equally preferred by inexperienced larvae and consisted of 10 µl 1-butanol and 10 µl of propyl acetate diluted in paraffin oil (1:300). Between each training session, focal larvae were rinsed with a fresh droplet of water. We recycled pairs of training food disks for 2

trials but used a new agar dish for each test. Following the 6 training sessions, focal larvae were placed in the center of a 100 mm Petri dish and given the choice between two odour cups containing 10 µl of the respective odours placed atop 1 cm diameter axenic fresh food disks on opposite sides located 3 cm from the midline. We randomized the sides of the odours by shuffling the odour cups and perforated the lids of the test Petri dishes along the midline. We recorded the choice of focal larvae, as indicated by physical contact with a food disk, within a maximum time window of 10 minutes. We excluded from the analyses the focal larvae that did not make a choice within 10 mins. We analyzed larval odour choices using generalized linear models with a binomial distribution, logit link function, and included the order of training, the odour, side chosen, and relevant interactions as factors. We also compared the frequency of choices between treatments using generalized linear models with a binomial distribution, logit link function, and included the order of training, the odour, side chosen, and relevant interactions as factors.

Results

While flies showed significant preference for novel odours previously paired with standard used food over novel odours previously paired with standard fresh food (70.3%, N = 37, GzLM: $\chi^2_{1} = 5.2$, p = 0.02), they showed no preference for novel odours previously paired with standard used food over novel odours previously paired with axenic used food (54.3%, N = 35, GzLM: $\chi^2_{1} = 0.221$, p = 0.6). Larval preference for novel odours previously paired with standard used food was significantly higher when it was paired against standard fresh food than axenic used (GzLM: $\chi^2_{1} = 3.9$ p = 0.047), but they showed no significant side preference (GzLM: $\chi^2_{1} = 1.7$, p = 0.2).

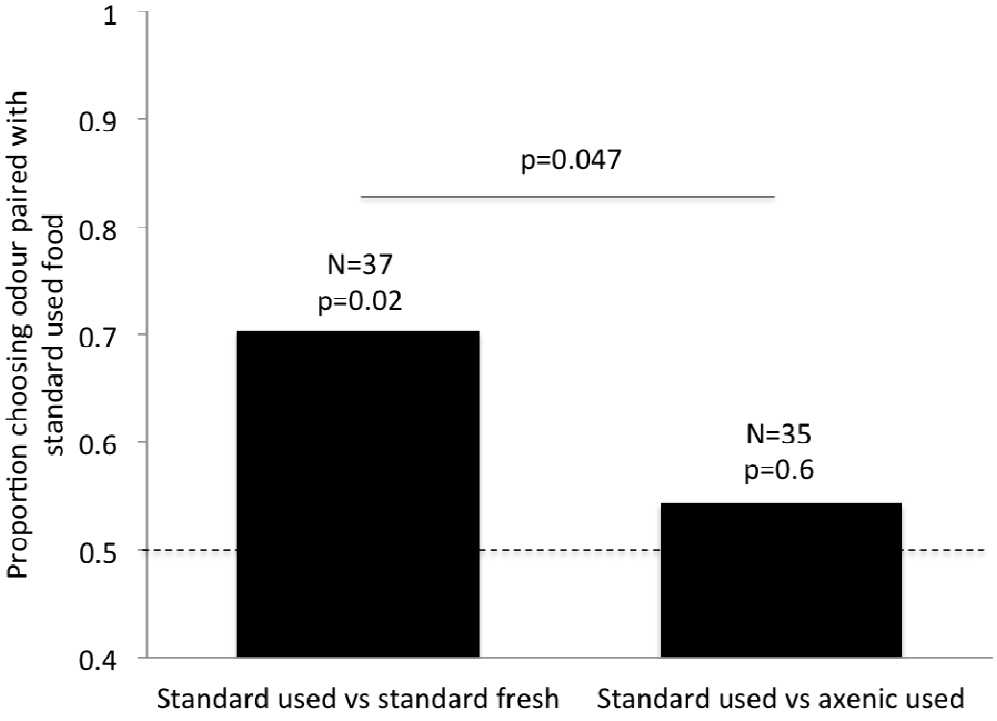

Figure 6. Focal larvae were observed in a learning task. Depending on the treatment, focals were given a choice between novel odours paired with standard used food over novel odours paired with standard fresh food (left) or novel odours paired with standard used food over novel odours paired with axenic used food (right).

