Effects of microbe deposition, pheromone deposition, and the social environment on dietary choice in mated female *Drosophila melanogaster*.

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Abstract

Dietary variation is key to health and organismal fitness. In the model organism used throughout this thesis, the fruitfly *Drosophila melanogaster*, the strength and nature of dietary preferences can be differentially influenced by sex and mating status. Individuals of both sexes will usually feed and develop as larvae through to adulthood within shared dietary environments. This will expose individuals to gut microbes, pheromones and digestive byproducts from others, which may have significant impacts on health and development. The potential costs and benefits of feeding on or developing in shared ('conditioned') environments are not known. Therefore, it is of great interest to investigate whether flies prefer conditioned diets when given this choice and the consequences for fitness of any such decisions. In this thesis, I used choice assays to test the preference of mated females to feed and lay eggs on 'conditioned' versus 'non-conditioned' diets. I then tested the developmental consequences of being reared on either type of diet. I first investigated the dietary preferences of mated females for high protein and high carbohydrate diets that were conditioned by males, virgin females or Ovo^{D1} (eggless) females. I tested these preferences in both "absolute" (two-choice) and "relative" (four-choice) assay environments. The results showed that there were clear preferences for feeding and laying on conditioned diets, especially on the high protein diet for feeding, and the high carbohydrate diet for oviposition. I then investigated how being reared on a conditioned or non-conditioned diet impacts pre-adult survival and body weight at emergence. I found that conditioned diets slowed developmental speed, though there were no immediate effects on pre-adult survival or body weight at emergence. Therefore, the fitness benefits of preferences for conditioned diets have not yet been identified. Finally, I discuss the potential next steps for investigating the mechanisms behind diet conditioning, including demonstrating dose-response effects of conditioning. I also discuss the broader context and importance of my results for understanding life-history traits, and suggest future experiments to investigate the wider significance of diet conditioning.

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1. Chapter 1 – Fitness effects of diet, dietary choices, microbiomes, pheromones and the social environment

1.1 Introduction

Diet has been shown to be a fundamental aspect of an organism's biology, and the diet consumed has been shown to influence various life history traits such as longevity, reproductive success and overall fitness (Caprara, 2018; Narayan *et al.*, 2024). Studies into the effects of diet have demonstrated important consequences affecting life histories of many invertebrates, including the study organism used in this thesis, *Drosophila melanogaster* (Lee, 2015). What is less studied, however, is whether flies make adaptive choices for diets that optimise specific life history traits and improve fitness. This highlights the effects of dietary choice as an important topic of study. This review investigates the effect of diet and how various factors such as the microbiome can influence dietary preferences and behaviours. The aim is to understand the flexibility of the microbiome and its role in modulating feeding preferences (Call et al., 2022), mating behaviour (Leftwich et al., 2018) and life history traits (Lesperance and Broderick, 2020). In addition, I aim to highlight the impact of pheromones on dietary preferences, illustrating how chemical signals deposited by individuals can alter feeding behaviour (Everaerts et al., 2010) and show the effect of the presence of pre-digested diets and digestive enzyme secretion in shaping dietary preferences (Gregg et al., 1990). Overall, this review aims to synthesise an understanding of the mechanisms underlying dietary choice, in the context of the social environment, microbe deposition, pheromone deposition, and its implications for life-history traits in *Drosophila melanogaster.*

1.2 The importance of diet in humans and other animals

The concept of maintaining a well-balanced diet including specific dietary components has long been advocated for optimal health and longevity in humans (Caprara, 2018). However, in humans, significant inequalities persist globally, with socioeconomic factors often dictating the accessibility of diets on a global scale (Wickramasinghe et al., 2020). Studies conducted on humans have investigated the effects of caloric restriction on ageing, showing that reducing calories while still maintaining the consumption of the major nutrients, could potentially reduce ageing while also improving health span and quality of life (Flanagan et al., 2020). Furthermore, dietary consumption has been found to exert intergenerational effects across the human lifespan through epigenetic modifications (Kanherkar et al., 2014). For example, a study in Gambia showed that the mother's periconceptional diet led to differential birth weights in offspring, with these changes persisting into adulthood (Waterland and Jirtle, 2003). These studies demonstrate that dietary consumption impacts not only

immediate health outcomes but also exerts long-term effects across lifespan, highlighting the significant influence of diet on human development, health and longevity.

In addition to humans, studies on various organisms, including many experimental studies in invertebrates, have investigated the interactions of diet, lifespan and health. For example, it has been shown in *Drosophila serrata* fruit flies, that lower carbohydrate consumption in females initially led to a decreased lifespan, but over multiple generations, this diet resulted in an evolved increase in lifespan, demonstrating that long-term manipulation of major dietary components can drive evolutionary adaptions in longevity over multiple generations (Narayan et al., 2024). Similarly, in solitary insects such as the black garden ant (*Lasius niger*), it was found that consuming excess protein relative to carbohydrates can shorten lifespan, an effect observed after just one day of exposure to high-protein diets (Dussutour and Simpson, 2012). This highlights how even minor adjustments in dietary consumption can affect important fitness-related traits such as lifespan.

1.3 The mechanisms linking diet and lifespan

There is vast amount of literature on the links between nutrient sensing and lifespan across many different taxa (Dabrowska et al., 2022; Pignatti et al., 2020). Many studies provide important insights into the mechanisms underlying longevity, and how organisms from different species respond to nutritional cues. For example, in model organisms such as the roundworm *Caenorhabditis elegans*, specific mechanisms which mediate the interaction of diet and lifespan have been described (Yen and Curran, 2016). Dietary restriction, which limits standard dietary intake without causing malnutrition, has been found to increase the lifespan of *C. elegans* by influencing the activity of TOR (Target of Rapamycin), a key nutrient sensor (Hansen et al., 2007). Modulation of TOR facilitates lifespan extension, at least in part, by triggering autophagy, a cellular recycling process (Hansen et al., 2008). Autophagy can be upregulated through increased exercise and dietary restriction (Escobar et al., 2019) and can result in lifespan extension by regulating glucose metabolism and oxidative stress (Cabo and Mattson, 2019). Similarly, in *Drosophila melanogaster*, inhibiting the TOR signalling pathway by altering the expression of TOR pathway genes has been shown to extend lifespan, mirroring the known effects of dietary restriction on longevity (Kapahi et al., 2004). The findings in these model organisms show evidence of a molecular mechanism that may be influenced by the dietary intake of not only humans but also other animals.

1.4 Age-dependent impact of dietary consumption on life-history traits

Consistent with the studies mentioned above, dietary restrictions such as intermittent fasting in *Drosophila melanogaster* show rapid activation of neuronal autophagy. In middle-aged male flies, this intervention led to an improved neuronal autophagic profile, resulting in youthful behaviours and longer lifespans compared to flies not subjected to intermittent fasting (Ratliff et al., 2016). Such findings highlight the potential significance of dietary restrictions across various life stages. Similarly, other studies also in *Drosophila melanogaster* have shown that the consequences of optimal nutritional conditions can differ for larvae and adult flies. For instance, adult fruit flies fed diets known to be poor for larvae, do not exhibit negative responses, demonstrating the possibility of divergent dietary requirements at different life stages, that may have differential fitness consequences (May et al., 2015).

1.5 Diet, diet choice and the microbiome

Males and females of many species may exhibit specific dietary preferences, and these can be dependent on factors such as nutrient content, physical condition and microbial presence. An example is that the presence of microbes changes dietary choice in humans, to avoid foods which appear mouldy (Gram et al., 2002). Similarly, other organisms can also exhibit direct attraction to substrates containing specific microbes. One study showed that *Drosophila melanogaster* may favour diets which contain microbes, over control diets which lack microbes (Leitao-Goncalves et al, 2017). The association of microbes and diet is therefore an important factor to consider in the study of dietary consumption. Diets consumed are also a key factor in shaping the composition of the gut microbiome across various organisms. The nutrients provided by the diet can serve as essential substrates to the gut microbiome and may also shape microbiome composition indirectly by modulating immune cell efficiency of the host (Zhang, 2022). This highlights that the gut microbiome composition can be altered by both internal and external stimuli, and that the diet consumed is key (Gentile and Weir, 2018; Leeming et al., 2019) and can alter microbial ecology (Herter and Kendall, 1910). The influence of factors such as diet on microbiome composition is explained in more detail below.

1.6 The flexibility and resilience of the insect microbiome

Diet has been found to be an important factor in structuring the bacterial community of insect hosts (Colman et al., 2012). For example, a study comparing the evolution of gut microbes in mammals and insects found that the microbe, *Lactoplantibacillus plantarum* (*L. plantarum*) coming from an external environment, such as diet, significantly shapes the composition of this microbe in the gut of *Drosophila melanogaster*. However, *L. plantarum* in a mouse's diet has minimal impact on this microbe colonising in the gut (Maritan et al., 2022). These studies highlight the importance of understanding the microbiome's flexibility and the influence of external factors such as diet. In some insects, the presence of microbes in an environment can modify feeding preferences and change organismal physiology (Engel and Moran, 2013). In many species in which hosts have relatively loose and flexible associations with microbes, external factors such as the dietary environment can be a strong predictor of the host's microbiome, as seen in *Drosophila suzukii* fruit flies (Bing et al., 2018). Wild populations of *Drosophila suzukii* have gut microbiomes that vary significantly across different wild populations. This was also demonstrated by comparing the gut microbiome of wild flies to flies reared in lab conditions and the results showed that both the abundance of microbes and bacterial diversity in lab-reared flies was lower than for wild populations. This same study demonstrated that an insect's ancestral environment may also predict its subsequent survival. Under natural conditions, *D. suzukii* will naturally feed on fresh fruit with a low microbial titre and when exposed to microbes, flies with their standard microbiomes had greater developmental success and survival than axenic flies lacking a microbiome. This showed that *D. suzukii's* response to a microbial environment depended on its previous microbial environment (Bing et al., 2018), giving an understanding of the complex interaction between insects, their microbiomes, their environments, and the adaptability and resilience of the composition of the microbiome in insects.

While there is ample evidence supporting the idea of a flexible microbiome in insects, some insects maintain highly obligate associations with their microbiome, harbouring large gut communities of specialised bacteria. For example, the olive fruit fly (*Bactrocera oleae*) have evolved to harbour a transmitted obligate symbiont, *Candidatus Erwinia dacicola*. This symbiont plays an important role in facilitating the fly's efficiency in exploiting olive fruit resources, thereby contributing to its survival (Engel and Moran, 2013). However, rather than this type of strong association between insects and their microbiomes, my primary focus for this thesis is on understanding the looser, non obligate, associations between insects and their microbiomes.

1.7 The effect of the insect microbiome on feeding behaviour

The insect microbiome has been shown to influence a variety of behaviours. As well as feeding behaviour, symbiotic gut bacteria can have important influences on factors such as host mating behaviour (Leftwich et al., 2018). For instance, the presence of microbes can be associated with feeding preferences or mating choices in some beetles and fruit flies. One example is a study using the Colorado Potato beetle (*Leptinotarsa decemlineata*), in which it was found that changes in the abundance of the host's gut microbiome can influence the olfactory system, which in turn may affect host feeding behaviour. This was shown with beetles manipulated to be axenic (microbe-lacking) through antibiotic treatment. Results showed that feeding behaviour diverged between axenic and control beetles (Li et al., 2023) indicating that the presence of a gut microbiome can influence dietary preferences. Similarly, foraging differences were also found to be influenced by the microbiome in the oriental fruit fly (*Bactrocera dorsalis*). Here, suppressing the microbiome resulted in altered foraging behaviours in both male and female flies. Individuals that lacked a microbiome had faster foraging movements and spent more time feeding. It was proposed that the absence of the gut microbiome might lower the response thresholds for visual and olfactory stimuli associated with food, hence resulting in an advantageous change in feeding behaviour (Akami et al., 2019).

1.8 The effect of the microbiome on fitness in insects

As well as the presence of microbes changing feeding behaviour, it has also been found that gut microbiome may be directly linked with the expression of fitness-related life-history traits. For example, in studies on the polyphagous fly (*Bactrocera tryoni)*, it was found that the presence of a gut microbiome, as well as the presence of microbes on a diet in which they are feeding, may promote fitness and development. Research demonstrated that flies subjected to microbial removal from the egg surface during their egg stage in the life cycle exhibited a lighter weight and decreased fecundity when they were adult flies compared to controls with an intact egg microbiome (Nguyen et al., 2021). Similarly, in the bean bug (*Riptortus pedestris*), having a gut symbiont resulted in an increased body size and weight of male adult insects, when compared to aposymbiotic individuals. This in turn resulted in positive fitness effects for competition against other male insects (Jung and Lee, 2023). These studies show that the presence of a microbiome, and having microbes to feed on can result in direct beneficial life-history effects on the host.

1.9 The effect of pheromones on dietary preferences in insects

Pheromones deposited or disseminated by other individuals can act as attractants or repellents and thus alter feeding behaviour (Engl and Kaltenpoth, 2018). In all animals, pheromones can serve as chemical signals between individuals and are responsible for robust innate social behaviours (Liberles, 2014). In insects, pheromones are responsible for a wide range of olfactory communication. One example is found in ants foraging for food, in which the ants deposit a pheromone which allows for the recruitment of nestmates (Dussutour et al., 2009). Studies in other Hymenoptera, such as honeybees (*Apis mellifera*), have shown that the reproductive individuals (queens) in a colony produce essential pheromones for maintaining a dominant reproductive status over the workers, showing that pheromones are critical for colony behaviour and organisation (Kocher and Grozinger, 2011). Pheromones can also play a crucial role in resource exploitation. Pharaoh ants (*Monomorium pharaonis*), utilise pheromone trails to communicate the location of food sources, influencing foraging behaviour (Jackson et al, 2007). Similarly, in the ant species *Lasius niger*, the abundance of pheromone in a trail is correlated with the exploitation of sugar sources (Beckers et al., 1993). Furthermore, Australian stingless bees (*Meliponini*) detect pheromones left behind by conspecifics, as well as those deposited by competing species such as the honey bee, *Apis mellifera* (Gloag et al., 2021). This suggests a complex influence of pheromones on foraging ecology and exemplifies how pheromones serve as vital chemical signals and influence various social and foraging behaviours, proving important for dietary preference behaviours among insects.

1.10 Dietary preferences in *Drosophila melanogaster*

Differences in dietary choice, due to nutritional, or compositional factors have been investigated in the model organism, *Drosophila melanogaster,* which is the experimental organism used in the research in this thesis. Multiple studies have shown *D. melanogaster* has specific dietary preferences when allowed to choose between a variety of diets. Previous research into the optimal diet for maximising fitness benefits for *D. melanogaster* has indicated that a diet high in carbohydrates is best in optimising both mated males and females' longevity and that a diet high in protein is optimal for promoting reproductive success (Lee, 2015). However, when given a choice, mated female flies will favour feeding on diets high in protein (Almeida de Carvalho and Mirth, 2017), despite this diet not maximising their lifespan. For feeding, it has also been found that when mated female flies have been presented with various diet options differing in both food texture (e.g., hard or soft) and nutrient content (e.g., differing protein: carbohydrate ratios), mated female flies will exhibit a preference for their preferred nutrient composition (e.g., a diet high in protein) over their preferred food texture (e.g., a softer diet) (Millar, Chapman, unpublished).

Behavioural assays such as these have also shown feeding and oviposition preferences may be distinct. For example, mated females exhibit a preference for diets rich in protein for feeding, yet they prefer to lay their eggs on diets high in carbohydrates, despite a diet high in carbohydrates being suboptimal for larval survival (Lihoreau et al., 2016a). In a previous unpublished study done in this laboratory (Millar, Chapman, unpublished), quantitative PCR (qPCR) analysis was used to investigate the consequences of female oviposition preference for a high carbohydrate diet. Specifically, the expression of transcripts associated with nutritional stress namely dFOXO and dilp3 were tested (Kramer et al., 2003). Results from the qPCR analysis suggested the activation of these transcripts in larvae reared on a high carbohydrate diet with a P: C ratio of 1:8, which was also the maternal preference for oviposition. While this diet was preferred by flies for egg laying, there was a higher expression of these transcripts compared to the P: C 8:1 diet. Showing that despite this being the preferred maternal oviposition choice, the high carbohydrate diet would not be as favourable for offspring fitness (Millar, Chapman, unpublished). Consistent with other studies, which have also demonstrated that a high carbohydrate diet may hinder offspring development (Klepsatel et al., 2020), these results show that maternal selection of a high carbohydrate diet for egg laying may induce gene expression in larvae associated with nutritional stress, potentially impairing offspring growth.

These studies, using dietary choice assays, show that there are distinct preferences of *D. melanogaster* that may be guided by olfactory signals, for both feeding and oviposition. As well as this, some studies have demonstrated that oviposition preference may depend on other factors such as the size of the experimental substrate, which can counteract the choice of a preferred nutrient composition (Schwartz et al., 2012). It has also been shown that when given the choice between different nutrient compositions and different textures of food, flies consistently preferred to lay their eggs on a soft diet, even if the diet was a usually non-favoured nutrient composition for oviposition choice (Millar, Chapman, unpublished). Demonstrating that other factors beyond olfactory signals play a part in dietary choice in mated female *D. melanogaster*.

The preferences shown may also be different for females, males and larvae, hence reflecting potential conflicts of interest. Dietary preferences may also be dictated by the gut microbiome in *D. melanogaster* (Wong et al., 2017) as discussed below.

1.11 External influences on the gut microbiome of *Drosophila melanogaster*

The composition of a fly's gut microbiome can change depending on the diet they consume (Chandler et al., 2011) and this can differ even according to different laboratory diets used. One study used a standard *Drosophila* diet, CMY (Cornmeal Molasses Yeast) and found that *Acetobacteriaceae* bacteria comprised over 50% of the microbiome of flies reared on this diet. However, in flies maintained on a Starch diet, those same bacteria were far less abundant. These results show that *D. melanogaster* has a flexible microbiome, that can be largely determined by environmental factors such as diet (Leftwich et al., 2017). When comparing flies reared in similar environments, a dissimilarity analysis showed limited evidence for a sex-specific microbiome, though higher levels of *Enterococcus* bacteria were found in females (Han et al., 2017). Some studies have demonstrated the impact of the social environment on the microbiome of *D. melanogaster*. For example, the presence of adult males during the larval stage can lead to alterations in the microbiome of both male and female pupae, and the stress induced by the presence of adult males during the larval stage could potentially influence the microbiome (Leech et al., 2021). These findings demonstrate the influence of the external social environment on the microbiome while revealing limited evidence for sex-specific effects.

Despite many studies showing evidence of the flexibility of the microbiome. One study showed that upon studying the *D. melanogaster* gut microbiome for evidence of the influence of external factors, it was found that *Lactobacillus* bacteria (a common *Drosophila* bacterial symbiont) colonises stably to the *Drosophila* host, and is resistant to disturbance (Dodge et al., 2023). Although it is known that external factors such as diet and the social environment can influence the *D. melanogaster* gut microbiome, it has also been shown that certain members of the *D. melanogaster* gut microbiome can remain stable across different *D*. *melanogaster* populations and strains.

1.12 The effect of microbes in a diet on *Drosophila melanogaster*

As well as the finding that diet can change the host's gut microbiome, it has also been found that the presence of microbes on a diet can change its nutrient or moisture content, which in turn can change the host's dietary preferences. Using a bacterial cocktail of 4 known *D. melanogaster* gut microbes, one study found that inoculating these microbes into diets resulted in reduced carbohydrate, and increased protein contents in the diet, as well as increasing the moisture content. From looking at the effect of increased moisture content on diets on life-history effects, it was found that increased moisture in a diet was correlated with increased host developmental time and lifespan (Lesperance and Broderick, 2020). This showed that the presence of additional microbes on a diet can have an indirect positive

effect on host fitness through dietary changes to nutrition or moisture, and could link to the previously described preference for high protein as well as softer diets. The presence of gut microbes can also have positive consequences on development. Guilhot et al (2020) showed, through isolating four different microbial strains from *D. melanogaster* faeces, that the inoculation of *Enterobacteriaceae* induced faster larval development and larger larvae (but not larger adults) than controls.