CHAPTER 4: POTENTIAL BENEFIT OF *L. BREVIS*

4.1 Does *L. brevis* improve food quality?

Rationale

The purpose of this experiment was to test whether *L. brevis* improves food quality and to assess whether standard larvae placed on standard used food (used by standard larvae) perform better than standard larvae placed on axenic used food (used by axenic larvae). We predicted that the standard focal larvae would perform the worst on standard fresh food because food wouldn't be pre-dug as in other treatments, which may be beneficial to forage through. We predicted that standard focal larvae would perform best on artificially used food because the food would be pre-dug without an accumulation of toxic waste products such as ammonia (Borash et al., 1998). We predicted that standard focal larvae would perform equally on the standard used food and axenic used food because a potential benefit of *L. brevis*, if any, for larvae might be counteracted by an accumulation of toxic waste products such as ammonia.

Protocol

The experimental setup and design is a modified version of the protocol described by Durisko and Dukas (in press) to assess the developmental effects of foraging density. We adopted the three fitness parameters (larval development rate, egg-to-adult survival, and adult body mass) used by Durisko and Dukas (in press). We randomly used 720 standard eggs as focal individuals for this experiment. The experiment consisted of 4 treatments each containing 15 2.5 ml food disks of standard used, axenic used, artificially used, and standard fresh. We began by collecting eggs for the standard and axenic conditions as described above under General methods.

Following the development of axenic and standard eggs into mid third-instar larvae, depending on the used treatment (standard used or axenic used) we transferred 1) 30 standard larvae to 2.5 ml of standard food and 2) 30 axenic larvae to 2.5 ml of autoclaved standard food supplemented with 1 ml and 5 ml of fluconazole and methyl paraben, respectively. For the artificially used treatment, we scratched 2.5 ml food disks with a sterilized needle as in the previous experiment to imitate the texture of foraging larvae and increase the surface area of the food. The standard fresh food disks were not manipulated and were transferred with the other treatment disks to a high humidity Tupperware chamber maintained at 25°C and in total darkness. We removed larvae from the two used treatments 24 hours after transferring. We collected standard eggs as our focal individuals 2 hours prior to the removal of larvae from the used treatments. Following the removal of all larvae from the used treatments, we used sterilized brushes to transfer 12 standard focal eggs to each food disk. Prior to the expected beginning of pupation (at approximately 118 hours after egg laying), we recorded larval developmental rate by noting the number of larvae developing into pupae every 2 hours for 4 days. We also counted the number of adults eclosing in order to calculate the survival rate from eggs to adults. Throughout the monitoring of pupation and eclosion, we collected vials completely eclosed with adults and stored them in the freezer. Following the collection of all adults, we sexed adult flies, dried them in the oven at 70°C for 3 days and weighed 5 flies at a time on a microbalance for our measure of adult body mass. Larval developmental rate was analyzed using a generalized linear model with a gamma distribution and log link function. Egg-to-adult survival was analyzed using a univariate ANOVA. Adult body mass was analyzed using a generalized linear model with a gamma distribution, log link function, and sex as an independent factor. Post-hoc pairwise comparisons utilized the sequential Bonferroni method adjusting for multiple comparisons.

Results

Development rate

Larvae developed significantly fastest in the axenic used and standard fresh treatments, intermediate in the standard used treatment, and then slowest in the artificially used treatment (Figure 7). The generalized linear model showed a significant effect of treatment (Wald χ^2_2=259.5, p=0.000). Post-hoc pairwise comparisons showed that each treatment was significantly different from the other three (all p<0.012) except the axenic used and standard fresh treatments (p=0.159).

Egg-to-adult survival

Analysis of egg survival to adulthood revealed no overall significant effect of treatment (F=1.23, p=0.307, Figure 8).

Adult body mass

The generalized linear model revealed a significant effect of treatment (Wald χ^2_2=31.7, p=0.000). As expected, analysis of adult body mass showed a significant effect of sex since sex-specific differences in body mass exists in fruit flies. Post-hoc pairwise comparisons showed that flies in the axenic treatment were significantly heavier than flies in all other treatments (all p<0.02). Post-hoc pairwise comparisons showed that flies of the standard used treatment, standard fresh treatment, and artificially used treatment were not significantly different from one another (all p>0.07).