Fly preferences for specific microbes can be mediated by olfactory mechanisms. One study, which used three different microbes associated with the *D. melanogaster* gut microbiome, showed that flies were attracted to the compounds associated with *Saccharomyces cerevisiae* and *Lactobacillus plantarum* but were repelled by *Acetobacter malorum* bacteria. As well as testing preferences between different microbes, microbial choice against a bacteria-free control was also tested for oviposition preferences. It was found that females preferred to lay their eggs on media containing any of the three microbes used in the experiment over the axenic control (Qiao et al., 2019). It has also been found that a *D. melanogaster's* ancestral environment, and the microbes they are exposed to in early life, can determine future microbial preferences. Wong et al. (2017) found that flies preferred to feed on *Acetobacter*, but only if they were exposed to this microbe in early life. This shows that microbial preference can be determined by the maternal microbiome, potentially promoting a symbiotic association through signalling their microbiome deposition on eggs. Similar results have been found for *Drosophila suzukii* (Bing et al 2018). In testing for trade-offs between microbes and a usually preferred nutrient composition of a diet, it was found that for feeding preferences, the consumption of microbes may be more important to a fly than the nutrient content (Wong et al., 2017)

Another factor to consider is the presence of essential amino acids in a diet. Essential amino acids and gut bacteria are both key modulators of protein appetite, which is an important determinant for lifespan and reproduction in *D. melanogaster*. Essential amino acids can act to lessen the effect of nutritional deprivation (Leitão-Gonçalves et al., 2017) and it has been found that the microbes can act as a protein-rich component, which is especially beneficial in poor-quality diets. Studies have also shown that having several microbes on a diet can rescue lifespan, but the quality or specificity of microbes on the diet can outweigh the effects of an abundance of microbes. This effect has only been found on poor-quality diets, and conversely, the addition of microbes in diets which are nutrient-sufficient can result in a reduced lifespan (Keebaugh et al., 2018). This is consistent with nutritional studies which report that too much protein can result in a reduced lifespan (Lee, 2015)

Foraging behaviour is an additional factor which can be affected by the presence of microbes. As well as microbial presence dictating dietary preferences, one study found the amount of time a fly is feeding can depend on the specific microbes present (Call et al., 2022), which will in turn can influence life-history effects.

1.13 Pheromones and dietary preferences in *Drosophila melanogaster*

The studies mentioned above have shown that microbes present on a diet may change both nutritional compositions of a diet, and dietary preferences in *Drosophila melanogaster*, both directly and indirectly. Pheromones may also change dietary preferences. Both male and female *D. melanogaster* are known to release several pheromones and cuticular hydrocarbons (CHCs) including a male-specific pheromone (cVA) (Everaerts et al., 2010). One study found that females display a preference for diets rich in the male pheromone cVA, with no such effect seen in males (Cazalé-Debat et al., 2019). Tolassy et al. (2023) showed that the presence of pheromones on diets may change olfactory behaviours. In this study, induced flight frequency was altered from diets with fly pheromones present, compared with control diets. When further investigating the effect of pheromones on behaviour, it has been discovered that some odorants were able to enhance mating signals. Pheromones methyl laurate (ML) and methyl myristate (MM) were found to attract both males and females, demonstrating signalling involved in courtship (Dweck et al., 2015). Furthermore, pheromones have been implicated in influencing specific oviposition preferences among females. Studies have revealed that females exhibit a preference for oviposition sites with higher pheromone concentrations, which is indicative of prior occupation by other flies. The pheromones cVA and 7-Triscone (7-T) served as a cue for oviposition site selection, and the attraction to these pheromones was mediated through odorant receptors Or67d and Or65a, showing the importance of olfactory signals in oviposition site selection (Verschut et al., 2023). There is also a suggested correlation between pheromones and diet. When the male-specific pheromone cVA was offered to female flies, females were continuously attracted. However, this attraction was only seen in starved females and disappeared when they were fed. This suggested that females may only be attracted to this pheromone under a malnourished status (Lebreton et al., 2015).

1.14 A digested diet, enzymes and dietary preferences in *Drosophila melanogaster*

In addition to the presence of microbes and pheromones, flies could also be attracted to diets if they have already been pre-digested by microbes or digestive enzymes. The *Drosophila melanogaster* genome is known to possess a large array of genes coding for digestive enzymes for processing different proteins, carbohydrates and lipids (Lemaitre and Miguel-Aliaga, 2013). When a *Drosophila* feeds on diets, they will excrete various enzymes

and will change the physical condition of a diet. Gregg et al. (1990) showed that when rearing *Drosophila hydei* flies on other *Drosophila hydei* carcasses, the larvae were able to digest various enzymes from these adult carcasses. A similar study, that looked into the influence of enzymes on food substrates, showed that the amylase enzyme excreted by *D. melanogaster* will influence the composition of a food substrate, which could, in turn, affect dietary choices from other flies (Haj-Ahmad and Hickey, 1982). As well as this, in a study aimed at better understanding external digestion in *Drosophila*, it was found that *Drosophila* larvae adjust their amylase secretion in response to the texture of food; harder food resulted in increased amylase secretion into diets. In addition, through this study, it was found that increased amylase content can result in delayed pupation which could be associated with increased glucose content (Sakaguchi and Suzuki, 2013). These studies show that *Drosophila* will excrete enzymes on a food surface, which can change food conditions, and can affect dietary choice.

1.15 Conclusion

The synthesis described above reveals that dietary choice can be dependent on key factors such as nutritional content, food texture, and the presence of other components such as microbes, pheromones and additional digestive enzymes. These can all, in turn, have consequential outcomes for life history effects. Therefore, it is important to understand whether *Drosophila melanogaster* chooses a diet that optimises their lifespan and fitness (reproductive success).

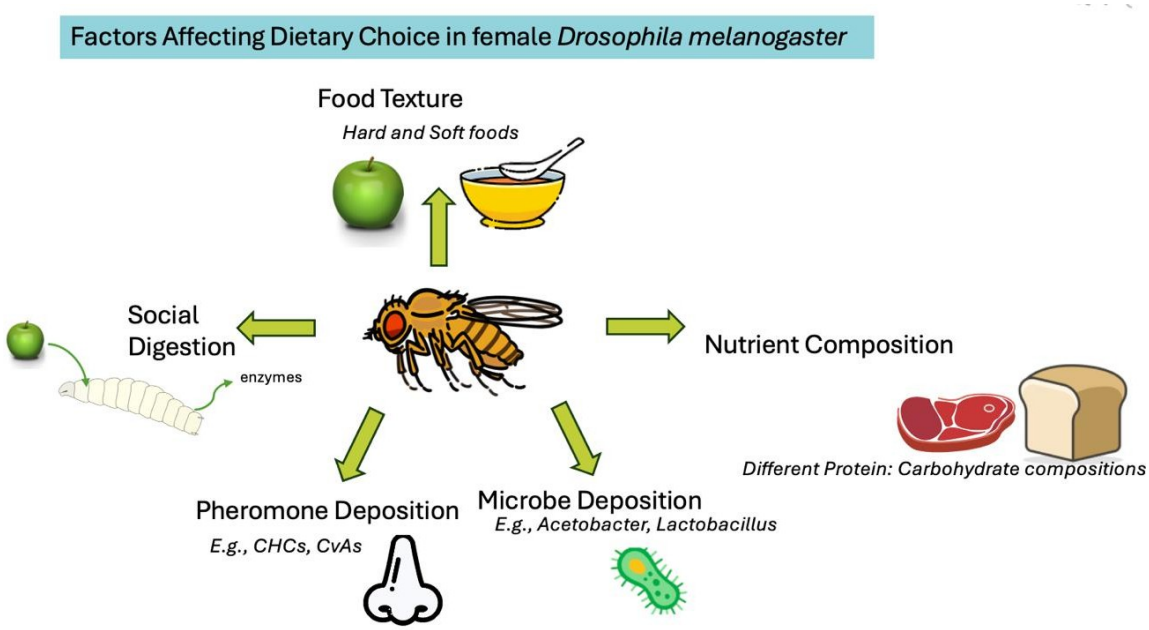

Figure 1.1: Illustration of factors affecting dietary choice in female Drosophila melanogaster. This diagram illustrates the various factors influencing the dietary

preferences of female *Drosophila melanogaster*. Factors influencing dietary choice include: food texture (e.g., hard or soft foods) nutrient compositions (e.g., varying levels of protein and carbohydrates), microbe deposition, pheromone deposition, and social digestion.

1.16 Outline of thesis

This thesis explores the effects of fly conditioned diets on the dietary choices of mated female *Drosophila melanogaster* and investigates the consequences for offspring of these choices. In addition, it proposes potential experiments to investigate the mechanisms underlying these dietary preferences. In both wild and laboratory settings, *D. melanogaster* often share environments with other flies, which can lead to the exchange of gut microbes, pheromones, and the influence of social cues such as shared digestion. Currently, the impact of such a diet on mated female flies and whether they preferentially select this type of diet when given a choice is not well understood. The aim of this thesis is to investigate how a fly conditioned diet affects the dietary preferences of mated female flies and to assess the developmental outcomes of being reared on conditioned or non-conditioned diets. By gaining insights into how microbial exposure and environmental cues can influence dietary choice and development in flies, this research can also provide a deeper understanding of similar effects in humans and other animals.

In **Chapter 2** I investigated how conditioning influences dietary choice in mated female *D. melanogaster*. To test this, I used diets conditioned by: males, virgin females and Ovo^{D1} (eggless) females. This approach allowed me to test for any sex-specific effects of conditioning, as well as the impact of the presence or absence of eggs on dietary preferences. Flies of different sexes and mating statuses are known to harbour distinct microbiomes and pheromones, which could also influence their dietary choices. I used diets that are both typically favoured by females for feeding (high protein, P: C 4:1) and oviposition (high carbohydrate, P: C 1:4). This allowed me to test how nutrient composition might influence dietary preferences. I also used two different assay designs: an absolute two-choice assay, which allowed a direct comparison of conditioned and unconditioned diets of the same P:C ratio, and a relative four-choice assay, which allowed for the comparison of both P:C nutrient compositions and conditioning treatments simultaneously. This was to test whether the dietary choices of mated females differed when additional diet choices were available. The results demonstrated that conditioning preferences were observed for both feeding and oviposition, but the extent of these preferences varied with conditioning treatment, nutrient composition and assay design.

Having determined that mated females exhibited distinct preferences for conditioned diets,

I further explored the developmental consequences of larvae reared on these diets. I investigated effects on developmental speed, pre-adult survival and body weight emergence. In **Chapter 3**, I used no-choice vials of P: C, 1:4 diets that were either conditioned or unconditioned with male flies. I set these up in two separate experiments: (i) an uncontrolled density experiment, where females were allowed to lay eggs naturally, and (ii) a controlled density experiment, where I added a known number of larvae (n = 63) to all vials. Results showed that in both the uncontrolled and the controlled density experiments, larvae reared in unconditioned vials developed faster than those in conditioned vials. However, there were no differences in survivability seen for pupae in either experiment, but in the uncontrolled density experiment, significantly more flies emerged in conditioned vials, suggesting this was a favoured diet and could be due to a density effect, as this was not found in the controlled density experiment. In addition, there were no differences in the body weight of flies between treatments in either experiment, suggesting the need for further experimentation beyond the adult stage.

Chapter 4 is a general discussion of the thesis. I discuss the wider implications of the findings of this thesis, with potential explanations for the results and behaviours I found. I demonstrate a protocol for a microbial wash experiment designed to isolate potential factors of fly conditioning, to better understand the underlying mechanisms.

Chapter 4 Supplementary Material outlines the experiment undertaken to identify any dose-dependent response of conditioning on mated female dietary choice, through which I found a dose-dependent response of conditioning for both feeding and oviposition behaviours. This experiment was completed as a preliminary experiment to a possible future microbial wash experiment, which was also described in **Chapter 4**.

Attribution statement

The work in this thesis was part of my Masters of Science by Research. Professor Tracey Chapman (primary supervisor), Professor Alexei Maklakov (secondary supervisor) and Oonagh Barker (supervisory team) supervised this project.

The author carried out all work unless stated below. Professor Tracey Chapman helped devise experiments in Chapter 2 and Chapter 3. Dr Emily Fowler helped with larvae picking in Chapter 3, and with devising the proposed experiment in Chapter 4. Dr Philip Leftwich helped with statistical analyses in Chapters 2 and 3.

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Chapter 2

2 The effect of fly-conditioned diets on dietary choice in *Drosophila melanogaster*

(Hannah Davis, CCASA-4.0)

2.1 Abstract

Animals utilise their environments in various ways and may often leave behind traces of their occupation that can be detected, for a variety of reasons, by others. For example, larval and adult fruitflies present in an environment may (i) deposit microbes or digestive enzymes from their guts with faeces, (ii) leave residual pheromones, or (iii) change the physical condition of the diet through feeding. These all represent examples of dietary 'conditioning'. The objectives of this chapter were to identify whether diets that have been conditioned by flies of either sex and by females that can and can't lay eggs were preferred or not preferred as feeding or oviposition substrates by mated females. I tested the effect of 'conditioning' (e.g. deposition of microbes, pheromones, digestate) on mated female dietary choice for feeding and oviposition. I performed 3 diet conditioning experiments (with diets exposed for 24h to males, to virgin wild type females that could lay eggs, or to mated females that could not lay eggs (OvoD1 strain)) each with 2 different test diets that differed in their overall attractiveness (4:1 or 1:4 Protein: Carbohydrate mixture). Two types of assay set-up were deployed. In the first, females were given a 2-way choice between 'conditioned' vs 'non conditioned' treatments of each diet. In the second, females were presented with a 4-way choice of 'conditioned' and 'non conditioned' patches of both food types simultaneously. I hypothesised that females would exhibit preferences for conditioned diets across all treatments on the basis that previous reports suggest that flies can sometimes be attracted to specific microbes or pheromones. The key findings were: (1) an overall preference for conditioned diets, which was more pronounced in high protein diets for both feeding and oviposition preferences; (2) that preferences for conditioned diets were more pronounced in the 4-way choice assays, in which flies could sample both diets and conditioning treatments; (3) conditioning preferences were stronger in diets conditioned by *Ovo*^{D1} (eggless) females than for diets conditioned by males or by wild type virgin females.

The results suggest that diets conditioned by previous exposure to conspecifics are more attractive to females for feeding and oviposition than non-conditioned diets. The potential benefits are not yet clear, which suggests that it is important to investigate the potential fitness consequences of female preferences for conditioned diets (Chapter 3).

2.2 Introduction

Dietary consumption throughout lifespan has been shown to influence life-history traits including reproductive success and overall fitness (Caprara, 2018). For example, in humans, it has been reported that reducing calorie intake while maintaining sufficient nutrient consumption may slow ageing and improve both health span and quality of life (Redman and Ravussin, 2011). Even during prenatal stages, a mother's dietary intake can impact offspring health and can be associated with differential birth weights, resulting in effects that persist into adulthood (Waterland and Jirtle, 2003). A large body of research also highlights key effects of dietary consumption on life history traits in many insects. For example, in *Drosophila serrata* fruit flies, a carbohydrate-rich diet has been shown to drive lifespan across generations (Narayan et al., 2024). Similarly, in the black garden ant (*Lasius niger*) a diet containing excess protein relative to carbohydrates was found to shorten lifespan (Dussutour and Simpson, 2012). These studies highlight the importance of dietary choice, and how the selection of diet can dictate different life-history outcomes.

Drosophila melanogaster, the model organism used throughout this thesis, can exhibit distinct dietary preferences. When given the choice from a variety of diets, mated females often favour feeding on a high-protein diet (Almeida de Carvalho and Mirth, 2017), despite this diet not maximising lifespan (although it does optimize reproductive success) (Lee, 2015). In addition, *D. melanogaster* females have been shown to exhibit different dietary preferences for feeding and oviposition, favouring high-carbohydrate diets for laying eggs (Almeida de Carvalho and Mirth, 2017b), even though high-carbohydrate diets appear suboptimal for larval survival (Lihoreau et al., 2016a). These findings suggest that females may exhibit distinct dietary preferences, for feeding and oviposition, guided by olfactory signals for specific nutrient compositions. I therefore predicted that in this experiment, nutrient composition would override preferences for conditioning, in the presence of high protein diets. However, other factors beyond just olfactory signals for nutrient composition may also affect dietary choice. For example, some dietary preferences may even be dictated by the composition of the gut microbiome in *D. melanogaster* (Wong et al., 2017).

In addition to a fly's microbiome, the presence of microbes on a diet itself can also affect choice. For example, one study showed that *D. melanogaster* gut microbes could indirectly affect dietary preferences by altering the nutrient composition of the diet. When a cocktail of *D. melanogaster* gut microbes was added to a diet, it led to a reduction in carbohydrate content and an increase in protein content (Lesperance and Broderick, 2020). Microbes present on a diet have also been shown to influence dietary preferences through olfaction. In a study testing preferences for commonly known *D. melanogaster* gut microbes, flies were particularly attracted to *Saccharomyces cerevisiae* and *Lactibacillus plantarum*, but were repelled by others (*Acetobacter malorum*). The study also revealed that females preferred to

lay their eggs on media containing any of these three microbes over a microbe-free control diet (Qiao et al., 2019).

Pheromones present on a diet may also influence dietary preferences. For example, females have been shown to favour diets rich in the male-specific pheromone (cVA) (Cazalé-Debat et al., 2019). This pheromone has also been identified as a cue for selecting oviposition sites (Verschut et al., 2023). Both males and females are attracted to another pheromone Z4-11AI, produced by females. The attractions of sex-specific pheromones, with blends of food volatiles, have been identified as a mechanism of olfactory signalling, shaping traits such as premating communication (Borrero-Echeverry et al., 2022) and upwind flight behaviours towards food sources (Lebreton et al., 2012). However, larval pheromonal cues have been shown to reduce larval attraction to certain diets (Farine et al., 2014), demonstrating how dietary choices can differ between males, females and larvae.

The extent to which a diet is pre-digested, i.e. its nutritional availability, is another factor that may influence dietary choices in *D. melanogaster*. The texture of the food may lead to differing levels of the digestive enzyme amylase secretion by *D. melanogaster* larvae, with harder diets resulting in increased amylase secretion onto the food (Sakaguchi and Suzuki, 2013). Adults could be attracted to these foods according to the enzymes present in the diet. Similarly, other studies indicate that *Drosophila* may be attracted to food substrates, where they can digest various enzymes. For example, in an experiment where *Drosophila* carcasses were used as a food source for rearing other *Drosophila*, the flies raised on the carcass-based diets were observed to digest enzymes from the carcasses (Gregg et al., 1990).

These studies show that several key factors could influence dietary preferences in flies, and in particular, the exposure of diets to other flies is expected to be able to alter dietary preferences. When flies are maintained on a diet, they may change the composition of it by leaving behind microbes from their gut microbiome, pheromones, or by changing the diet composition through digestate (social digestion), which may change the food texture of the diet. This is known throughout this thesis as fly conditioning. In this chapter, I used patch preference assays (Churchill et al., 2021) to test the effects of dietary conditioning on mated female dietary choices. This allowed me to monitor dietary choice preferences for both feeding and oviposition by placing different diet patches within a single arena and allowing females to choose the diet which they prefer to feed and oviposit on.

My three main hypotheses were:

1. Females show dietary preferences for conditioned diets.
    1.1 Females show different dietary preferences for diets conditioned by males or females.
        1.1.1 Female dietary preferences are affected by the presence or absence of already laid eggs on a substrate.

I developed these hypotheses based on findings from the relevant literature (Chapter 1). I reasoned that, based on the possible mechanisms of conditioning, mated female flies may be attracted to diets containing elevated levels of microbes (Qiao et al., 2019) or pheromones (Dweck et al., 2015). My hypotheses for sex-specific conditioning effects were based on studies showing that each sex has a distinct microbiome (Han et al., 2017) and pheromone complement (Borrero-Echeverry et al., 2022).

2.3 Materials and Methods

2.3.1 Investigating choice for Conditioned / Unconditioned media by using patch preference assays

I tested how media conditioned by males or females can influence dietary preferences (feeding and oviposition) in mated females. Different patch preference assays were designed to analyse whether a mated female prefers a conditioned or an unconditioned diet for feeding and oviposition. The assays were set up by allowing flies to roam freely for 24 hours across diet patches of specific Protein: Carbohydrate (P: C) ratios (4:1 and 1:4, both prepared at the same overall concentration of 120g/L). The 4:1 diet ratio tested is known from previous work to be a diet that females prefer to feed on (Almeida de Carvalho and Mirth, 2017b), whereas the 1:4 diet is a preferred diet for oviposition (Lihoreau et al., 2016a). The preferences of mated wild-type females for the different types of conditioned patches were then tested in three separate experiments in which diets were conditioned for 24h by:

(i) Wild type Males.
(ii) Wild-type Virgin Females (with eggs).
(iii) *Ovo*^{D1} Females (no eggs).

These three distinct conditioning experiments allowed for tests of sex-specific conditioning effects. They were also expected to provide insights into the effects of the presence and absence of eggs on conditioning, thus allowing for a better understanding of the mechanisms of any preferences observed.

Choice assays were designed in each of these experiments to test preferences by allowing absolute (2 patch) and relative (4 patch) preference tests, using 4:1 and 1:4 Protein: Carbohydrate (P: C) ratios (Figure 2.1). This allowed me to investigate if preference was stronger when females could sample all 4 treatment conditions simultaneously:

(i) Absolute test – 2 patch tests - females can choose between “Conditioned” versus “Unconditioned” patches of 4:1 or 1:4 diets separately.
(ii) Relative test – 4 patch tests - females can choose between “Conditioned” and “Unconditioned” patches of 4:1 AND 1:4 diet patches simultaneously.

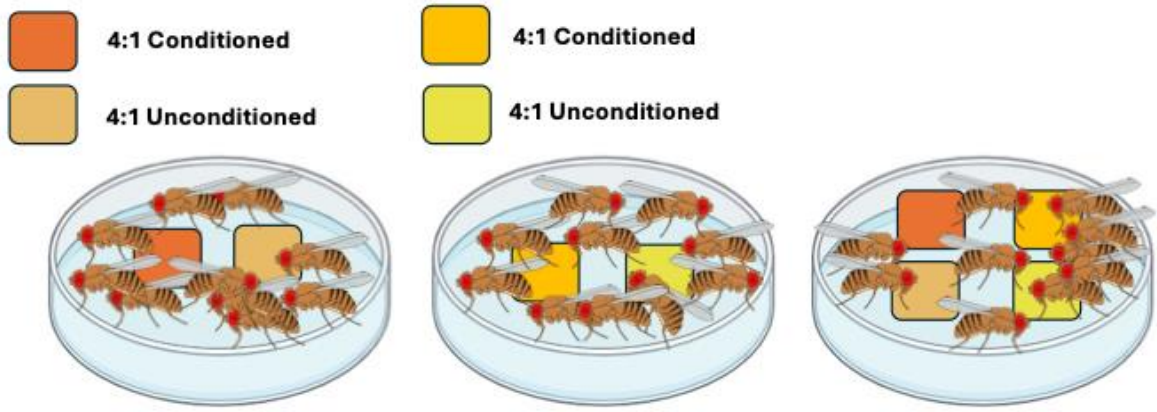

Figure 2.1: Illustration of the dietary preference choice assays. The figure illustrates the experimental setup used to test the dietary choices of mated female flies under different conditioning treatments. There were two types of dietary choice assays: 2-way choice (panels a,b); 4-way choice (panel c)**.** From left to right: (a) Flies were exposed to two types of food: 4:1 conditioned (dark orange) and 4:1 unconditioned (light orange). (b) Flies were exposed to two types of food: 1:4 conditioned (dark yellow) and 1:4 unconditioned (light yellow) (c) Flies were exposed to a combination of all four types of food: 4:1 conditioned (dark orange), 4:1 unconditioned (light orange), 1:4 conditioned (dark yellow), and 1:4 unconditioned (light yellow) ("Scientific Image and Illustration Software | BioRender," n.d.).

An alternative four-way choice design could have been to have each of the conditioning types in one plate (males, virgin females, OvoD1 females), as well as an unconditioned control, this could be considered for future to allow for a direct test of different fly conditioning.

2.3.2 Fly rearing

All flies were reared, and experiments took place, in a controlled temperature room of 25 °C, 50% RH on a 12:12 light: dark regime. Fly rearing was conducted in vials of sugar yeast agar medium (SYA; **Appendix 2**). Eggs were collected from Dahomey wild type population cages using purple agar plates (**Appendix 2**) with yeast paste in the centre to attract ovipositing females. These plates were placed in the population cages from the start of lights on, for 3 hours, then stored in a pillowcase to incubate for 24 hours to allow first instar larvae to hatch. After this, first instar larvae were picked and placed into SYA vials (50 larvae/vial, to standardise density and minimise any environmentally driven differences in body size). The vials were left in the 25 °C CT room for rearing, and after 9 days, the flies started to eclose.

2.3.3 4:1 and 1:4 Protein: Carbohydrate meridic diet treatments

To investigate the effect of the Protein: Carbohydrate (P: C) diet ratio with and without conditioning on dietary preference, two different P: C diets were used (4:1 and 1:4). These allowed an investigation of the effect of nutrient composition for both high carbohydrate / low protein and high protein / low carbohydrate. Fly food media was prepared using an autoclave, to ensure consistency across batches. Different Protein and Carbohydrate levels were controlled using differing levels of Casein and Sucrose (Table 2.1), with 0.3 g of Cholesterol, 4 g of Lecithin and 20 g of Agar as standard for both diets. After dry ingredients were added, liquid salt solutions (100 ml of KH_2PO_4, K_2HPO_4, $MgSO_4$, $NaHCO_3$, nucleic acid and 200 ml of distilled water) were added. The diets were then autoclaved, and after cooling to 60 °C, 10 ml of nipagin solution, 3 ml of propionic acid and 150 ml of a vitamin mix were added (**Appendix 2**). These diets were then poured into Petri dishes to be used for dietary choice assays.

P:C diet	Casein	Sucrose
1:4	24 g	96 g
4:1	96 g	24 g

Table 2.1. Table of casein and sucrose used for Protein: Carbohydrate ratios. The table shows the amount of Casein and Sucrose (g / L) added for the appropriate Protein: Carbohydrate ratio diets used.

2.3.4 Conditioning diet experiments

2.3.4.1 Experiment 1: The effect of diet conditioning by m ales on mated female dietary choice

2.3.4.1.1 Male fly collection

The first conditioning experiment was done using wild-type mated Dahomey males. When flies started to eclose, they were left in SYA vials for a day. Flies were then tipped into new SYA vials and were left for a further 24 hours. After 3 days from the initial eclosions, the flies were separated. The dietary choice experiment, using male conditioned flies, was done in two separate blocks. For the first block, mated males were allocated into groups of n = 40 vials containing n = 10 males each, for the conditioning, while mated females were sorted into n = 30 vials, each containing n = 10 females, to be used as the focals for the

experimental diet choice observations. In the second block, n = 20 vials containing n = 10 males were collected for the conditioning and n = 15 vials containing n = 10 mated females as the focals for the dietary choice observations.

2.3.4.1.2 Conditioning of diets by males

One day after flies had been sex separated as described above, the conditioning treatments were set up. Diet patches of the 4:1 and 1:4 diets were cut out using a 2cm x 2cm square cutter. These food patches were then individually placed into 90 mm Petri dishes. Petri dishes containing single diet patches were to be used for conditioned treatments, while those containing two diet patches were to be used for the unconditioned treatments.

The n = 10 mated Dahomey males were then added to each of the Petri dishes containing 4:1 or 1:4 single diet patches. Petri dishes containing both 4:1 and 1:4 patches were placed and handled alongside the conditioned dishes but left unconditioned (no males added). In block 1, n = 20 Petri dishes each containing 4:1 or 1:4 single patches (conditioned treatment), and n = 20 Petri dishes containing 4:1 and 1:4 patches (unconditioned) were set up. For the second block, n = 10 conditioned and n = 10 unconditioned dishes were set up. For the experimental observation part of this experiment, this would result in n = 10 repeats of each treatment assay in block one, and n = 5 in block 2.

These Petri dishes were all left in the 25 °C CT room for 24 hours. For the conditioning treatments, this allowed males to roam around the patches and condition them (potentially with microbes, pheromones and/or digestate). After 24 hours, males were removed using CO_2 and were discarded. The conditioned and unconditioned diet patches were then used in the patch preference tests, described below (Figure 2.2).

2.3.4.2 Experiment 2: The effect of diet conditioning by virgin females on mated female dietary choice

2.3.4.2.1 Virgin female fly collection

Wild-type Dahomey virgin females, laying unfertilised eggs, were used to condition diets in the second experiment, to explore potential sex-specific effects of conditioning. Flies were reared as stated above and individuals eclosing overnight from standard density cultures were transferred into fresh SYA vials and designated for use as the focal mated females in the experimental dietary choice observations. Approximately 6 hours later, newly emerged flies were sex separated on ice, and virgin females were separated and stored in SYA vials

of n = 10 females. The experiment was conducted in 4 blocks. In the first and second, n = 16 vials of virgin females were collected, in the third, n = 20 vials, and in the fourth, n = 24 vials.

From the overnight emerging flies, vials of n = 10 mated females were collected to serve as the focal choosing females to be used in the patch preference tests. There were n = 4 vials for blocks one and two, n = 5 for block three and n = 6 for block four.

2.3.4.2.2 Conditioning of diets by virgins

One day following the separation of flies into vials of virgin females (for the conditioning treatment) and vials of mated females (for experimental observations), experimental conditioning and unconditioned patches were set up. Using the 2cm x 2cm cutter, the appropriate number of diet patches for each diet ratio were placed into standard 90 mm Petri dishes. Two match the vials of flies collected, in both the first and second blocks, n = 8 Petri dishes were set up containing separate 4:1 or 1:4 patches (for conditioning), while n = 8 Petri dishes contained 4:1 and 1:4 patches (unconditioned). In the third block, there were n = 10 Petri dishes, and in block 4 there were n = 12 Petri dishes of each type. For the experimental observation experiment, this would result in n = 4 repeats of each assay in blocks one and two, n = 5 in block three, and n = 6 in block four.

Virgin females were added to the conditioning treatment dishes and were left in the 25 °C CT room for 24 hours, alongside the unconditioned Petri dishes. After 24 hours, virgin females were removed from dishes using CO_2 and were discarded (Figure 2.2). The number of eggs present on each diet patch, laid by the virgin females was counted under a microscope, this allowed for the subtraction from the total egg count after the addition of mated females in the dietary choice assays, to sum the exact number of eggs subsequently laid on each patch by the mated females.

2.3.4.3 Experiment 3. The effect of *Ovo*^{D1} female conditioning (without eggs) on female dietary choice

2.3.4.3.1 *Ovo*^{D1} female fly collection

Ovo^{D1} (eggless) females (Chanut-Delalande *et al.*, 2006) were used to investigate the effects of female conditioning in the absence of eggs, on mated female dietary preferences. Following the rearing protocol described above, wild-type Dahomey flies that had emerged overnight were discarded. Approximately 6 hours later, newly eclosed flies were sexed on ice, virgin females were collected and stored in SYA vials of n = 5 females. The collected virgin females were then crossed with *Ovo*^{D1} males, by placing n = 5 Dahomey Virgin

females together with n = 5 *Ovo^{D1}* males in SYA vials for 24 hours. All flies were then removed, and the cultures were incubated at 25˚C until the adults emerged. Concurrently, larvae were picked from Dahomey population cages and placed into SYA vials, to generate the focal mated females for the experimental choice tests that would emerge at the same time as the *Ovo^{D1}* females.

After 10 days, newly emerged flies from the *Ovo^{D1}* crosses were separated and *Ovo^{D1}* females were placed into new SYA vials to be used for conditioning, while *Ovo^{D1}* males were discarded. The Dahomey flies emerging were separated into males and females, and n = 40 mated females were placed in new SYA vials (containing n = 10 females each in both blocks one and two). In both blocks, n = 32 *vials of Ovo^{D1}* females were collected.

2.3.4.3.2 Conditioning of diets by *Ovo^{D1}* females

One day after flies had been separated into the appropriate SYA vials, the conditioning treatments were set up. Using standard 90 mm Petri dishes, square cuts of 4:1 and 1:4 media were taken as above, using a 2cm x 2cm cutter. In both blocks, n = 8 Petri dishes contained 4:1 or 1:4 patches (conditioned), while n = 8 Petri dishes contained 4:1 and 1:4 patches (unconditioned). For the experimental observation experiment to follow, this would result in n = 8 assay repeats in both blocks one and two.

Ovo^{D1} females were added to the conditioning treatment dishes and were left in the 25 °C CT room for 24 hours, alongside the unconditioned Petri dishes. After 24 hours, *Ovo^{D1}* females were removed from dishes using CO_2 and were discarded, the treatment assays were created using these patches and were then used for dietary choice observations (Figure 2.2).

2.3.5 Testing for dietary choice preferences (experiments 1-3):

After diets had been conditioned by the appropriate treatments for each of the 3 experiments, as described above (i.e. conditioned by males, wild type virgin females or by *Ovo^{D1}* (eggless) females) all conditioning flies were discarded, and experimental Petri dishes were set up. Single conditioned patches were removed from and added to fresh clean 90 mm petri dishes, and unconditioned patches were then added. This dietary choice design allowed me to test for any direct effects of diet conditioning. n = 10 mated Dahomey females were then added to each Petri dish to measure choice for conditioned versus non conditioned diets under 2-way and 4-way choice scenarios (Figure 2.2).

For male conditioned diets, there were n = 10 assay repeats in block one, and n = 5 in block 2. While for virgin female conditioned diets, there were n = 4 in blocks one and two, n = 5 in block 3, and n = 6 in block 4. Finally, for *Ovo*^{D1} conditioning, there were n = 8 assay repeats in both of blocks one and two.

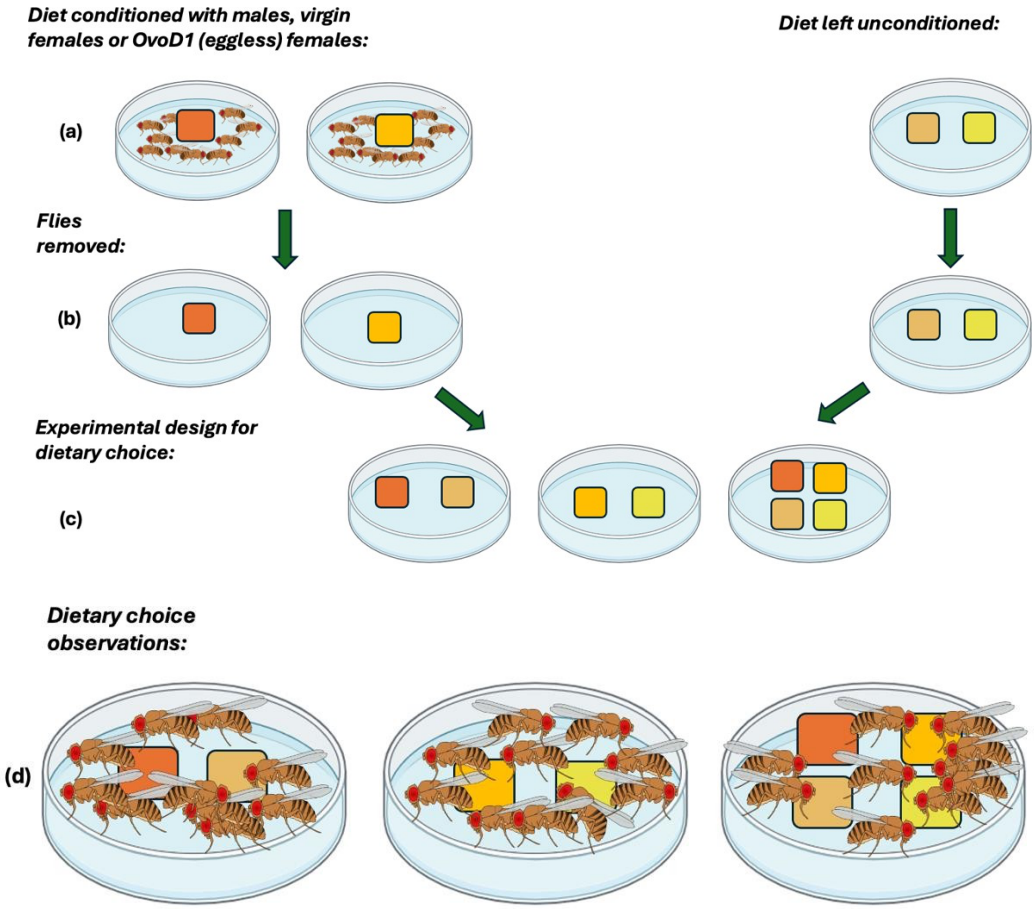

Figure 2.2. Illustration of experimental design for testing dietary preferences in flies. The figure illustrates the procedure for conditioning diets and testing dietary preferences in mated female flies. From top row to bottom row: (a) Diet patches were conditioned by exposing them for 24 hours to males, virgin females, or OvoD1 (eggless) females. Control diet patches were left unconditioned. (b) After conditioning, the flies were removed. (c) The experimental setup for dietary choice, with 2-way preference tests (4:1 conditioned versus non conditioned, or 1:4 conditioned versus unconditioned) and 4-way preference tests (4:1 AND 1:4 conditioned and unconditioned diets). (d) Mated female flies were then added to the Petri dishes to record dietary and oviposition preferences.

2.3.5.1 Mated female feeding preference behaviour

The dietary choice observations were conducted in the controlled environment of the 25 °C CT room. I monitored the number of females present across all patches every 30 minutes for 5 hours, from 13.00 – 18.00, by photographing all Petri dishes from approximately 1 metre distance. The number of females per patch every 30 minutes was recorded to give the measure of feeding preference, I referred to this as “observation” and used as a random effect when analysing mixed models.

2.3.5.2 Mated female oviposition preference behaviour

To determine oviposition preference, the dietary choice assay Petri dishes were retained, lids secured with tape, and placed in a pillowcase. These dishes were then returned to the 25 °C CT room overnight to allow the focal females to lay eggs. The following day at 12.00 all Petri dishes were put in a - 20 °C freezer to halt further oviposition and egg hatching and allow counting of the eggs laid on each food patch. For the collection of oviposition preference data, an Eagle M microscope with a GXCAM screen was used. The sides were cut from each face of the 2cm diet squares and placed horizontally to facilitate the counting of all eggs laid on the sides and top of each diet patch. Pictures of the sides and top of each food patch were captured using the microscope, and eggs were later counted from the images using a clicker counter.

2.3.5 Statistical analyses

All statistical analyses were conducted using R version 4.4.1, with the tidyverse package (Wickham et al., 2024). Assumptions of all models were checked using DHARMa (Hartig and Lohse, 2022) and performance (Lüdecke (@strengejacke) *et al.*, 2024) packages. As there were two separate assay designs within each treatment, a separate analysis was done for 2-way (absolute) and 4-way (relative) diet choice assays. The treatments were analysed with the best model fit for each assay and treatment type.

Two-choice feeding assays

Binomial Generalised Linear Mixed Models (GLMMs) were used to analyse the effects of male, virgin and Ovo^{D1} conditioning for feeding and oviposition. Random effects used in the model were “plate” (Petri dish / the assay) and “observation” (the 30-minute time period in which flies on a patch were counted).

Four-choice feeding assays

Poisson GLMM was used for the male conditioning experiment, a Negative Binomial Generalised Linear Model (GLM) for virgin conditioning and Ovo^{D1} conditioning for the feeding assays. Negative Binomial GLMs were used for the oviposition assays for all three conditioning experiment.

2.4 Results

2.3.6 Male Conditioning

2.3.6.1 Feeding behaviour

To investigate mated female preferences for conditioned versus unconditioned 4:1 and 1:4 diets in two-choice assays (an absolute environment), I initially tested for a two-way interaction effect between diet ratio (4:1 or 1:4) and block (one or two). No such interaction effect was found (Binomial GLMM: $n = 329$, $X^2 = 0.296$, $P = 0.586$) so I dropped this interaction from the model (Table 2.2).

	cbind(Conditioned, Unconditioned)		
Predictors	*Odds Ratios*	*CI*	*p*
(Intercept)	4.55	3.01 – 6.90	**<0.001**
ratio1 × 4	0.33	0.23 – 0.47	**<0.001**
block [two]	0.87	0.59 – 1.27	0.463
Random Effects			
σ^2		3.29	
$\tau_{00\ observation}$		0.04	
$\tau_{00\ plate}$		0.12	
ICC		0.05	
N_{plate}		10	
$N_{observation}$		11	
Observations		329	
Marginal R^2 / Conditional R^2		0.083 / 0.125	

Table 2.2. Results from a Binomial Generalised Linear Mixed model, testing the diet ratio and block on the number of female flies feeding on conditioned versus unconditioned diets in two-choice tests. The intercept represents flies on a conditioned diet in the 4:1 ratio in block one. The table shows the predictors, odds ratio, confidence intervals and p-values.

Following this, I tested the effects of diet ratio and block on whether a female was feeding on a conditioned or an unconditioned diet. In the 4:1 diet two-choice assays, there was a significant preference for a conditioned diet, with a marginal probability mean preference of 0.81 [95% CI 0.751 – 0.856] compared to an unconditioned diet. On average, approximately 1.5 [95% CI 0.94 – 2.4] more flies per observation were found on conditioned compared to unconditioned patches (Binomial GLMM: $n = 418$, $z = 6.162$, $P < 0.0001$). However, in the 1:4 diet assays, there was a significant decrease in the number of flies feeding on a conditioned diet compared to the 4:1 assays (Binomial GLMM: $n = 418$, $z = 4.026$, $P < 0.001$). The marginal probability of flies preferring a conditioned diet in the 1:4 assays was only 0.59 [95% CI 0.491 – 0.670] (Figure 2.3). No significant block effects were found

between blocks one and two, with a difference of only 0.2 [95% CI -0.6 – 0.382] fewer flies observed on a 4:1 conditioned patch in block two (Binomial GLMM: *n = 418, z = 0.734, P = 0.463*).