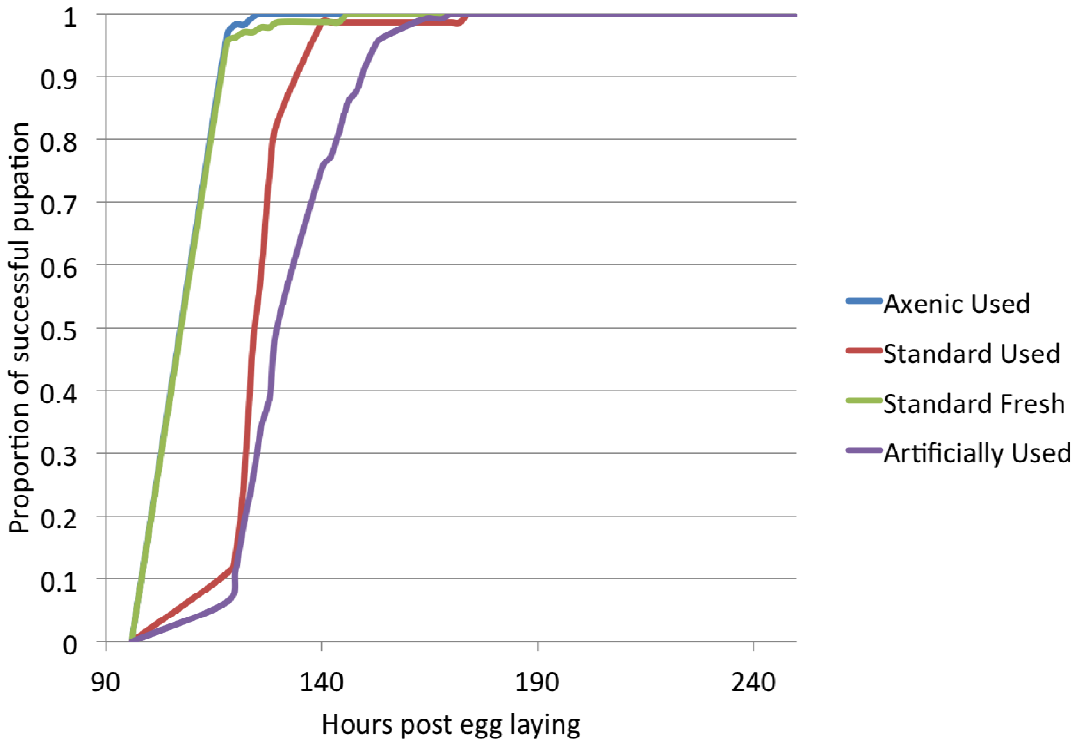

Figure 7. Larval development was observed for focal larvae reared in axenic used, standard used, standard fresh, and artificially used food.