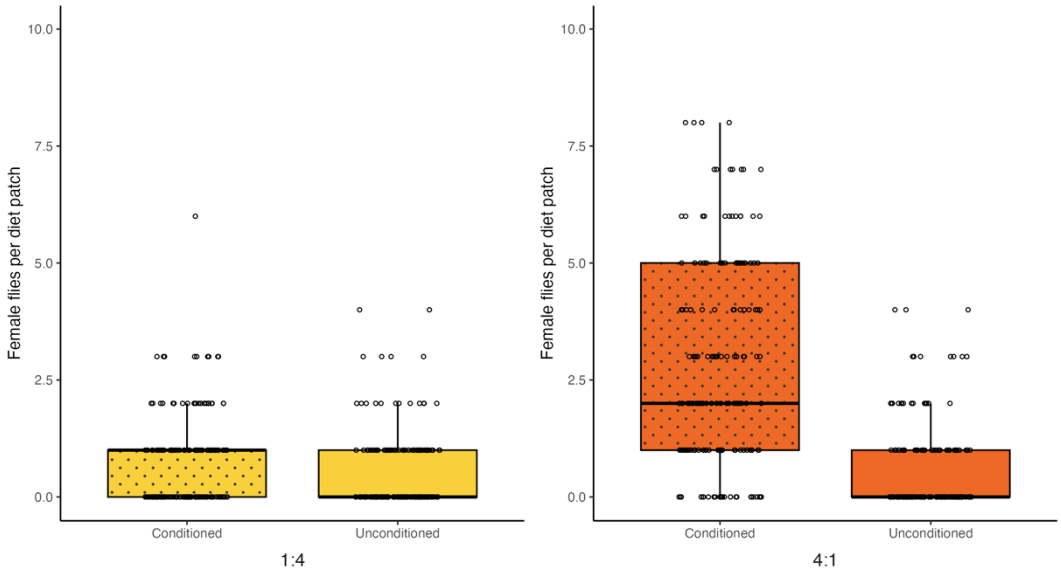

Figure 2.3. Box plots comparing the number of female flies per diet patch per observation for different Protein: Carbohydrate diet ratios conditioned or not by males, in two-choice tests. 1:4 diet ratio (yellow) and 4:1 diet ratio (orange) with conditioned (dots) and unconditioned (no pattern) diets. Each boxplot represents the interquartile range (IQR), with the median indicated by the horizontal line within the box. Whiskers extend to 1.5 times the IQR, individual points represent each observation of flies on a diet.

I then tested the diet choices of mated females in four-choice assays. There was no significant 3-way interaction effect between diet ratio (4:1 or 1:4), conditioning treatment (conditioned / unconditioned), and block (one or two) (Poisson GLMM: *n = 660*, *LRT = 0.217, P = 0.642*), so this was dropped from the model. However, there was a 2-way interaction between conditioning treatment and block (Poisson GLMM: *n = 660, X^2 = 5.336, P = 0.02*) which was retained, but no significant interaction effects between diet ratio and block (Poisson GLMM: *n = 660, X^2 = 2.21, P = 0.137*) or diet ratio and conditioning treatment (Poisson GLMM: *n = 660, X^2 = 2.05, P = 0.151*) so these were dropped from the model (Table 2.3).

	fly_numbers		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	2.27	1.65 – 3.14	**<0.001**
ratio1 × 4	0.34	0.28 – 0.41	**<0.001**
condition [Unconditioned]	0.66	0.53 – 0.84	**0.001**
block [two]	0.60	0.44 – 0.83	**0.002**
condition [Unconditioned] × block [two]	0.66	0.48 – 0.93	**0.016**
Random Effects			
σ^2	0.75		
$\tau_{00\ plate:block}$	0.06		
$\tau_{00\ block}$	0.00		
$\tau_{00\ plate:observation}$	0.00		
$\tau_{00\ observation}$	0.10		
ICC	0.18		
N_{plate}	10		
N_{block}	2		
$N_{observation}$	11		
Observations	660		
Marginal R^2 / Conditional R^2	0.371 / 0.482		

Table 2.3 Results from a Poisson Generalised Linear Mixed Model, testing the diet ratio, conditioning treatment and block on where a female fly chooses to feed in the four-choice tests. The intercept represents the number of flies on a conditioned diet in the 4:1 diet ratio in block one. The table shows the predictors, odds ratio, confidence intervals and p-values.

Following the removal of interaction terms, the effect of diet ratio, conditioning treatment, block, as well as the interaction of conditioning treatment and block, were tested. There were significant preferences for conditioned over unconditioned diets in the 4:1 diets (Poisson GLMM, $n = 660, z = 3.463, P < 0.0001$). On average 2.27 [95% CI, 1.65 – 3.14] flies fed on a 4:1 conditioned diet, whereas only 1.5 [95 % CI 0.86 – 2.62] flies fed on a 4:1 unconditioned diet, showing a difference of 0.77 [95 % CI, 0.79 – 1.39] more flies feeding on a conditioned diet. In addition, differences between diet ratios for feeding preferences were found (Poisson GLMM, $n = 660, z = 11.65, P < 0.0001$). A 4:1 conditioned diet was significantly preferred over a 1:4 conditioned diet, where only 0.77 [95% CI, 0.46 – 1.27] flies per average observation were found feeding on a 1:4 conditioned diet, compared to 2.27 [95% CI, 1.65 – 3.14] on 4:1. This revealed a difference of 1.5 [95 % CI, -1.1 – 1.9] more flies feeding on a 4:1 conditioned diet, showing diet ratio preferences were also observed (Figure 2.4).

The analysis showed that the number of flies feeding on a 4:1 conditioned diet decreased from block one to block two, with an average of 1.66 fewer flies [95 % CI 1.2 – 2.3] on a 4:1

conditioned diet in block two compared to block one (Poisson GLMM, *n = 660, z = 2.306, P = 0.02*), this suggested that the intensity of conditioning preferences differed between blocks. The interaction effect also showed flies feeding on a 4:1 unconditioned diet to decrease between blocks one and two (Poisson GLMM, *n = 660, z = 2.398, P = 0.016*) where an average of only 1 fly [95 % CI, 0.4 – 2.4] fed on a 4:1 unconditioned diet in block two (Table 2.6) (Figure 2.4). Although there were some effects of block present, diet preferences were consistent, with the block effect likely arising from different intensities of the preferences, rather than opposing preferences (Figure S2.1).

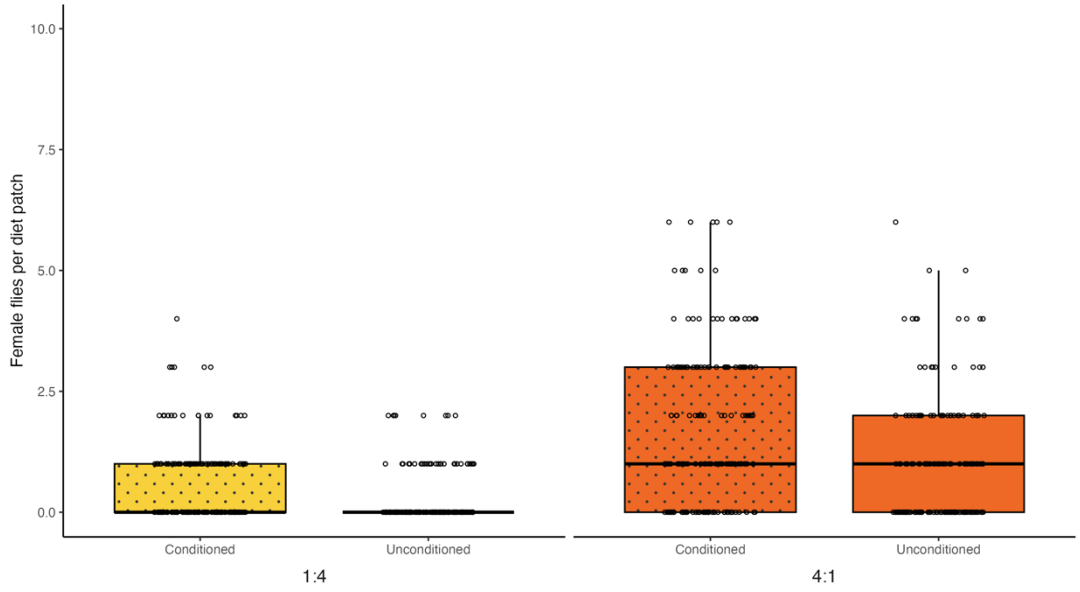

Figure 2.4 Box plots comparing the number of female flies feeding on a diet patch per observation for different Protein: Carbohydrate diet ratios conditioned or not by males, in four-choice tests. 1:4 (yellow) and 4:1 (orange) diet ratios with conditioned (dots) and unconditioned (no pattern) diets.

2.3.6.2 Oviposition behaviour

I next tested the effects of male conditioning on mated female oviposition preferences in the two-choice assays. There was a significant two-way interaction between diet ratio and block (Binomial GLMM, *n = 30, X^2 = 38.9, P < 0.0001*), which was retained in the model (Table 2.4).

	cbind(Conditioned, Unconditioned)		
Predictors	*Odds Ratios*	*CI*	*p*
(Intercept)	5.68	2.78 – 11.61	**<0.001**
ratio1 × 4	0.10	0.07 – 0.14	**<0.001**
block [two]	0.76	0.32 – 1.80	0.531
(ratio1 × 4) × block [two]	4.35	2.70 – 7.01	**<0.001**
Random Effects			
σ^2		3.29	
$\tau_{00\ plate:block}$		0.51	
$\tau_{00\ block}$		0.00	
ICC		0.13	
N_{plate}		11	
N_{block}		2	
Observations		30	
Marginal R^2 / Conditional R^2		0.145 / 0.259	

Table 2.4 Results from a Binomial Generalised Linear Mixed Model, testing the effects of diet ratio and block on whether a female lays their eggs on a conditioned or unconditioned diet in the two-choice tests. The intercept represents the number of eggs on a conditioned diet in the 4:1 ratio in block one. The table shows the predictors, odds ratio, confidence intervals and p-values.

For the 4:1 diets, there was a significant preference for females to lay their eggs on conditioned over unconditioned diets (Binomial GLMM, *n = 30, z = 4.761, P < 0.0001*), with a marginal means probability of 0.832 [95% CI, 0.762 – 0.884] preferring to lay on conditioned over unconditioned diets. This was shown with an average difference of 5.7 [95% CI, 2.8 – 11.6] more eggs laid on a conditioned diet. However, there was a significant decrease in females preferring a conditioned diet in the 1:4 diets compared to the conditioning preferences in the 4:1 diets (Binomial GLMM, *n = 30, z = 11.64, P < 0.0001*), with a marginal means probability of only 0.5 [95% CI, 0.4 – 0.56] preferring to lay on conditioned over unconditioned (Figure 2.5).

There was an interaction effect between the 1:4 diet ratio and block, showing a significant increase in preferring a 1:4 conditioned diet in block two compared to block one (Binomial GLMM, *n = 30, z = 6.055, P < 0.0001*) (Figure 2.5). Although this block effect was observed, similar to the findings of the male conditioning feeding results, it is likely to have come from different intensities of preferences, rather than differences in preferences (Figure S2.2).

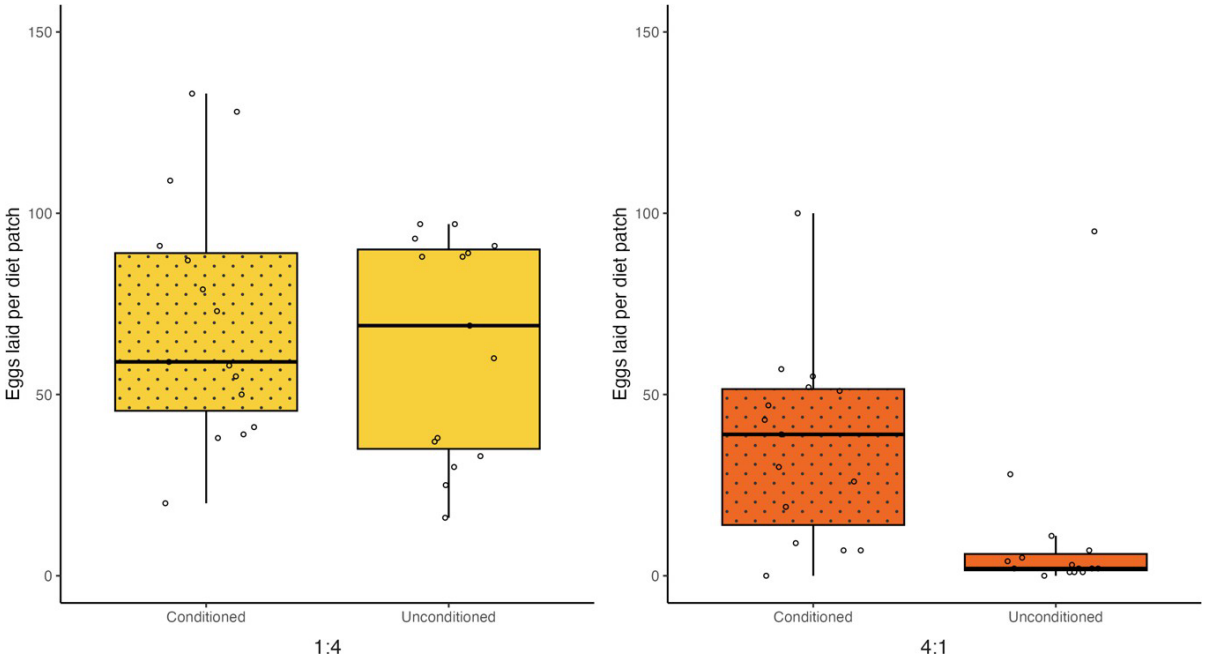

Figure 2.5. Box plots comparing the number of eggs on different Protein: Carbohydrate diet ratios conditioned or not by males, in two-choice tests. 1:4 assay (yellow) and 4:1 assay (orange) with conditioned (dots) and unconditioned (no pattern) diets.

For the four-choice tests, there was no significant 3-way interaction between diet ratio, conditioning treatment and block (Negative Binomial GLM: *n = 60, z = 10.1, P < 0.0001*), so this was dropped from the model. In addition, no two-way interactions between diet ratio and block, block and conditioning treatment or diet ratio and conditioning treatment were found (Table 2.5).

	egg_numbers		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	11.85	7.50 – 19.40	**<0.001**
ratio1 × 4	7.69	4.95 – 11.97	**<0.001**
condition [Unconditioned]	0.70	0.45 – 1.09	0.109
block [two]	0.75	0.46 – 1.18	0.214
Observations	60		
R^2 Nagelkerke	0.791		

Table 2.5. Results from a Negative Binomial Generalised Linear Model, testing the effects of diet ratio, conditioning treatment and block on where a female fly chooses to lay their eggs in the four-choice tests. The intercept represents the number of eggs on a conditioned diet in the 4:1 ratio in block one. The table shows the predictors, odds ratio, confidence intervals and p-values.

No significant differences were observed between conditioning treatments (Negative Binomial GLM, $n = 60$, $z = 1.604$, $P = 0.109$), females laid an average of 11.85 [95% CI, 7.5 - 19] eggs on the 4:1 conditioned diet, and 8.9 [95% CI, 3.2 - 21] on the 4:1 unconditioned diet. However, there was a significant oviposition preference according to diet ratio within the conditioned diets (Negative Binomial GLM: $n = 60$, $z = 9.260$, $P < 0.001$). Females preferred to lay on a 1:4 conditioned diet, with an average of 91 [95% CI, 37 - 232] eggs laid per patch, and only 11.85 [95% CI, 7.5 - 19] on the 4:1 conditioned diet (Figure 2.6). No significant block effects were observed (Negative Binomial GLM: $n = 60$, $z = 1.243$, $P = 0.214$).

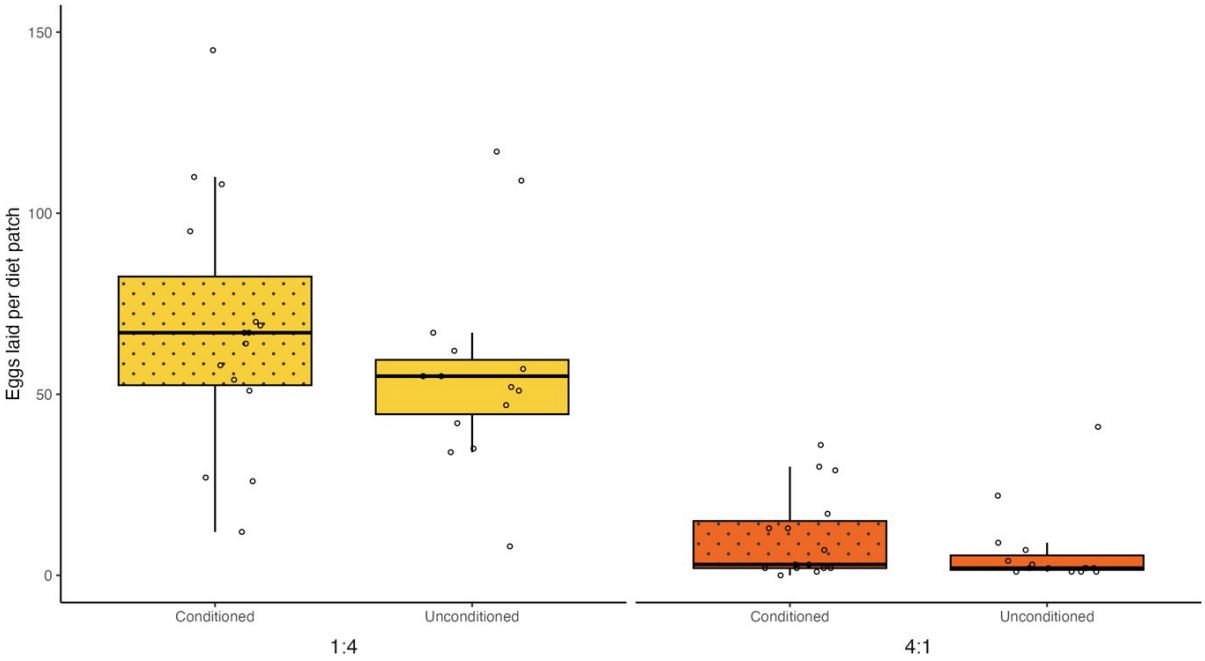

Figure 2.6. Box plots comparing the number of eggs laid on different Protein: Carbohydrate diet ratios conditioned or not by males in four-choice tests. 1:4 (yellow) and 4:1 assay (orange) with conditioned (dots) and unconditioned (no pattern) diets.

2.4.2. Virgin Conditioning

2.4.2.1 Feeding Behaviour

Using two-choice assays and diets conditioned by virgin females, I initially tested for a two-way interaction effect between diet ratio and block (one, two, three and four). No significant interaction effect was found (Binomial GLMM: $n = 418$, $X^2 = 2.736$, $P = 0.434$) so I dropped this interaction from the model (Table 2.6).

	cbind(Conditioned, Unconditioned)		
Predictors	*Odds Ratios*	*CI*	*p*
(Intercept)	1.73	1.07 – 2.79	**0.025**
ratio1 × 4	0.57	0.43 – 0.75	**<0.001**
block [two]	0.58	0.29 – 1.16	0.125
block [three]	0.78	0.41 – 1.46	0.430
block [four]	2.34	1.25 – 4.39	**0.008**
N_{plate}		6	
N_{block}		4	
$N_{observation}$		11	
Observations		418	

Table 2.6. Results from a Binomial Generalised Linear Mixed Model, testing the diet ratio and block whether a female feeds on a conditioned or unconditioned diet in the two-choice tests. The intercept represents flies on a conditioned diet in the 4:1 ratio in block one. The table shows the predictors, odds ratio, confidence intervals and p-values.

Following this, I investigated how different diet ratios with block affect whether a fly feeds on a conditioned or an unconditioned diet. In the 4:1 two-choice assays, flies showed a significant preference for the conditioned diet (Binomial GLMM: $n = 418$, $z = 2.245$, $P = 0.025$), where per average observation, 1.73 more flies [95% CI: 1.02 – 2.79]) fed on the conditioned diet compared to the unconditioned diet. However, this differed with the diet ratio assay being tested, in the 1:4 assays, the number of flies feeding on a conditioned diet decreased significantly, compared to the 4:1 assays (Binomial GLMM: $n = 418$, $z = 4.026$, $P < 0.001$), with a marginal probability means of preferring a conditioned diet of only 0.5 [95% CI, 0.43 – 0.57] compared to 0.637 [95% CI 0.577 – 0.692] in the 4:1 diets.

No significant block effects were found between block one and blocks two and three. However, there was a significant increase in the number of flies feeding on a 4:1 conditioned diet in block four, compared to block one (Binomial GLMM: *n = 418, z = 2.647, P = 0.008*) with an increase of 2.34 [(95% CI 0.26 – 0.75)] flies on average feeding on a diet (Figure 2.7). However, this block effect likely reflects varying intensities of preferences, rather than different preferences (Figure S2.3).

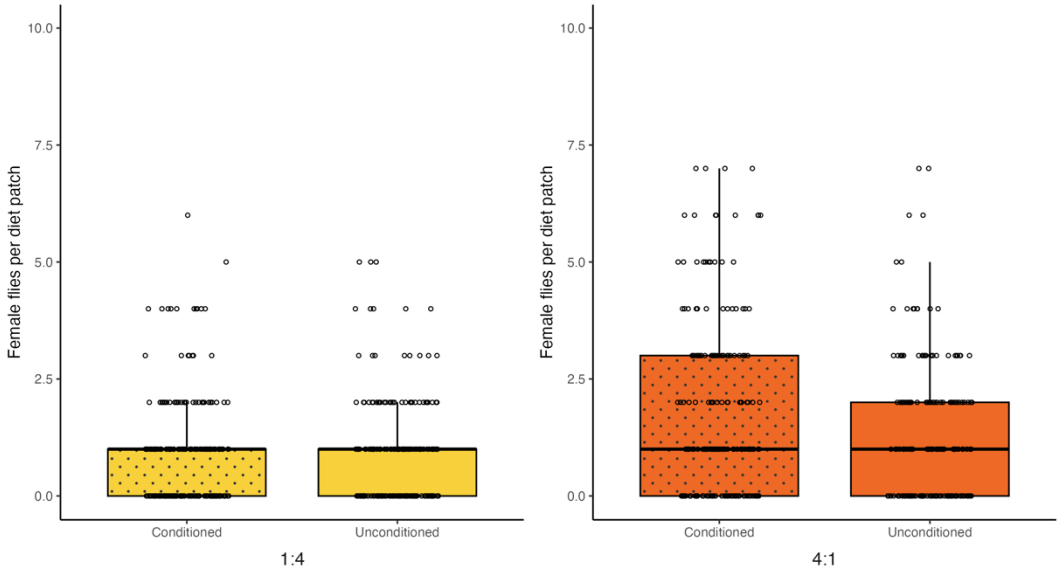

Figure 2.7. Box plots comparing the number of females feeding on different Protein: Carbohydrate diet ratios per observation conditioned or not by virgin females in two-choice tests. 1:4 (yellow) assay and 4:1 assay (orange) with conditioned (dots) and unconditioned (no pattern) diets.

Following this, mated female feeding preferences of virgin conditioned diets were analysed using four-choice assays. There was no 3-way interaction between diet ratio, conditioning treatment and block (Negative Binomial GLM, *n = 660,* $F_{3,840}$ *= 1.362, P = 0.253*), so, this was dropped from the model. Following this, 2-way interactions were tested, and a significant interaction between conditioning treatment and block was found (Negative Binomial GLM: *n = 791,* $F_{3,858}$ *= 6.22, P < 0.0001*). However, there were no significant interactions between diet ratio and conditioning treatment (Negative Binomial GLM, *n = 660,* $F_{1,840}$ *= 1.12, P = 0.28*) or diet ratio and block (Negative Binomial GLM, *n = 660,* $F_{3,845}$ *= 2.2, P = 0.09*) (Table 2.7).

	fly_numbers		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	2.11	1.72 – 2.57	**<0.001**
condition [Unconditioned]	0.73	0.55 – 0.99	**0.042**
block [four]	0.85	0.65 – 1.11	0.229
block [three]	0.85	0.65 – 1.11	0.229
block [two]	1.08	0.82 – 1.42	0.566
ratio1 × 4	0.36	0.31 – 0.42	**<0.001**
condition [Unconditioned] × block [four]	1.27	0.85 – 1.89	0.246
condition [Unconditioned] × block [three]	1.27	0.85 – 1.89	0.246
condition [Unconditioned] × block [two]	0.53	0.34 – 0.83	**0.006**
Observations	791		
R^2 Nagelkerke	0.328		

Table 2.7. Results from a Negative Binomial Generalised Linear Model, testing the effects of diet ratio, conditioning treatment and block on where a female fly chooses to feed in the four-choice tests. The intercept represents flies on a conditioned diet in the 4:1 ratio in block one. The table shows the predictors, odds ratio, confidence intervals and p-values.

Subsequently, I tested the effects of diet ratio, conditioning treatment, block, and the interaction between conditioning treatment and block. Effects of conditioning were observed, a 4:1 conditioned diet was significantly preferred over a 4:1 unconditioned diet (Negative Binomial GLM*, n = 791, z = 2.034, P = 0.04*). A conditioned diet had an average of 2 [95% CI, 1.8 – 2.2] flies present on a patch per average observation, while an unconditioned diet only had an average of 1.4 [95% CI, 1.23 – 1.58] flies, showing a significant difference of 0.584 [95% CI, 0.544 – 0.624] flies. In addition, preferences in diet ratio were also found, while there was an average of 2 [95% CI, 1.8 – 2.2] flies in a 4:1 conditioned diet, there was an average of only 0.714 [95% CI, 0.62 – 0.83] flies in a 1:4 conditioned diet, showing a difference of 1.27 [95% CI, 1.165 – 1.379] flies (Negative Binomial GLM, *n = 791, z = 12.79, P < 0.0001*) (Figure 2.8).

There were no significant preferences for a 4:1 conditioned diet, between block one and blocks two, three and four in this experiment. However, interaction effects were found between a 4:1 unconditioned diet in block one, and a 4:1 unconditioned diet in block two (Negative Binomial GLM, *n = 791, z = 2.755, P < 0.006*), where an average of 1.7 [95% CI, 0.54 – 2.1] flies fed on an unconditioned diet in block two, significantly different from the

average of 1.5 [95% CI, 0.94 – 2.5] flies in block one (Table 2.7) (Figure S2.3). The interaction effect between conditioning and block may be due to different intensities of choosing an unconditioned diet, rather than conditioning preferences differing between different blocks (Figure S2.3).

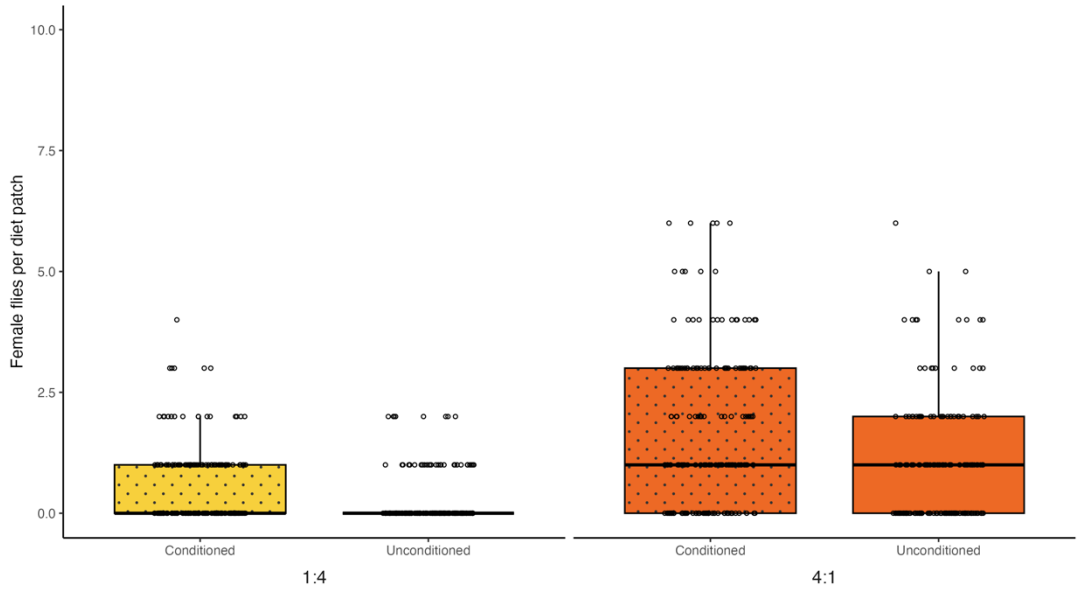

Figure 2.8. Box plots comparing the number of females feeding on different Protein: Carbohydrate diet ratios per observation conditioned or not by virgin females, in four-choice tests. 1:4 (yellow) and 4:1 assay (orange) with conditioned (dots) and unconditioned (no pattern) diets.

2.4.2.2 Oviposition behaviour

The effects of virgin conditioned diets on female dietary oviposition choice in two-choice assays were analysed. There was a significant interaction effect between diet ratio and block (Binomial GLMM: $n = 30, X^2 = 124, P < 0.0001$), which was retained in the model (Table 2.8).

	cbind(Conditioned, Unconditioned)		
Predictors	*Odds Ratios*	*CI*	*p*
(Intercept)	0.17	0.08 – 0.38	**<0.001**
ratio1 × 4	9.35	5.80 – 15.08	**<0.001**
block [four]	6.57	2.42 – 17.81	**<0.001**
block [three]	1.72	0.62 – 4.78	0.301
(ratio1 × 4) × block [four]	0.08	0.04 – 0.13	**<0.001**
(ratio1 × 4) × block [three]	0.24	0.14 – 0.40	**<0.001**
Random Effects			
σ^2		3.29	
$\tau_{00\ block:plate}$		0.50	
$\tau_{00\ plate}$		0.00	
ICC		0.13	
N_{block}		3	
N_{plate}		6	
Observations		30	
Marginal R^2 / Conditional R^2		0.124 / 0.240	

Table 2.8. Results from a Binomial Generalised Linear Mixed Model, testing the diet ratio and block whether a female lays their eggs on a conditioned or unconditioned diet in the two-choice tests. The intercept represents the number of eggs on a conditioned diet in the 4:1 ratio in block one. The table shows the predictors, odds ratio, confidence intervals and p-values.

A model testing the effects of diet ratio and block, as well as their interaction on whether females choose to feed on a conditioned or unconditioned diet was used. In the 4:1 diets, I found a significant preference for an unconditioned diet (Binomial GLMM: $n = 30, z = 4.31, P < 0.0001$), showing a marginal means probability of only 0.28 [95% CI, 0.21 – 0.37] for laying their eggs on conditioned diet over an unconditioned diet, highlighting the preference for an unconditioned diet. There was also a significant increase in preferring a conditioned diet over an unconditioned diet in the 1:4 diets compared to the 4:1 diets (Binomial GLMM: $n = 30, z = 9.2, P < 0.0001$) indicating that for oviposition, conditioning may be more preferred in a high carbohydrate diet – a known preferred oviposition diet (Figure 2.9).

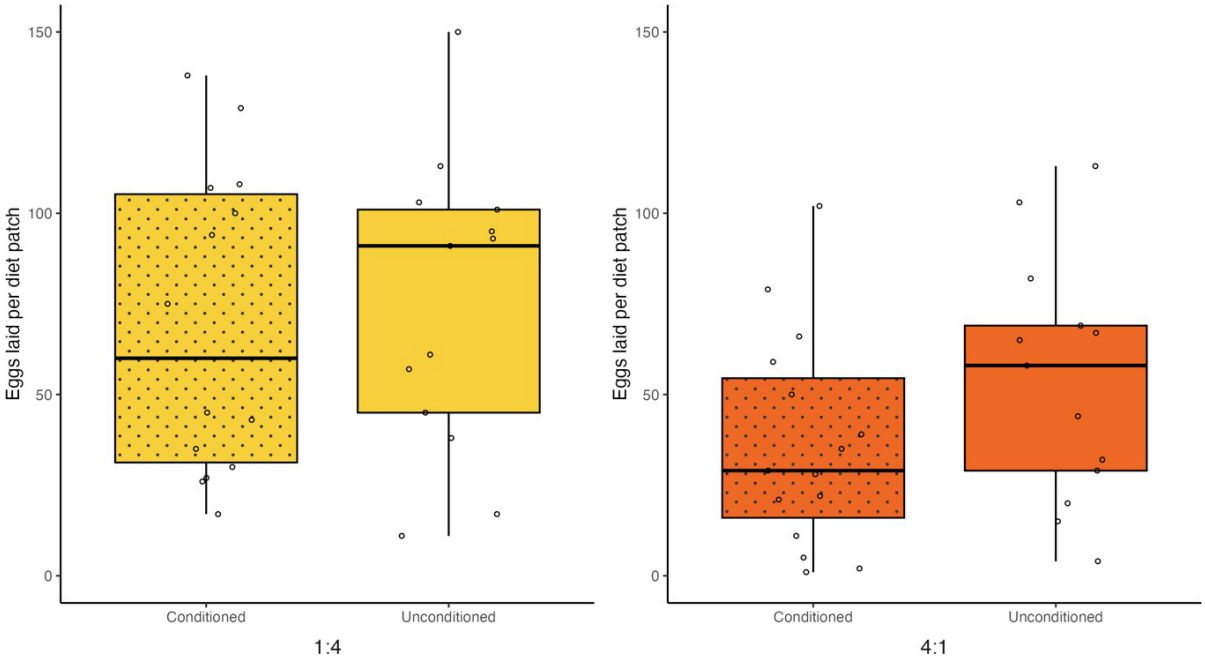

Figure 2.9. Box plots comparing the number of eggs laid per patch on different Protein: Carbohydrate diet ratios conditioned or not by virgin females, in two-choice tests. 1:4 (yellow) assay and 4:1 assay (orange) with conditioned (dots) and unconditioned (no pattern) diets.

I then analysed the effects of virgin-conditioned diets on oviposition preferences in four-choice assays. There was no 3-way interaction between diet ratio, block and conditioning treatment (Negative Binomial GLM, $n = 60$, $F_{2,73} = 2.11$, $P = 0.132$), which was removed the model. Following this, I found an interaction effect between block and conditioning treatment (Negative Binomial GLM, $n = 60$, $F_{2,77} = 3.55$, $P = 0.036$), but no two-way interactions between diet ratio and conditioning treatment or diet ratio and block (Table 2.9).

	egg_numbers		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	32.67	19.91 – 56.20	**<0.001**
condition [Unconditioned]	0.51	0.27 – 0.99	**0.042**
block [four]	1.32	0.73 – 2.37	0.339
block [three]	1.26	0.68 – 2.31	0.449
ratio1 × 4	1.12	0.80 – 1.56	0.509
condition [Unconditioned] × block [four]	0.95	0.41 – 2.18	0.895
condition [Unconditioned] × block [three]	2.90	1.22 – 6.86	**0.014**
Observations	60		
R^2 Nagelkerke	0.444		

Table 2.9 Results from a Negative Binomial Generalised Linear Model, testing the diet ratio, conditioning treatment and block on where a female fly chooses to lay their eggs in the four-choice tests. The intercept represents the number of eggs on a conditioned diet in the 4:1 ratio in block one. The table shows the predictors, odds ratio, confidence intervals and p-values.

A model containing diet ratio, block, and conditioning treatment, as well as the interaction of conditioning treatment and block was used to test for effects on oviposition preferences of diet patches. A preference for conditioned diets was observed (Negative Binomial GLM, *n = 60, z = 2.031, P = 0.04*) with an average of 16.8 [95% CI, 5.3 - 55] eggs laid on the 4:1 unconditioned diets, compared to an average of 32.7 [95% CI, 19.9 - 56] eggs laid on the 4:1 conditioned diet. In addition, within the conditioned diets, there were no diet ratio preferences observed; with an average of 36.5 [95% CI, 15.8 – 87.8] eggs in a 1:4 conditioned diet (Negative Binomial GLM, *n = 60, z = 0.661, P = 0.51*) compared to the 32.7 [95% CI, 19.9 - 56] eggs laid on the 4:1 conditioned diet (Figure 2.10).

There was no interaction effect between the blocks in preference for a conditioned 4:1 diet, but an interaction effect between unconditioned and block three was observed (Negative Binomial GLM, *n = 60, z = 2.451, P = 0.014*). However, this block effect is likely due to varying intensities of preferences, rather than differences in preferences (Figure S2.4).

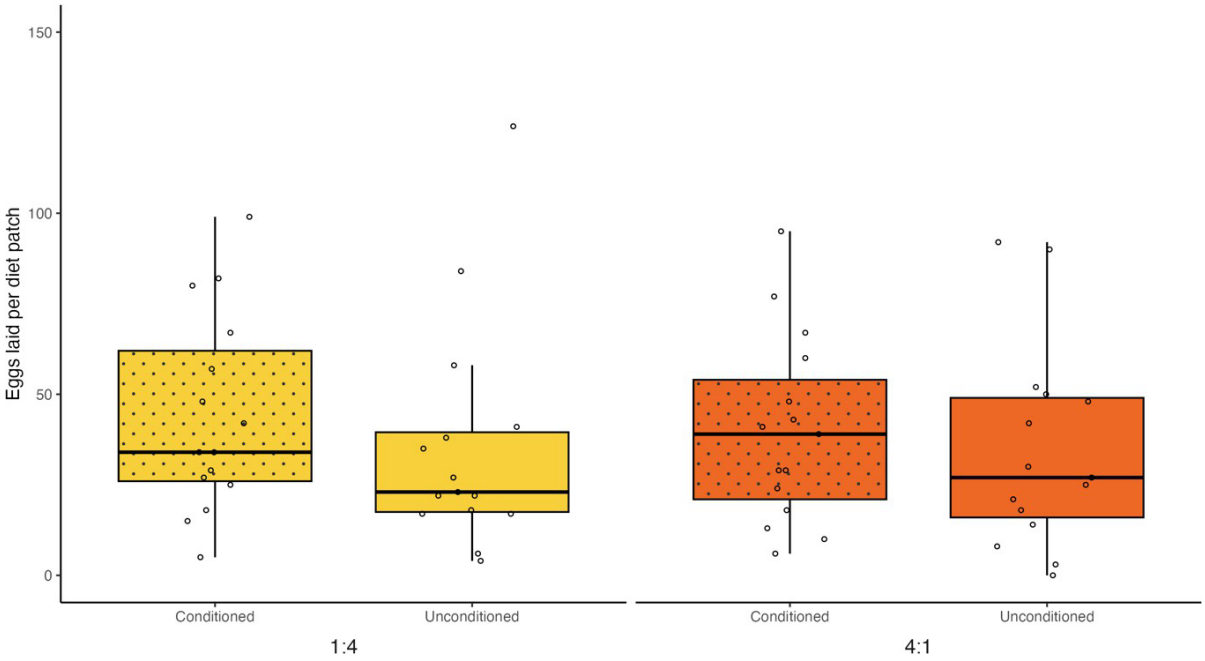

Figure 2.10. Box plots comparing the number of eggs laid per patch on different Protein: Carbohydrate diet ratios conditioned or not by virgin females, in four-choice tests. 1:4 (yellow) and 4:1 assay (orange) with conditioned (dots) and unconditioned (no pattern) diets.

2.4.3 *Ovo*^{D1} Conditioning

2.4.3.1 Feeding behaviour

To investigate the effects of *Ovo*^{D1} (eggless) female conditioned 4:1 and 1:4 diets within an absolute environment, two-choice assays were used. A significant interaction effect was found between diet ratio and block (one and two) (Binomial GLMM: $n = 330, X^2 = 27.96, P < 0.0001$) so this was kept in the model (Table 2.10).

	cbind(Conditioned, Unconditioned)		
Predictors	*Odds Ratios*	*CI*	*p*
(Intercept)	0.59	0.31 – 1.11	0.099
ratio1 × 4	6.91	4.26 – 11.18	**<0.001**
block [two]	3.51	1.33 – 9.25	**0.011**
(ratio1 × 4) × block [two]	0.13	0.06 – 0.28	**<0.001**
N_{plate}		8	
N_{block}		2	
$N_{observation}$		11	
Observations		330	

Table 2.10. Results from a Binomial Generalised Linear Mixed Model, testing the effects of diet ratio and block on whether a female feeds on a conditioned or unconditioned diet in the two-choice tests. The intercept represents flies on a conditioned diet in the 4:1 ratio in block one. The table shows the predictors, odds ratio, confidence intervals and p-values.

In the 4:1 two-choice assays, there was no significant preference for the conditioned diet. The marginal means probability of preferring a conditioned diet was 0.523 [95% CI 0.4 – 0.64], and on average, only 0.59 [95% CI: 0.31 – 1.11] more flies fed on the conditioned diet compared to the unconditioned diet (Binomial GLMM: $n = 329, z = 1.651, P = 0.098$). In contrast, within the 1:4 assays, there was a significant increase in the number of flies feeding on the conditioned diet compared to the 4:1 assays (Binomial GLMM: $n = 329, z = 7.854, P < 0.001$). The average number of flies feeding on the conditioned diet increased by 4 [95% CI, 1.32 – 12.3] with a marginal means probability of 0.73 [95% CI 0.62 – 0.82] for preferring a conditioned over an unconditioned diet (Figure 2.11).

In addition, significant block effects were found between block one and two for the 4:1 conditioned diet (Binomial GLMM: *n = 329, z = 2.542, P = 0.01*). However, this block effect is likely due to the intensity of preferences rather than differences in conditioning preferences (Figure S2.7).

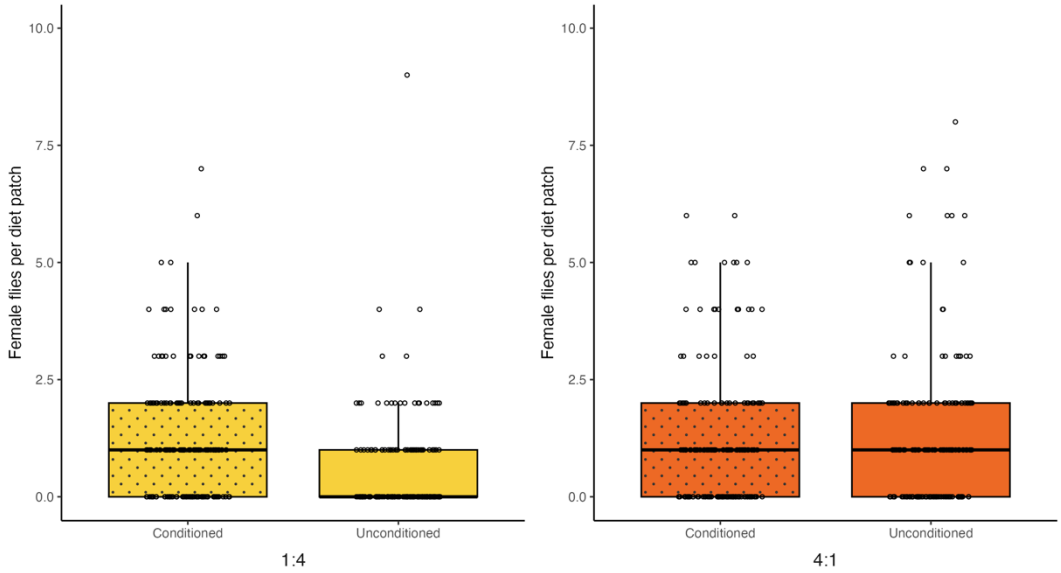

Figure 2.11. Box plots comparing the number of females feeding on different Protein: Carbohydrate diet ratios per observation conditioned or not by *Ovo^{D1}* females in two-choice tests. 1:4 (yellow) and 4:1 assay (orange) with conditioned (dots) and unconditioned (no pattern) diets.