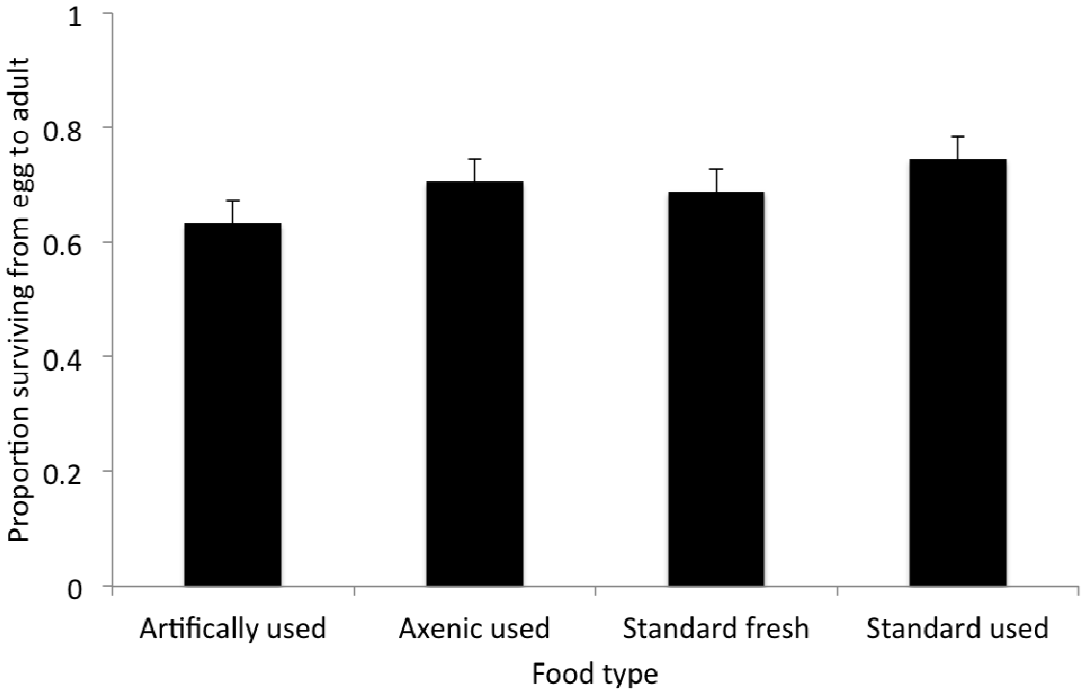

Figure 8. The proportion of focal larvae surviving to adulthood was observed for focal larvae reared in axenic used, standard used, standard fresh, and artificially used food.

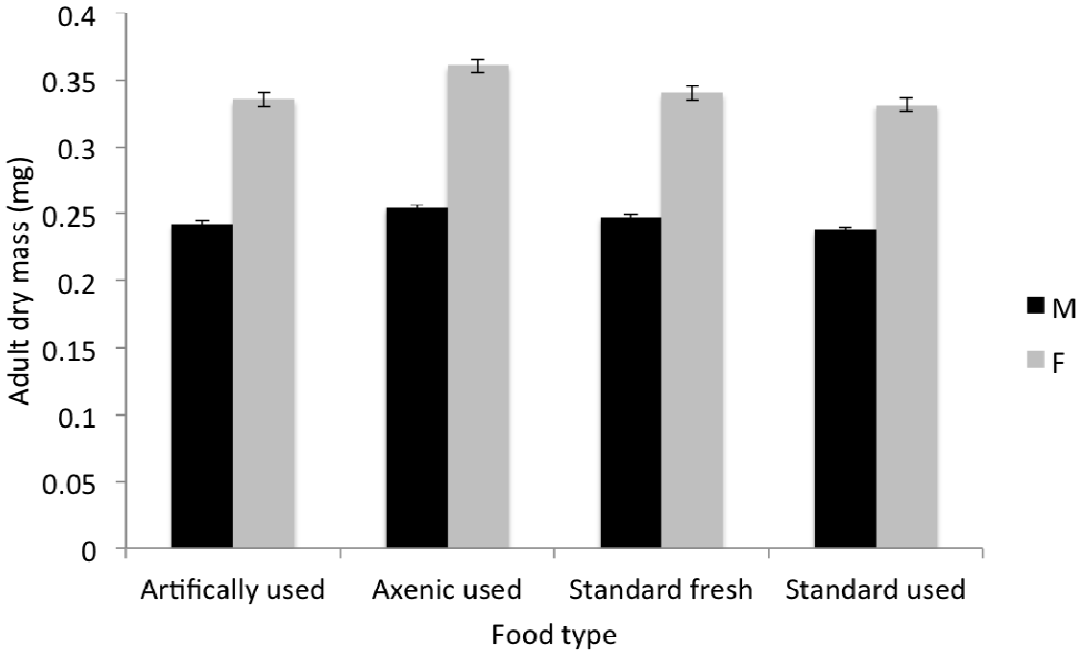

Figure 9. The adult dry mass of focal larvae surviving to adulthood was observed for focal larvae reared in axenic used, standard used, standard fresh, and artificially used food.

DISCUSSION

Major findings

In our first experiment investigating the origin of the volatiles that are attractive to flies, we tested whether larvae are attracted to bacterial volatiles. As in previous research, focal larvae were more attracted to standard used food with larvae than to standard fresh food but showed no difference in attraction to axenic used food with axenic larvae versus axenic fresh food (Figure 1). Consistent with our prediction, removal of the bacteria via sterilizations had abolished the social attraction observed in previous work (Durisko & Dukas, in press). This indicates that focal larvae do not merely show an attraction to food that has been occupied and consumed by conspecifics; rather the attractive odour responsible for the social attraction is the bacterial volatiles. Storelli et al. (2011) observed a significant delay in adult emergence between fruit flies experimentally deprived of bacteria compared to conventional flies when larvae were reared on poor-nutrient conditions. Furthermore, slower development of larvae was noted for adults of the axenic treatment compared to their conventionally reared siblings (Ridley et al. (2012). These results suggest that larvae are able to pay attention to volatiles of the food consumed by others.