I tested the effects of *Ovo^{D1}* fly-conditioned diets on female dietary choices in a relative environment, using four-choice assays. I found no significant three-way interaction effect between diet ratio, conditioning treatment, and block (Negative Binomial GLM: *n = 616, X^2 = 0.817, P = 0.366*), so I dropped this interaction from the model. However, significant two-way interaction effects were found between diet ratio and conditioning treatment (Negative Binomial GLM: *n = 616, X^2 = 4.2, P = 0.041*) (Table 2.11), which was retained in the model.

	fly_numbers		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	3.07	2.62 – 3.60	**<0.001**
ratio1 × 4	0.31	0.24 – 0.38	**<0.001**
condition [Unconditioned]	0.33	0.25 – 0.44	**<0.001**
block [two]	0.90	0.74 – 1.11	0.326
(ratio1 × 4) × condition [Unconditioned]	1.55	1.04 – 2.29	**0.031**
condition [Unconditioned] × block [two]	0.69	0.47 – 1.00	0.054
Observations	616		
R^2 Nagelkerke	0.504		

Table 2.11. Results from a Negative Binomial Generalised Linear Model, testing the diet ratio, conditioning treatment and block on where a female fly chooses to feed in the four-choice tests. The intercept represents flies on a conditioned diet in the 4:1 ratio in block one. The table shows the predictors, odds ratio, confidence intervals and p-values.

A model containing diet ratio, conditioning treatment, block, as well as the interactions of diet ratio and conditioning treatment, and conditioning treatment and block was used to test female feeding preferences. A significant preference for a 4:1 conditioned diet was found, with an average of 2.9 [95% CI, 2.58 – 3.3] compared to 0.811 [95% CI 0.668 – 0.985] females per patch on the conditioned, versus unconditioned diets, respectively (Negative Binomial GLM: $n = 616, z = 7.689, P < 0.0001$). Significant differences were also observed between feeding on a 1:4 conditioned diet and a 4:1 conditioned diet (Negative Binomial GLM: $n = 616, z = 10.428, P < 0.0001$), with an average of 0.895 [95% CI, 0.73 – 1.1] females on 1:4 conditioned patches compared to an average of 2.9 [95% CI, 2.58 – 3.3] for the 4:1 diet.

There was no significant interaction between a conditioned diet and block. However, an interaction effect between 1:4 conditioned and 1:4 unconditioned diets was observed (Negative Binomial GLM: $n = 616, z = 2.159, P = 0.031$). This indicated that the preference for a diet ratio will change depending on the conditioning treatment (Figure 2.12).

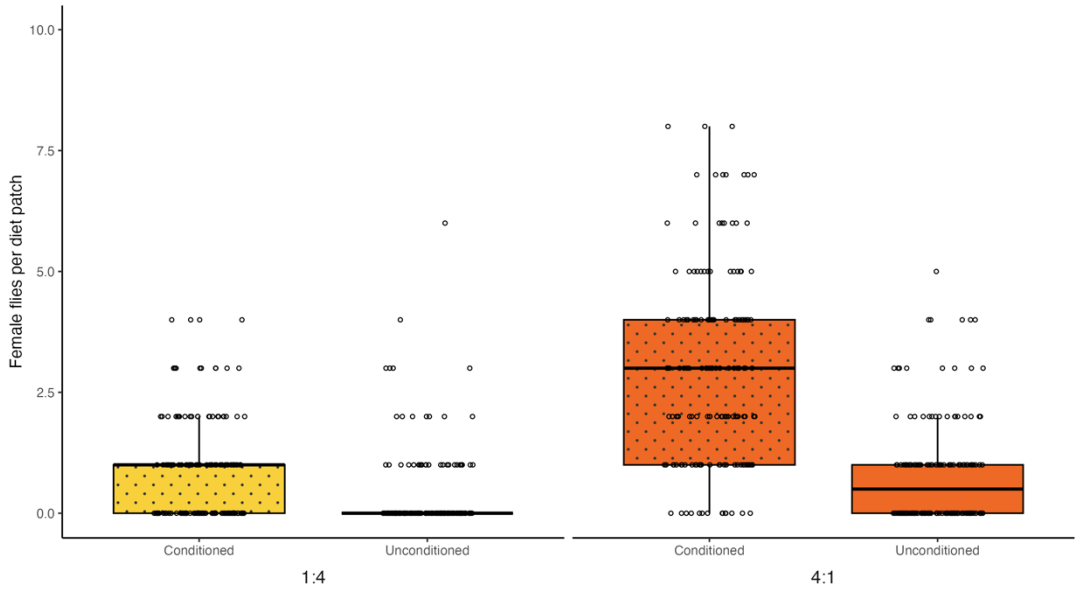

Figure 2.12. Box plots comparing the number of females feeding on different Protein: Carbohydrate diet ratios per observation conditioned or not by *Ovo*^{D1} females in four-choice tests. 1:4 (yellow) and 4:1 assay (orange) with conditioned (dots) and unconditioned (no pattern) diets.

2.4.3.2 Oviposition behaviour

To analyse the effects of diets conditioned by *Ovo*^{D1} females on dietary oviposition choice in mated females, using two-choice assays, I analysed the effects of diet ratio and block, on whether mated females choose to lay their eggs on a conditioned diet or an unconditioned diet. I first found a two-way interaction between diet ratio and block (Binomial GLMM: *n = 30, X*^{2} *= 79, P < 0.0001*) which was retained in the model (Table 2.12)

	cbind(Conditioned, Unconditioned)		
Predictors	*Odds Ratios*	*CI*	*p*
(Intercept)	2.65	0.97 – 7.24	0.057
ratio1 × 4	4.57	3.36 – 6.21	**<0.001**
block [two]	0.41	0.09 – 1.78	0.234
(ratio1 × 4) × block [two]	0.19	0.13 – 0.27	**<0.001**
Random Effects			
σ^2		3.29	
$\tau_{00\ block:plate}$		2.00	
$\tau_{00\ plate}$		0.00	
N_{block}		2	
N_{plate}		8	
Observations		30	
Marginal R^2 / Conditional R^2		0.248 / NA	

Table 2.12. Results from a Binomial Generalised Linear Mixed Model, testing the effects of diet ratio and block on whether a female lays their eggs on a conditioned or unconditioned diet in the two-choice tests. The intercept represents the number of eggs on a conditioned diet in the 4:1 ratio in block one. The table shows the predictors, odds ratio, confidence intervals and p-values.

There was no significant effect of conditioning within the 4:1 diets (Binomial GLMM: *n = 30, z = 1.9, P = 0.057*) with a marginal means probability of females preferring to lay on a conditioned diet of 0.629 [95% CI, 0.45 – 0.78] with an average of 2.65 [95% CI, 0.91 – 7.6] more eggs laid on a conditioned diet over an unconditioned diet. Despite no conditioning differences observed between the 4:1 diets, they were observed between the 1:4 conditioned and unconditioned diets (Binomial GLMM, *n = 30, z = 4.9, P < 0.0001*), with a probability of 0.771 [95% CI, 0.62 – 0.88] for choosing to lay on the conditioned diet over the unconditioned diet. In addition, significant preferences were observed between diet ratio preferences (Binomial GLMM: *n = 30, z = 9.7, P =< 0.0001*), with females laying an average

of 12.1 [95% CI, 3.1 – 49] more eggs on a 1:4 conditioned diet compared to a 4:1 conditioned diet (Figure 2.13).

There was no effect of block observed in 4:1 conditioned diets across blocks (Binomial GLMM, *n = 30, z = 8.7, P = 0.234*). An interaction effect between a 1:4 conditioned diet and block was observed (Binomial GLMM, *n = 30, z = 8.7, P < 0.0001*) which is likely due to varying intensities of preferences rather than differences in preferences (Figure S2.6).

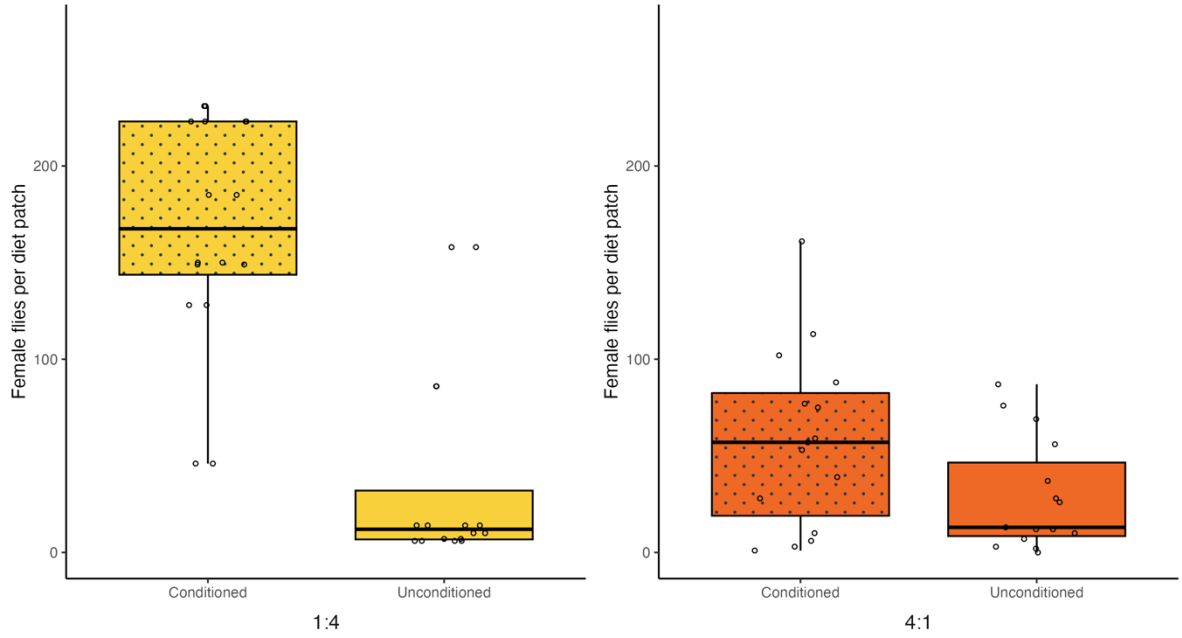

Figure 2.13. Box plots comparing the number of eggs laid on different Protein: Carbohydrate diet ratios conditioned or not by *Ovo*^{D1} females in two-choice tests. 1:4 (yellow) assay and 4:1 assay (orange) with conditioned (dots) and unconditioned (no pattern) diets.

There was no 3-way interaction between conditioning treatment, block and diet ratio (Negative Binomial GLM, *n = 60,* $F_{1,63}$ *= 0.09, P = 0.76*), so this term was dropped from the model. Two-way interactions between conditioning treatment and block (Negative Binomial GLM, *n = 60,* $F_{1,92}$ *= 25, P < 0.0001*) and diet ratio and block (Negative Binomial GLM, *n = 60,* $F_{1,90}$ *= 23, P < 0.0001*) were found, and were retained (Table 2.13).

	egg_numbers		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	9.93	6.73 – 14.99	**<0.001**
condition [Unconditioned]	0.16	0.10 – 0.26	**<0.001**
block [two]	1.36	0.77 – 2.40	0.300
ratio1 × 4	19.81	12.23 – 32.39	**<0.001**
condition [Unconditioned] × block [two]	6.23	3.22 – 12.08	**<0.001**
block [two] × ratio1 × 4	0.17	0.09 – 0.34	**<0.001**
Observations	60		
R^2 Nagelkerke	0.987		

Table 2.13. Results from a Negative Binomial Generalised Linear Model, testing the effects of diet ratio, conditioning treatment and block on where a female fly chooses to lay their eggs in the four-choice tests. The intercept represents the number of eggs on a conditioned diet in the 4:1 ratio in block one. The table shows the predictors, odds ratio, confidence intervals and p-values.

I analysed the effects of conditioning treatment, diet ratio, and block as well as the interaction of treatment and block on where females choose to feed. Within the 4:1 diets, I first found a significant effect between a conditioned and unconditioned diet (Negative Binomial GLM, $n = 60, z = 7.5, P < 0.0001$). Females laid an average of 11.56 [95% CI, 8.68 – 15.4] eggs per patch on a conditioned diet, compared to an average of only 4.71 [95% CI, 3.42 – 6.5] eggs on an unconditioned diet. I also analysed the differences between the two treatments within the 1:4 diets and found a significant preference for a conditioned over unconditioned diets (Negative Binomial GLM, $n = 60, z = 5.35, P < 0.0001$), with an average of 95 eggs [95% CI, 72.9 – 124.6] on a 1:4 conditioned diet, and an average of 38.8 eggs [95% CI, 29.6 – 50.9] on a 1:4 unconditioned diet. In addition, I observed significant differences in diet ratio between the 4:1 and 1:4 conditioned diets (Negative Binomial GLM, $n = 60, z = 7.5, P < 0.0001$), with females laying an average of 95 [95% CI, 72.9 – 124.6] eggs on a 1:4 conditioned diet, and an average of 11.56 [95% CI, 8.68 – 15.4] eggs on 4:1 conditioned (Figure 2.14).

There was no significant effect of block observed with the 4:1 conditioned diets, but an interaction effect between 4:1 unconditioned in block one and two, and 1:4 conditioned in block one and block two was observed (Negative Binomial GLM, $n = 60, z = 5.2, P < 0.0001$) (Figure S2.6), likely due to varying intensities of preferences.

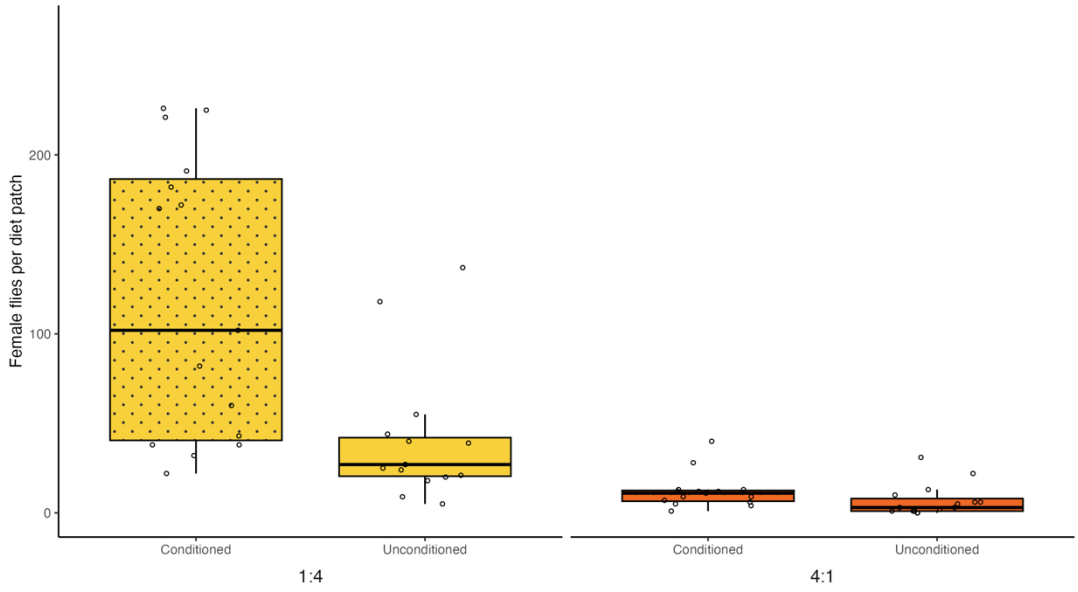

Figure 2.14. Box plots comparing the number of eggs laid on different Protein: Carbohydrate diet ratios conditioned or not by *Ovo*^{D1} females in four-choice tests. 1:4 (yellow) and 4:1 assay (orange) with conditioned (dots) and unconditioned (no pattern) diets.

	Absolute (two-choice)				Relative (four-choice)			
	Feeding		Oviposition		Feeding		Oviposition	
	4:1	1:4	4:1	1:4	4:1	1:4	4:1	1:4
Male	**Binomial GLMM, *z = 7.153, P < 0.0001* + conditioned**	Binomial GLMM, *z = 1.759, P = 0.079* + conditioned	**Binomial GLMM, *z = 4.761, P < 0.0001* + conditioned**	Binomial GLMM, *z = 1.795, P = 0.072 (-) conditioned*	**Poisson GLMM, *z = 5.552, P < 0.001* + conditioned**	**Poisson GLMM, *z = 4.848, P < 0.001* + conditioned**	Negative Binomial GLM, *z = 1.449, P = 0.468* + conditioned	Negative Binomial GLM, *z = 0.629, P = 0.9226* + conditioned
Virgin	**Binomial GLMM, *z = 6.232, P < 0.0001* + conditioned**	**Binomial GLMM, *z = 3.652, P < 0.001* + conditioned**	Binomial GLMM, *z = 4.308, P < 0.0001* (-) conditioned	Binomial GLMM, *z = 0.743, P = 0.457 (-) conditioned*	**Negative Binomial GLM, *z = 3.845, P = 0.0007* + conditioned**	Negative Binomial GLM, *z = 1.355, P = 0.5279* + conditioned	**Negative Binomial GLM, *z = 2.031, P = 0.042* + conditioned**	**Negative Binomial GLM, *z = 2.725, P = 0.00642* + conditioned**
Ovo^{D1}	Binomial GLMM, *z = 1.651, P = 0.098 (-) conditioned*	**Binomial GLMM, *z = 3.966, P < 0.0001* + conditioned**	Binomial GLMM, *z = 1.9, P = 0.057* + conditioned	**Binomial GLMM, *z = 4.852, P < 0.0001* + conditioned**	**Zero Inflated Negative Binomial, *z = 10.99, P < 0.0001* + conditioned**	**Zero Inflated Negative Binomial, *z = 4.674, P < 0.0001* + conditioned**	**Negative Binomial GLM, *z = 7.499, P < 0.0001* + conditioned**	**Negative Binomial GLM, *z = 3.668, P = 0.0014* + conditioned**

Table 2.14. Dietary choice feeding and oviposition results summary table. This table shows the statistical tests, the z values and the p-values, as well as if there is a positive (+) or negative (-) estimate value for a conditioned diet. A statistically significant preference for a conditioned diet is highlighted in green.

2.4 Discussion

In this chapter, I explored the effects of media conditioning on dietary feeding and oviposition choice in mated female *Drosophila melanogaster*. Dietary variation has been shown to lead to distinct dietary preferences in flies, influenced by factors such as sex and mating status. For example, one study found that virgin and mated females will have different nutrient composition preferences (Camus et al., 2018). Other factors, such as body weight (Almeida de Carvalho and Mirth, 2017) and gut microbiome (Leitão-Gonçalves et al., 2017) can also affect dietary choices, which in turn can result in varying life-history consequences. In many studies in the lab that use *D. melanogaster*, flies are raised in dietary environments shared with other flies, potentially depositing various fly cues throughout these environments. It is therefore important to understand the consequences of a diet like this, and whether flies would choose a similar diet when given the option. To further understand this, through this chapter, I focused on investigating the dietary choice effects of cues of habitat usage and the previous presence of flies of the same species. I hypothesised that conditioning of the diet by previous occupants could alter dietary composition or quality through mechanisms such as (i) the deposition of microbes from the flies' gut microbiome, (ii) the deposition of pheromones, or (iii) social digestion, where a fly has changed the physical condition of the diet through the deposition of dietary digestate or digestive enzymes. I predicted that fly-conditioned diets would impact feeding and oviposition preferences. Specifically, I predicted that mated females would prefer feeding and laying eggs on a conditioned diet over an unconditioned diet when given the choice. This hypothesis was based on the assumption that diet fly conditioning involves factors such as microbial and pheromonal deposition, supported by findings where mated female flies exhibit preferences for specific microbial content (Qiao et al., 2019) and pheromonal cues (Dweck et al., 2015). In addition, I predicted that the type of conditioning treatment (ie: the sex of the fly, and whether or not the flies lay eggs) would result in distinct dietary preferences. This prediction was further supported by studies indicating the existence of sex-specific microbiomes (Han et al., 2017) and pheromones (Borrero-Echeverry et al., 2022), suggesting different diet conditioning preferences could be exhibited. The results showed clear conditioning preferences amongst mated females, with this preference being more pronounced in high-protein diets (Protein: Carbohydrate, 4:1), especially for feeding behaviour. Conditioning preferences were most pronounced when Ovo^{D1} females had conditioned the diets, indicating the absence of eggs enhances a female's attraction to a diet. These divergent responses to different dietary conditions support findings from other studies, which show that mated females display varying behaviours when presented with different types of food, depending on factors such

as nutrient composition (Almeida de Carvalho and Mirth, 2017) and food texture (Millar and Chapman, unpublished)

I first tested the effects of diet conditioning by males. Significant preferences for conditioned diets were found, although the strength of the conditioning varied based on nutrient composition and whether the environment allowed for absolute (two-choice) or relative (four-choice) comparisons. In both assay types, a preference for conditioning was evident for 4:1 feeding, but this preference decreased in the 1:4 diets. For oviposition, conditioning preferences were observed only in the 4:1 two-choice assays. When diets were conditioned by virgin females, the results were similar - with a preference for conditioned diets observed in the 4:1 diets in both assays but decreased when in the 1:4 diets. Oviposition preferences were mixed, with females showing a preference for unconditioned diets in the 4:1 two-choice assays and an increased preference for conditioned diets in the 1:4 two-choice assays. These results suggest that females might avoid laying eggs on diets with existing eggs and that conditioning results differ depending on the assay type. The results from the experiments in which diets were conditioned by Ovo^{D1} females also showed distinct conditioning preferences. In the two-choice assays, no conditioning preference was found in the 4:1 diets, but there was a significant increase in flies feeding on a conditioned diet in the 1:4 assays, contrasting with male and virgin female conditioning results. However, significant conditioning effects were observed in the four-choice assays for feeding for both 4:1 and 1:4 diets, and for oviposition, in the four-choice assay for both 4:1 and 1:4, and the two-choice assay for the 1:4 diet.