We replicated the social attraction results using 3-day-old mated females, indicating that females as well show no preference between the axenic used food with axenic larvae and axenic fresh food and confirm that the attractive odour responsible for the social attraction is the bacterial volatiles (Figure 2).

Moreover, in our experiment testing whether larvae are attracted to volatiles of the internal bacterium we isolated from larvae, we predicted that the supplementation of the internal bacterium isolated from larvae to axenic used food would restore the social attraction observed in our previous experiment.

834 Consistent with our prediction, *L. brevi*s recapitulated the bacterial volatiles social
835 attraction effect (Figure 3).
836 In our other experiment, we tested a common internal bacterium isolated
837 from larvae in other labs, *L. plantarum*, as a potential attractant. Given the ability
838 of *L. plantarum* to accelerate larval development in germ free individuals upon
839 poor-nutrient conditions (Storelli et al., 2011) on its own, we predicted that the
840 supplementation of *L. plantarum* would also restore the social attraction observed
841 in our previous experiments. Consistent with our prediction, *L. plantarum* also
842 recapitulated the bacterial volatiles social attraction effect (Figure 4).
843 In sum, our results illustrate that *L. brevis* and *L. plantarum,* commensal
844 bacteria of D. melanogaster, are sufficient to elicit the social attraction observed
845 previously (Durisko & Dukas, in press). This suggests that a single bacterial
846 species, such as *L. brevis* and *L. plantarum*, can reestablish the social attraction
847 effect evident in previous work (Durisko & Dukas, in press).
848 In our second experiment investigating the attractiveness of *L. brevis*, we
849 tested whether *L. brevis* are attractive to larvae on their own, or only in the
850 presence of other larvae. We predicted that focal larvae would be more attracted
851 to a few types of media when supplemented with *L. brevis* than when they are
852 bacteria-free. Focal larvae showed a significant preference for *L. brevis* on
853 scratched MRS agar and axenic used food with axenic larvae, but did not show a
854 significant preference for *L. brevis* on scratched axenic food (Figure 5).
855 This indicates that focal larvae do not merely show an attraction to media
856 containing *L. brevis* only in the presence of larvae; rather *L. brevis* is also
857 attractive to larvae on its own. Plate counting revealed a statistically significant
858 difference in the number of colony-forming units between the axenic food with *L.*
859 *brevis* and MRS agar with *L brevis*. A plausible explanation for the absence of
860 attraction to axenic food with *L. brevis* over bacteria-free axenic food is that *L.*
861 *brevis* did not grow well enough on the axenic food to elicit an attraction. Despite

a lack of attraction of larvae to the axenic food with *L. brevis,* our results still suggest that *L. brevis* is sufficiently attractive on its own to larvae and that an interaction of the larvae and bacteria is not necessary.

In our third experiment, investigating why larvae are attracted to *L. brevis*, we tested whether larvae perceive food with *L. brevis* as better than axenic food. We predicted that focal larvae would be more attracted to novel odours previously paired with standard used food over novel odours previously paired with axenic used food. As per previous research, larvae showed significant preference for novel odours previously paired with standard used food over novels odours previously paired with standard fresh. Interestingly, larvae showed no preference for novel odours previously paired with standard used food over novel odours previously paired with axenic used food, suggesting that experiencing the presence of bacteria does not increase the apparent quality of food.

In our final experiment investigating whether *L. brevis* improves food quality, we tested whether standard larvae placed on standard used food (used by standard larvae) perform better than standard larvae placed on axenic used food (used by axenic larvae). Contrary to our predictions, the three fitness parameters observed, larval development rate, egg-to-adult survival, and adult body mass, revealed inexplicable findings. The egg-to-adult survival was significantly lower than observed in previous research (Durisko & Dukas, in press). The experimental setup and design used a modified version of the protocol described by Durisko and Dukas (in press). In the protocol described by Durisko and Dukas (in press), focal eggs were placed in dishes of 2.5 ml of food. For easier adult collection, we opted to place focal eggs into vials instead of dishes. It is possible that there was a problem placing focal eggs into the narrow vials, which may have in turn reduced their survival. While larval development rate partially mirrored previous work (Golden & Dukas, unpublished) with larvae in the standard fresh treatment developing significantly faster than larvae in the artificially used treatment, adult

body mass for males and females were much higher than previously observed (Golden & Dukas, unpublished). Since our observations of the fitness parameters revealed differences from previous research (Golden & Dukas, unpublished), we will need to replicate our results before being able to interpret our data.

Comparison to other species

Bacteria as attractants have been documented in several studies examining other species. Work by Verhulst (2009) et al., illustrates the role of skin microbiota in the attraction of African malaria mosquitos, *Anopheles gambiae*, to human hosts. Upon examining the attraction of *A. gambiae* to volatile organic compounds produced by human skin microbiota, experimenters observed significantly more *A. gambiae* caught on blood agar containing skin microbiota than sterile blood agar. Hasselschwert and Rockett's (1988) investigation of the attractiveness of bacteria to gravid mosquitos, *Aedes aegypti* revealed bacteria such as *Bacillus cereus* and *Pseudomonas aeruginosa* as ovipositional attractants. Bacterial filtrates from 11 strains of bacteria were significantly more attractive to Mexican fruit flies, *Anastrepha ludens* than uninoculated media. Chemicals, such as ammonia, aliphatic amines, pyrazines, imines and acetic acid, identified from the investigated bacteria were observed as attractants (Robacker et al., 1998). Work by Dillon et al. (2000) on desert locusts, *Schistocerca gregaria*, reveals the role of *Pantoea agglomerans* in the production of guaiacol, a volatile compound promoting locust aggregations. Analysis of the fecal pellets of axenic locusts revealed the lack of guaiacol as a reason for the significant difference in smell between locusts with and without their normal gut biota (Dillion et al., 2000). Work by Leroy et al. (2011) illustrate the role of bacterial volatiles, which function as semiochemicals between bacteria and insects, are plentiful in nature (Leroy et al., 2011). It is important to remember that these semiochemicals "encompass pheromones, allomones, kairomones, attractants and repellents"

(Leroy et al., 2011). Leroy et al.'s (2011) work on the pea aphid *Acyrthosiphon pisum*, reveals the role of a host-associated bacterium, *Staphylococcus sciuri,* as an effective attractant and ovipositional stimulants for the pea aphid's natural enemy. While the above mentioned literature describes bacterial volatiles which serve as pheromones, kairomones or attractants, work on *Drosophila melanogaster* illustrates the role of a microbial odorant, geosmin, in functioning as a repellent (Stensmyr et al., 2012). Work by Stensmyr et al. (2012) reveal geosmin to be a potent repellent activating a single class of sensory neurons sufficient to result in aversive behaviour. Our finding that social attraction is mediated by bacterial volatiles in fruit flies conforms to literature in which bacteria produce volatiles that can function as attractants.

Conclusions and Prospects

This thesis presents strong evidence that social attraction is mediated by bacterial volatiles in fruit flies. Prospective research can attempt to unravel the volatile chemicals produced by bacteria that are attractive to fruit flies. Work by Pripis-Nicolau et al (2004) illustrates the role of lactic acid bacteria in forming products of methionine metabolism with salient odours during malolactic fermentation of wine. One can conduct odour preference tests using chemicals such as 3-(methylsulphanyl) propionic acid as the attractant and can use a modified version of the ammonia protocol described by Durisko and Dukas (unpublished) to test the presence of any such preference. Another way to identify the volatile compounds is to identify volatile components of bacteria-produced-supernatants using a capillary gas chromatography and gas-chromatography-mass spectrometry like Lee et al. (1995) in their investigation of volatiles of bacterial fermentation, which are attractive to the Mexican fruit fly. Investigation of the chemical odours and identification of the potential volatiles will result in a better