These findings described above highlight how mated females will exhibit distinct dietary preferences for feeding and oviposition choice, which has been seen in previous studies (Lihoreau et al., 2016). The results showed that the cues of dietary usage and previous social environments can affect dietary choices. I hypothesised different potential benefits of fly conditioning, which are discussed in more detail below.

One potential benefit I proposed was due to microbe deposition. Microbes may enhance the protein content of the diet (Lesperance and Broderick, 2020), making it more attractive to females, which have previously been shown to favour high-protein diets for feeding (Almeida de Carvalho and Mirth, 2017b). In this chapter, I found that, for feeding, particularly within the 4:1 high protein diets, conditioned diets were consistently preferred, although this preference wasn't observed in Ovo^{D1} conditioned diets in the 4:1 two-choice assay. In addition, an attraction to conditioned diets due to microbe deposition directly, rather than just microbes acting as a protein component, may have also been observed. Previous studies show that

females are attracted to diets containing specific microbes, with preferences expressed for both feeding and oviposition (Qiao *et al.*, 2019; Lasa *et al.*, 2019). This has been suggested to be relevant to the natural context in which flies have naturally evolved to lay their eggs on ripening fruits, which are potentially rich in microorganisms (Karageorgi *et al.*, 2017; Günther *et al.*, 2019). The findings throughout this thesis, showed the possible consequences of a diet being naturally pre-conditioned with *D. melanogaster* gut microbes, possibly suggesting that *D. melanogaster* are attracted to both feeding and laying their eggs on a diet which has been pre-conditioned with another *D. melanogaster's* gut microbes.

Another potential explanation for the benefits of the observed conditioning preferences could be due to pheromonal deposition. As male, virgin and Ovo^{D1} female flies roam around on a diet, they may deposit different sex-specific pheromones. This could influence preferences for feeding and oviposition, as female flies have been shown to prefer feeding and laying their eggs at sites with a high concentration of pheromones (Verschut et al., 2023). cVA is a male-specific pheromone that is deposited by males and transferred to females during mating (Bartelt et al., 1985). Therefore, Ovo^{D1} females could potentially carry this pheromone, but not virgin females. This could explain why conditioning is more pronounced in males and Ovo^{D1} females for both feeding and oviposition, as mated females are known to be attracted to cVA (Lebreton et al., 2012). These studies support the hypothesis that conditioned diets are favoured over unconditioned diets due to pheromonal deposition, as unconditioned diets will likely lack these pheromonal cues.

In addition, it is possible that flies may be attracted to feeding on a diet that has already been fed on by other flies. This could be due to changes in texture and potentially bioavailability, possibly because of the presence of additional digestive enzymes excreted by the other flies. This may be a factor that increases the attractiveness of conditioned diets. As previous flies had fed on these diets, the texture may have become softer, which could make it more beneficial as both a feeding and egg-laying substrate, with previous studies showing flies prefer laying eggs on a softer substrate (Vijayan et al., 2022). However, there has been little research to date into how the physical condition of a diet is altered by previous fly activity and how this can affect the dietary choice of other flies. Further investigation is needed to explore this.

Overall, these findings demonstrate that conditioning preferences were seen for both feeding and oviposition dietary choices, but these preferences varied based on the fly conditioning treatment, nutrient composition, and whether the environment was a two-choice or four-choice assay. The decreased conditioning preferences for certain nutrient compositions, in

both feeding and oviposition, could be due to flies already having strong feeding and egg-laying behaviours towards specific nutrient compositions (Almeida de Carvalho and Mirth, 2017). In this experiment, I intentionally used both high-protein diets and high-carbohydrate diets, as females prefer feeding on and laying their eggs on these respective diets. This may explain why conditioning was generally stronger in the 4:1 ratio for feeding and in the 1:4 ratio for oviposition. In addition, conditioning preferences also varied depending on whether flies were choosing in a two-choice or four-choice assay, with preferences being more pronounced in a four-choice assay. The impact of increasing the number of choices for decision making was evident, and further investigation may be needed to understand why introducing additional options influences this decision making process. Further research is needed to investigate the fitness and developmental consequences of these conditioned diets, as well as the underlying mechanisms that make them more appealing to other flies. Potential mechanisms could be investigated by additional experiments (see Chapter 4).

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2.6 Chapter 2 – Supplementary Material

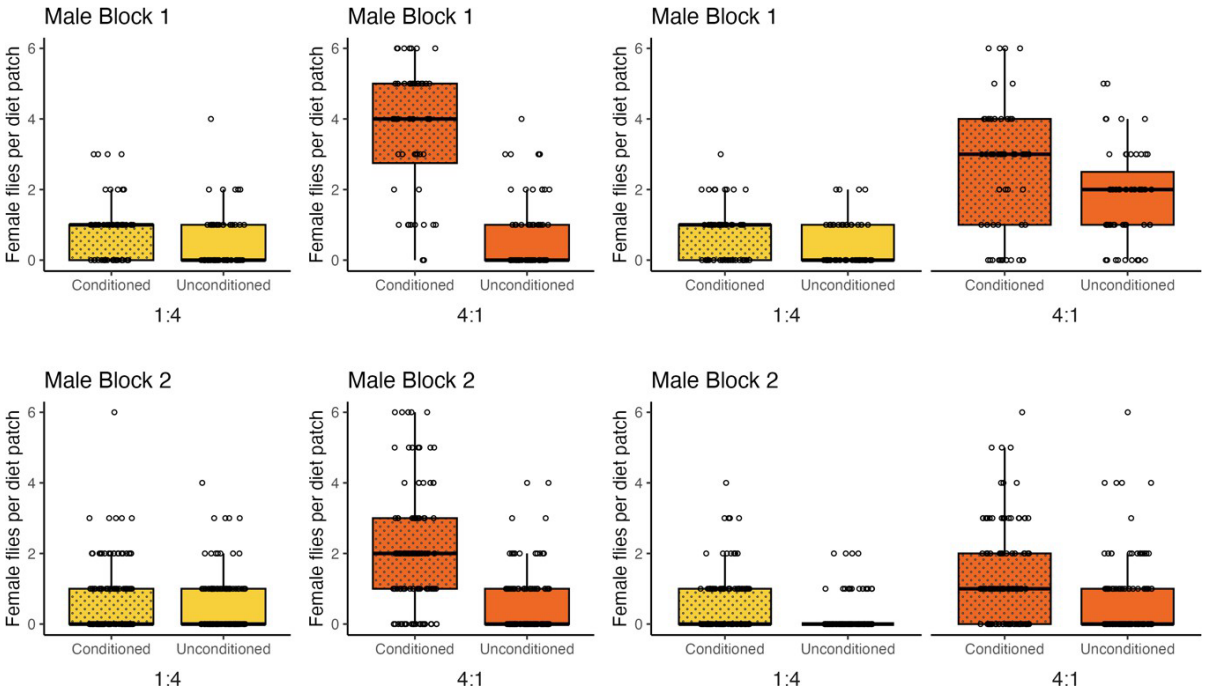


Figure S2.1: Box plots showing feeding results from male-conditioned diets. box plots show each block (one and two) under different conditioning treatments (conditioned and unconditioned) and diet ratios (4:1 and 1:4)

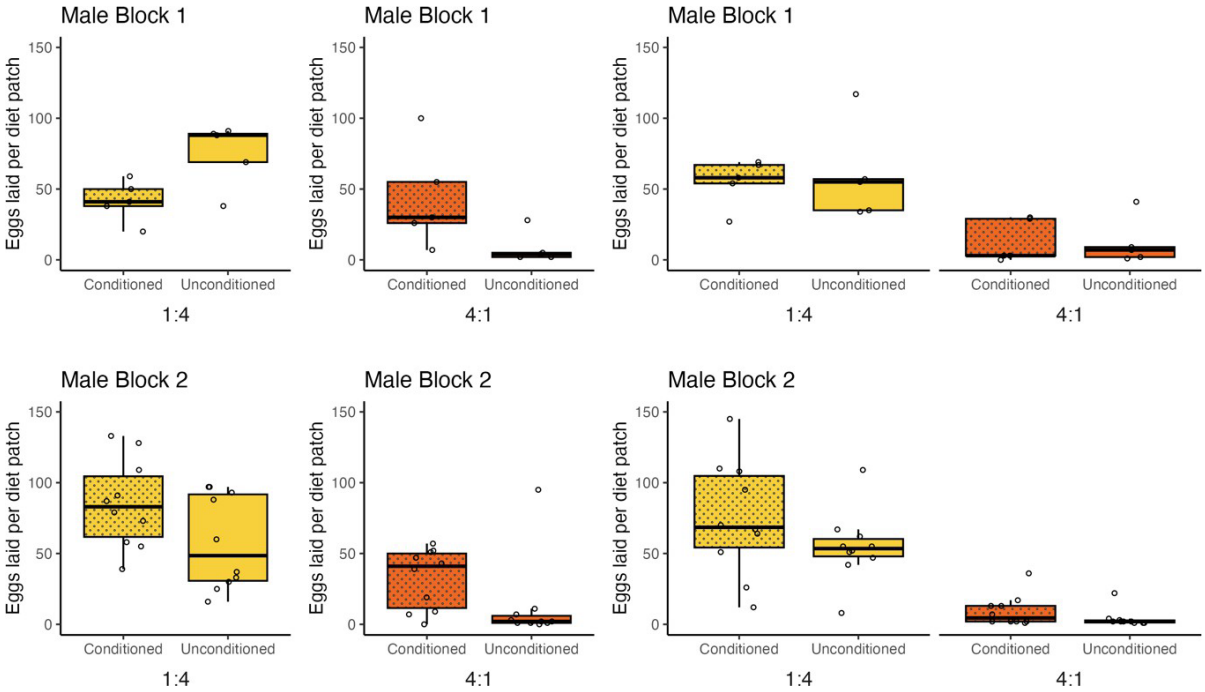
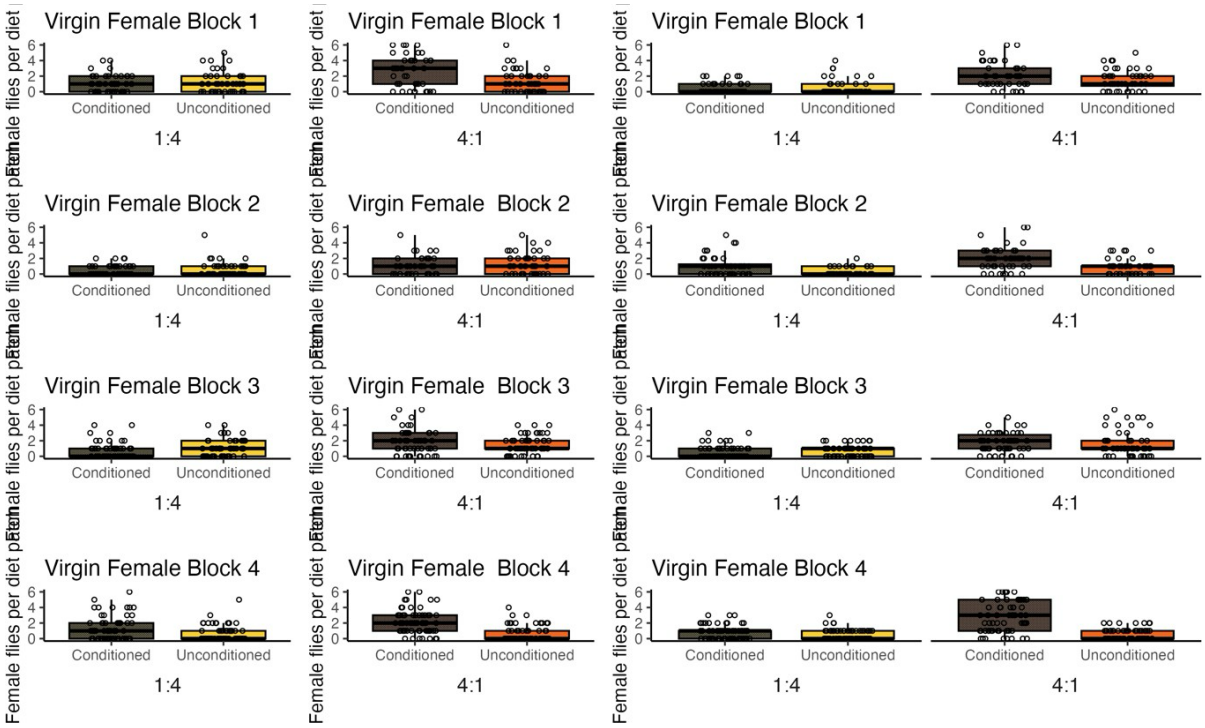


Figure S2.2. Box plots showing oviposition results from male-conditioned diets, box plots show each block (one and two) under different conditioning treatments (conditioned and unconditioned) and diet ratios (4:1 and 1:4)



Supplementary Figure 2.3. Box plots showing feeding results from virgin female conditioned diets, box plots show each block (one, two, three and four) under different conditioning treatments (conditioned and unconditioned) and diet ratios (4:1 and 1:4)

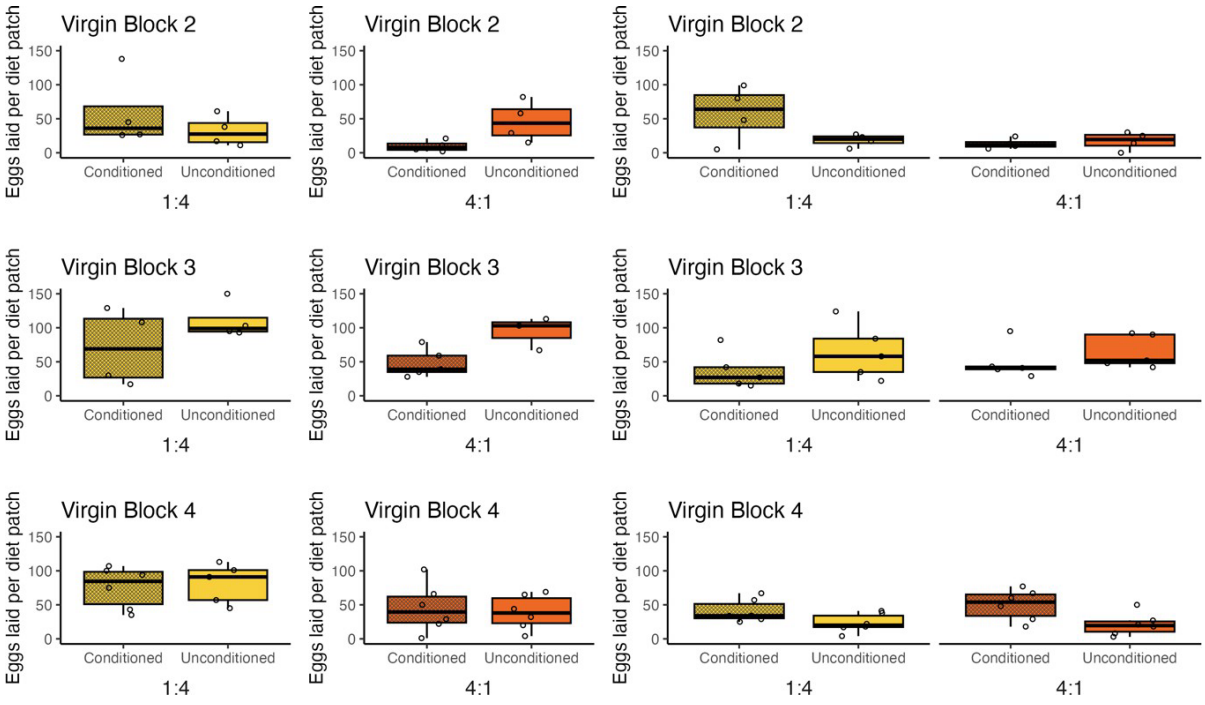


Figure S2.4. Box plots showing oviposition results from virgin female conditioned diets, box plots show each block (one, two, three and four) under different conditioning treatments (conditioned and unconditioned) and diet ratios (4:1 and 1:4)

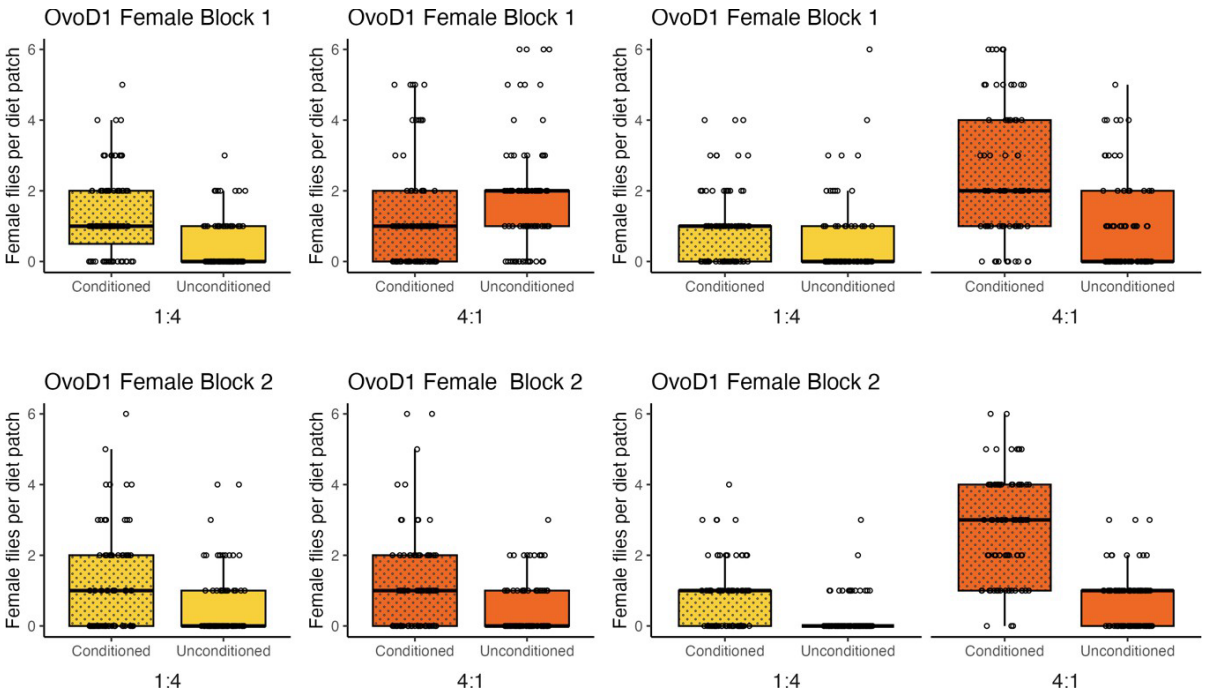


Figure S2.5. Box plots showing feeding results from *Ovo*^{D1} female conditioned diets, box plots show each block (one and two) under different conditioning treatments (conditioned and unconditioned) and diet ratios (4:1 and 1:4)