945 understanding of the cues involved in the social attraction exhibited by focal
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973 REFERENCES
974
975 Bartelt, Robert J., Angela M. Schaner, and Larry L. Jackson. 1985. cis-Vaccenyl
976 acetate as an aggregation pheromone in Drosophila melanogaster. *Journal*
977 *of Chemical Ecology* 11.12: 1747-1756.
978
979 Borash, D.J., Gibbs, A.G., Joshi, A., Mueller, L.D., 1998. A genetic
980 polymorphism maintained by natural selection in a temporally varying
981 environment. *American Naturalist* 151, 148–156.
982
983 Bouskra, D., Brézillon, C., Bérard, M., Werts, C., Varona, R., Boneca, I. G., &
984 Eberl, G. 2008. Lymphoid tissue genesis induced by commensals through
985 NOD1 regulates intestinal homeostasis. *Nature*, *456*(7221), 507-510.
986
987 Brummel, Ted, et al. 2004. Drosophila lifespan enhancement by exogenous
988 bacteria. *Proceedings of the National Academy of Sciences of the United*
989 *States of America* 101.35: 12974-12979.
990
991 Casadevall, A., & Pirofski, L. A. 2000. Host-pathogen interactions: basic concepts
992 of microbial commensalism, colonization, infection, and disease. *Infection*
993 *and Immunity*, *68*(12), 6511-6518.
994
995 De Man, J. C., Rogosa, M., & Sharpe, M. E. 1960. A medium for the cultivation
996 of lactobacilli. *Journal of Applied Microbiology*, *23*(1), 130-135.
997
998 Dillon, R. J., Vennard, C. T. & Charnley, A. K. 2000. Exploitation of gut bacteria
999 in the locust. *Nature*, 403, 851.
1000
1001 Durisko, Z., Anderson, B., Dukas, R. 2013. Effects of larvae on patch choice in
1002 fruit flies. Submitted.
1003
1004 Durisko, Z. & Dukas, R. 2013. Attraction to and learning from social cues in fruit
1005 fly larvae. *Proceedings of the Royal Society of London B-Biological*
1006 *Sciences*, 20131398.
1007
1008 Durisko, Z. & Dukas, R. 2012. In prep.
1009
1010 Dukas, R. 2010. Insect social learning. *In: Encyclopedia of Animal Behavior*
1011 (Breed, M. & Moore, J., eds). Academic Press, Oxford, pp. 176—179.
1012
1013 Galef, B. G. & Laland, K. N. 2005. Social learning in animals: empirical studies and theoretical models

BioScience 55, 489–499.

Galef B. G. & White D. J. 1998. Mate-choice copying in Japanese quail, *Coturnix coturnix japonica. Animal Behaviour* 55: 545–552.

Hasselschwert, D., & Rockett, C. L. 1988. Bacteria as ovipositional attractants for Aedes aegypti (Diptera: Culicidae). *Great Lakes Entomol*, *21*, 163-168.

Heyes, C. M., & Galef Jr, B. G. (Eds.). 1996. Social learning in animals: the roots of culture. Access Online via Elsevier.

Lancet, Y. & Dukas, R. 2012. Socially influenced behaviour and learning in locusts. *Ethology* 118: 302-310.

Leadbeater, E., & Chittka, L. 2007. Social learning in insects—from miniature brains to consensus building. *Current Biology*, *17*(16), R703-R713.

Lee, C. J., DeMilo, A. B., Moreno, D. S., & Martinez, A. J. 1995. Analysis of the volatile components of a bacterial fermentation attractive to the mexican fruit fly (Anastrepha ludens). *Journal of agricultural and food chemistry*, *43*(5), 1348-1351.

Keene, A. C. & Waddell, S. 2007. Drosophila olfactory memory: single genes to complex neural circuits. *Nat. Rev. Neurosci*. 8, 341–354. (doi:10.1038/nrn2098)

Kendal, R. L., Coolen, I. & Laland, K. N. 2009. Adaptive trade-offs in the use of social and personal information. *In: Cognitive Ecology II* (R., D. & Ratcliffe, J., eds). Univ. of Chicago Press, Chicago, pp. 249—271.

Leroy, P., Sabri, A., Verheggen, F., Francis, F., Thonart, P. & Haubruge, E. 2011a. The semiochemically mediated interactions between bacteria and insects. *Chemoecology*, 21, 113-122.

Leroy, P. D., Sabri, A., Heuskin, S., Thonart, P., Lognay, G., Verheggen, F. J., Francis, F., Brostaux, Y., Felton, G. W. & Haubruge, E. 2011b. Microorganisms from aphid honeydew attract and enhance the efficacy of natural enemies. *Nat Commun*, 2, 348.

Mery et al. 2009. Public Versus personal information for mate copying in an invertebrate. *Current Biology*. 19, 1-5.

Miller, J.H. 1992. A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria. Cold Spring Harbor Laboratory Press.

Munger, S. D., Leinders-Zufall, T., McDougall, L. M., Cockerham, R. E., Schmid, A., Wandernoth, P., Wennemuth, G., Biel, M., Zufall, F. & Kelliher, K. R. 2010. An olfactory subsystem that detects carbon disulfide and mediates food-related social learning. *Current Biology* 20, 1438–1444

Oliver, K. M., Russell, J. A., Moran, N. A., & Hunter, M. S. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences*, *100*(4), 1803-1807.

Pripis‐Nicolau, L., Revel, G., Bertrand, A., & Lonvaud‐Funel, A. 2004. Methionine catabolism and production of volatile sulphur compounds by Oenococcus oeni. *Journal of applied microbiology*, *96*(5), 1176-1184.

Prokopy, R. J. & Roitberg, B. D. 2001. Joining and avoidance behavior in nonsocial insects. *Ann. Rev. Entomol*. 46, 631–665. (doi:10.1146/annurev.ento.46.1.631)

Ridley, Emma V., et al. 2012. Impact of the Resident Microbiota on the Nutritional Phenotype of Drosophila melanogaster. *PloS one* 7.5:e36765.

Robacker, D. C., Martinez, A. J., Garcia, J. A., & Bartelt, R. J. 1998. Volatiles attractive to the Mexican fruit fly (Diptera: Tephritidae) from eleven bacteria taxa. *Florida Entomologist*, 497-508.

Sarin, S. & Dukas, R. 2009. Social learning about egg laying substrates in fruit flies. Proc. R. Soc. Lond. *B Biol. Sci*. 276, 4323—4328.

Schneider, J., Dickinson, M. H., Levine, J. D. 2012. Social structures depend on innate determinants and chemosensory processing in drosophila. *PNAS*. (doi: 10.1073/pnas.1121252109)

Sharon, Gil, et al. 2010. Commensal bacteria play a role in mating preference of Drosophila melanogaster. *Proceedings of the National Academy of Sciences* 107.46: 20051-20056.

Stensmyr, Marcus C., Dweck, Hany K. M., Farhan, A., Ibba, I., Strutz, A., Mukunda, L., Linz, J., Grabe, V., Steck, K., Lavista-Llanos, S., Wicher, D., Sachse, S., Knaden, M., Becher, Paul G., Seki, Y. & Hansson, Bill S. 2012. A conserved dedicated olfactory circuit for detecting harmful microbes in *Drosophila*. *Cell*, 151, 1345-1357.

Storelli, Gilles, et al. 2011. Lactobacillus plantarum Promotes Drosophila Systemic Growth by Modulating Hormonal Signals through TOR-Dependent Nutrient Sensing. *Cell metabolism* 14.3: 403-414.

Szentkuti, L., Riedesel, H., Enss, M. L., Gaertner, K., & Von Engelhardt, W. 1990. Pre-epithelial mucus layer in the colon of conventional and germ-free rats. *The Histochemical journal*, *22*(9), 491-497.

Tannock, Gerald W. 1992. The lactic microflora of pigs, mice and rats. *The Lactic Acid Bacteria Volume 1*. Springer US, 21-48.

Verhulst, N. O., Beijleveld, H., Knols, B. G., Takken, W., Schraa, G., Bouwmeester, H. J., & Smallegange, R. C. 2009 Cultured skin microbiota attracts malaria mosquitoes. *Malar J*, *8*(1), 302.

Von Frisch, K. 1967 The dance language and orientation of bees.

Walter, J. 2008 Ecological role of lactobacilli in the gastrointestinal tract: implications for fundamental and biomedical research. *Applied and environmental microbiology*, *74*(16), 4985-4996.

Wong, Chun Nin Adam, Patrick Ng, and Angela E. Douglas. 2011 Low‐diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. *Environmental microbiology* 13.7: 1889-1900.