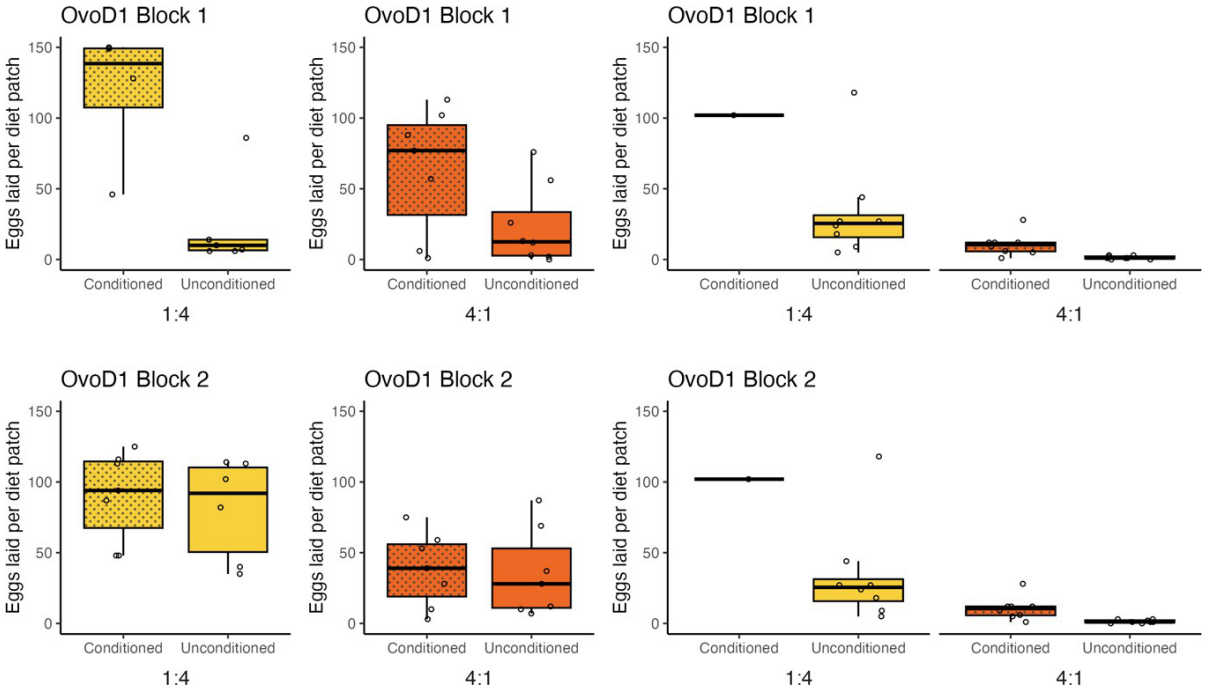


Figure S2.6. Box plots showing oviposition results from *Ovo*^{D1} female conditioned diets, box plots show each block (one and two) under different conditioning treatments (conditioned and unconditioned) and diet ratios (4:1 and 1:4

Chapter 3

3 The Impact of Fly Diet-Conditioning on Development in *Drosophila melanogaster*

(Picture: Nina, 2006, ASA 2.5 Generic)

3.1 Abstract

Variation in environmental conditions such as diet availability can influence the expression of key life history phenotypes. In the dietary choice experiments reported in Chapter 2, I showed that female *D. melanogaster* prefer to feed and lay their eggs on diets that have been 'conditioned' by exposure to conspecifics of both sexes. In this chapter, I tested for the fitness effects of such choice, by measuring the developmental speed and viability of larvae reared on conditioned versus non-conditioned diets. In the first experiment, I allowed females to express their natural preferences for conditioned over non-conditioned diets and then examined the development of the offspring on either diet. In this uncontrolled density experiment, the results showed that larvae reared in the non-favoured unconditioned diets exhibited faster development to pupae and adulthood. No significant differences were detected in survival to the pupal stage between the two diet treatments, or in adult body weight. However, significantly more flies emerged from the conditioned treatments. To disentangle the effect of diet conditioning versus density differences, I then conducted a second, controlled density experiment by adding an equal number of first-instar larvae to conditioned or non-conditioned diets. As in the first experiment, both pupae and adults reared on unconditioned diets emerged significantly faster than those on conditioned diets. However, there were no differences in overall survival to pupae or adulthood or in adult body weight between the two diet treatments. Overall, these results show that rearing on fly-conditioned diets results in a potential cost of significantly delayed development, though no costs in terms of developmental survival or adult body weight. Therefore, the potential fitness benefits of laying eggs on preferred, conditioned diets are not yet clear. Further tests of the fitness consequences of developing on conditioned versus unconditioned diets on adult-life history traits such lifespan and reproductive success would be useful to investigate this.

3.1 Introduction

The diet consumed during early life can play a crucial role in an individual's behaviour, development and lifespan, with these effects being evident across many invertebrates and vertebrates. Both the quantity and quality of a diet can play critical roles in shaping an organism's fitness. For example, a study in humans found that individuals that consumed less protein during early life tended to consume more protein in later life and had poorer perceived health and a higher probability of obesity. This suggested that early-life food exposure can be linked to both long-term behavioural and physiological effects (Adamopoulou et al., 2024). A second study, also in humans, found that infants who were fed nutrient-dense formula milk showed better developmental progression in later life compared to those fed less nutrient-dense formula milk (Morley, 1996). This effect was also observed in premature infants, in which dietary manipulation had significant consequences for later development, with individuals that received a less nutrient-dense diet showing evidence of developmental impairments (Lucas et al., 1990).

Similar studies have been conducted in many other mammal species. For example, a study on mice investigated the effects of different types of milk during infancy. Mice fed milk similar in composition to human milk had lower body weights and a higher preference for fatty food in later life, compared to control mice fed on standard mouse formula milk (Ronda et al., 2020). Similarly, a study on rats investigated the impact of litter size on early nutrition. Rats raised in small litters had less body fat, both relatively and absolutely, compared to those raised in larger litters that had lower access to nutritional resources (Faust et al., 1980).

In insects, early-life dietary conditions are also commonly reported to have significant effects on development and survival. For example, reduced diet concentration during the third instar nymph life stage in the brown plant hopper (*Nilaparvata lugens*), shortened the developmental period of adults but also decreased their survival rate (Kang et al., 2022). In the tenthredinid sawfly (*Nematus pravus*) individuals fed a stable diet had longer development times and greater body mass than another sawfly species (*Dineura puillor*) fed on a poorer diet (Kause et al., 2001). These and many other examples (reviewed in more detail in Thompson (1999)) show that early life diet can influence life-history trade-offs, affecting both developmental trajectories and adult fitness.

Fruitflies of the genus *Drosophila* have been particularly useful for studies of the effects of nutrition on life history and have revealed sometimes contrasting results. For example, a study of fitness trade-offs associated with larval diets in *Drosophila suzukii*, found that while larval development time was faster for individuals fed on high-protein natural fruit diets

versus artificial media, this nutritional variation did not alter other fitness traits body size, as has been observed in some other studies (Jaramillo et al., 2015). In the species used throughout this thesis, *Drosophila melanogaster,* multiple studies have demonstrated that developmental diet has significant effects on behaviour, development, lifespan and healthspan. For example, one study showed that developmental diet influenced adult male aggression. Males reared on low-resource development diets were less likely to engage in aggressive lunging against rivals, indicating that a developmental diet can have effects across the whole of the life history (Edmunds et al., 2021).

Several studies have also shown that nutritional variation during development can directly affect adult physiology (e.g. Klepsatel *et al.*, 2020). In addition, larval and adult diets may have contrasting effects. For example, larval diet is reported to affect all body size traits, while adult diet influences just body weight (Poças et al., 2022). Larval diets are also reported to alter adult fecundity-longevity relationships (Collins et al., 2023). Collectively, these studies show that the nutritional conditions experienced during development have pervasive effects on life history traits of larvae and adults, highlighting the importance of early nutritional environments.

In the previous chapter (Chapter 2), I demonstrated that female *D. melanogaster* predominantly prefer to feed and lay their eggs on diets that have been conditioned by other flies (by males, virgin females, and eggless Ovo^{D1} females). However, it is not yet clear whether these fly-conditioned diets provide any fitness benefits for the adults that prefer to eat them or for the development of their offspring emerging from eggs laid on those conditioned diets. Given the extensive body of research showing the importance of the developmental diets summarised above, I reasoned that adult female oviposition choices for conditioned diets could have significant fitness effects for the offspring reared on them (assuming that conditioned versus non-conditioned diets vary in their nutritional quality or quantity).

To test this prediction, I investigated the effect of rearing offspring on conditioned versus unconditioned 1:4 Protein: Carbohydrate (P:C) diets on larval developmental speed, survival and emerging adult body weight. The overall aim was to determine if the diet chosen by females for egg laying is beneficial for offspring fitness and development.

3.3 Materials and Methods

3.3.1 Fly diets for rearing

All flies were reared, and experiments took place in a controlled temperature room of 25 °C, 50% RH on a 12:12 light: dark regime. Standard fly rearing was conducted in sugar yeast agar medium (SYA). Containing water, sugar, yeast, nipagin solution and propionic acid (**Appendix 2**).

3.3.2 Fly rearing

Eggs were first sampled from wild type Dahomey population cages maintained in overlapping culture on bottles of SYA medium, using two purple agar plates (**Appendix 2**) with yeast paste in the centre to attract flies for oviposition. These plates were placed in the population cages for 4 hours, removed and then placed in a pillowcase for 24 hours to allow eggs to hatch, and to stop other flies laying in the dishes. First instar larvae on the plates were then placed into n = 40 SYA vials at a standardised density of 50 larvae/vial) and then returned to the 25 °C CT room. Experimental flies were then collected from these cultures 9 days later.

3.3.3 1:4 Meridic diet preparation

For the tests of the effects of conditioning, I used a meridic, semi-defined diet containing Protein: Carbohydrate (P:C) components in a ratio of 1:4. Protein was supplied as Casein and carbohydrate as Sucrose (**Appendix 2**).

3.3.4 Natural diet conditioning with uncontrolled larval density

3.3.4.1 Experimental set-up of diet treatments

In the first experiment, I tested the fitness impacts for offspring of being reared on naturally Conditioned versus Unconditioned diets. Vials containing a P: C 1:4 diet were used, as this diet is known to be preferred by mated females for egg-laying (Chapter 2). Two treatments were tested:

(i) 1:4 Conditioned diet, in which media vials were conditioned naturally by adult males for 24 hours.

(ii) 1:4 Unconditioned (control) diet, in which media vials from the same batch were handled identically, but not exposed to adult males for 24 hours.

Experimental flies were reared as described above. Upon eclosion, they were left in the vials for 2 days, to ensure all flies had been mated at least once. Flies were then separated into separate sexes using CO_2 anaesthesia. In total, n = 300 males and n = 600 females were collected. Males were used for the dietary conditioning, and n = 10 males were added to each of n = 30 1:4 vials. Concurrently, n = 30 empty 1:4 vials were handled in the same manner, but no males were added. All vials were left in the 25 °C Controlled Temperature (CT) room. After 24 hours, the males from the conditioning treatment vials were discarded, and n = 10 experimental females were added to both the conditioned and unconditioned treatment vials to lay eggs. Females were removed 24 hours later from the vials and the offspring emerging from the eggs laid were tested for developmental speed, survival and adult body size.

3.3.4.2 Developmental speed and survival to pupariation and adult emergence on Conditioned versus Unconditioned media (uncontrolled density)

Eight days (192 hours) after the eggs were laid in the conditions and unconditioned vials, pupae began to form and were counted twice daily by marking the locations of the pupae on the side of the vials. Twelve days after egg-laying (289 hours), adult flies started to eclose and were similarly monitored twice daily, by using CO_2 to remove and separate flies by sex, count and then store flies in 1.5ml Eppendorf tubes in a -20 °C freezer. Pupae counts were stopped four days after adult flies started to emerge, and adult fly counts were stopped ten days after flies first started to eclose from pupae, to avoid any second-generation counting.

3.3.4.3 Body weights of adults developing from Conditioned versus Unconditioned media (uncontrolled density)

Following the development counts and collections, flies were selected from the samples collected at peak emergence for body weight measurement. Flies, previously separated by sex and stored in the -20˚C freezer in 1.5 ml Eppendorf tubes were dried in an oven at 60 °C for 48 hours. Each fly was then individually weighed using an Analytical A&D microbalance BM-20.

3.3.5 Diet conditioning experiment with controlled larval density

3.3.5.1 Experimental set-up

In the second experiment, rather than, as above, allowing females to lay eggs freely on conditioned or unconditioned diets, thus at different densities, I placed known, controlled numbers of larvae on each type of diet. I then tested the developmental speed, survival and adult body weight of the offspring developing under these controlled density conditions.

Dahomey male flies for the conditioning treatment were collected as described above. On the second day of emergence, males were collected, and females were discarded. To condition the treatment, n = 10 males were added to n = 15 of the P: C 1:4 vials and n = 15 were left alone, without males added (these were the unconditioned control vials).

On the same day, experimental eggs were collected, by placing 8 egg collection plates into 4 Dahomey population cages. These were left in the cages for 4 hours and were then removed, placed within a pillowcase and left to incubate for 24 hours to allow the first instar larvae to hatch, n = 63 first instar larvae from these plates were picked into n = 15 conditioned vials, and n = 15 unconditioned vials. I used n = 63 larvae for this study, as this represented the median number of eggs laid in both the conditioned and unconditioned vials in the uncontrolled density experiment described above.

3.3.5.2 Developmental speed and survival to pupariation and adult emergence on Conditioned versus Unconditioned media (controlled density)

After first-instar larvae had been placed in the treatment vials, both treatments were returned to the 25°C CT room. Pupae began to emerge 165 hours later and were counted twice daily. Flies started to emerge 269 hours after first-instar larvae had been placed in the treatment vials and were collected twice daily, separated by sex, counted, and then stored in 1.5ml Eppendorf tubes and stored in the -20°C freezer, to be used for body weight measurement.

3.3.5.3 Body weights of adults developing from Conditioned versus Unconditioned media (controlled density)

Flies that were weighed in this experiment were sampled from the peak emergence timepoint of 341 hours after first-instar larvae were put into the vials. The sampled timepoint was the same for both conditioned and unconditioned treatments. Flies, previously separated by sex and stored in the -20˚C freezer in 1.5 ml Eppendorf tubes were dried in an

oven at 60 °C for 48 hours. Each fly was then individually weighed using an Analytical A&D microbalance BM-20.

3.3.6 Statistical analysis

All statistical analyses were completed using R v 4.4.1 through the tidyverse package (Wickham et al., 2024) . Model assumptions for all analyses were tested with DHARMa (Hartig and Lohse, 2022) and performance (Lüdecke (@strengejacke) *et al.*, 2024) packages.

Experiment 1 (uncontrolled density)

Developmental speed

Pupae and fly development time were both analysed using Poisson Generalised Linear Mixed Models, although similar results and model assumptions were obtained when using a Gaussian model.

Survival

Total pupae and total fly emergence were analysed using Negative Binomial Generalised Linear Models.

Body weight

Body weight was analysed for males and females using a Negative Binomial Generalised Linear Model.

Experiment 2 (controlled density)

Developmental speed

Pupae development time was analysed using a Negative Binomial Generalised Linear Model, and fly development time was analysed using a Poisson Generalised Linear Mixed Model.

Survival

Total emergence of pupae was analysed using a Poisson Generalised Linear Mixed Model and total fly emergence was analysed using a Zero-Inflated Poisson Model.

Pupae survivability was analysed using a Negative Binomial Generalised Linear Model, and fly survivability was analysed using a Zero-Inflated Poisson Model. Survivability from the pupa-fly stage was analysed using a Zero-Inflated Negative Binomial Model.

Body weight

Body weight was analysed for males and females using a Negative Binomial Generalised Linear Model.

3.4 Results

3.4.1 Uncontrolled density experiment

3.4.4.1 The effects of conditioned and unconditioned developmental diets on pupal developmental speed

The effect of conditioned and unconditioned treatments on the time of emergence of pupae in the first, uncontrolled density experiment was investigated by comparing conditioned and unconditioned treatments (summary of the analysis, Table 3.1).

	time_hours		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	266.88	264.03 – 269.75	**<0.001**
treatment [unconditioned]	0.93	0.93 – 0.93	**<0.001**
Random Effects			
σ^2	0.00		
$\tau_{00\ vial}$	0.00		
ICC	0.18		
N_{vial}	30		
Observations	5308		
Marginal R^2 / Conditional R^2	0.209 / 0.353		

Table 3.1. Results of pupal emergence time from a negative binomial Generalised Linear Model. Summary of analysis of the time of emergence of pupae across conditioned and unconditioned diets (uncontrolled density). The intercept represents the time of pupal emergence in the conditioned treatment. The table presents the incident rate ratios, the confidence intervals and the p-values for the predictors in the model.

The analysis revealed a significant difference in time to pupariation between the two treatment conditions. Unconditioned treatment larvae pupated significantly faster, compared to the conditioned treatment larvae (Poisson GLMM: $n = 5308$, $z = 39.3$, $P < 0.0001$). On average, pupae in conditioned vials formed at 267 hours [95% CI 264 - 269], which was approximately 18.3 hours later [95% CI 17.5 – 18.9] than those in unconditioned vials, which emerged at 249 hours [95% CI 245 - 252] (Figure 3.1).

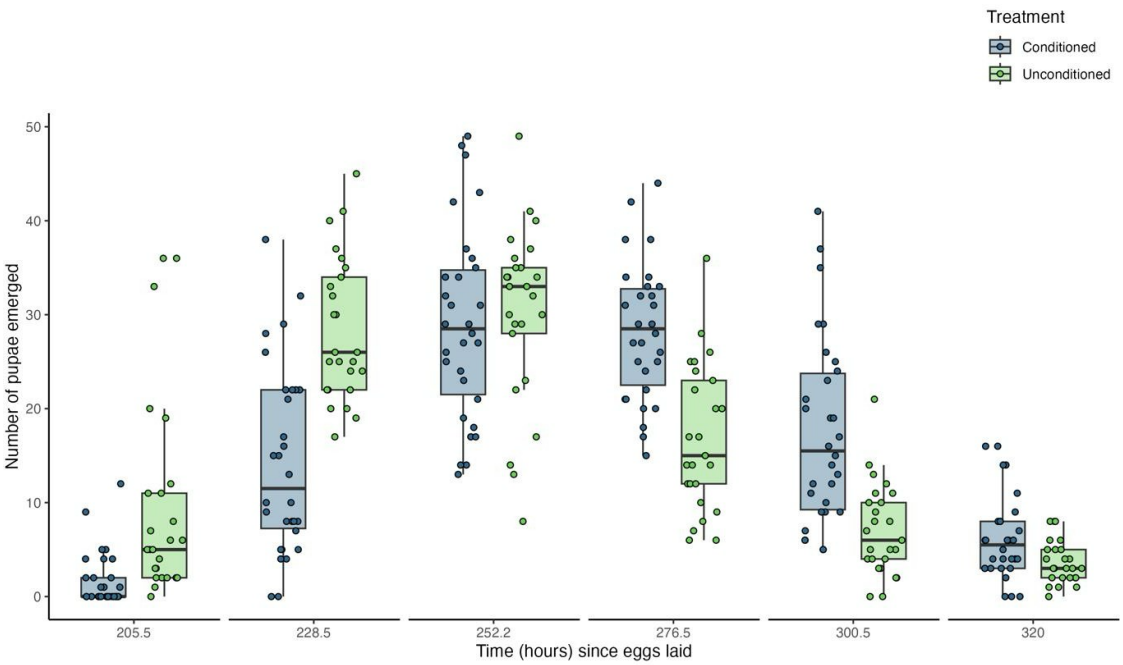


Figure 3.1. Box plot showing the number of pupae emerging over time in conditioned and unconditioned treatments (uncontrolled density). Time (in hours) since eggs were laid in the treatment vials, and the number of pupae forming, in two different diet treatments; conditioned (blue) and unconditioned (green). Each boxplot represents the interquartile range (IQR), with the median indicated by the horizontal line within the box. Whiskers extend to 1.5 times the IQR, individual points represent each pupa count.

3.4.1.2 The effects of conditioned and unconditioned developmental diets on fly developmental speed

Consistent with the findings for pupal development, adult flies reared in unconditioned vials also developed significantly faster than those reared in conditioned vials. A significant two-way interaction (Poisson GLMM: $n = 3560$, $\chi^2 = 20.2$, $P < 0.0001$), retained in the model, showed that this effect was different across the sexes (Table 3.2).

	time_hours		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	391.28	387.35 – 395.25	**<0.001**
treatment [unconditioned]	0.92	0.92 – 0.93	**<0.001**
sex [males]	0.98	0.97 – 0.99	**0.006**
treatment [unconditioned] × sex [males]	1.02	1.01 – 1.02	**<0.001**
Random Effects			
σ^2	0.00		
$\tau_{00\ vial:sex}$	0.00		
$\tau_{00\ sex}$	0.00		
ICC	0.21		
N_{vial}	30		
N_{sex}	2		
Observations	3560		
Marginal R^2 / Conditional R^2	0.268 / 0.425		

Table 3.2. Results of adult emergence time from a Poisson Generalised Linear Mixed Model. Summary of analysis of the time of emergence of adult flies reared on conditioned versus unconditioned treatments (uncontrolled density). The intercept represents the time of emergence of female flies in a conditioned treatment. The table presents the incident rate ratios, the confidence intervals and the p-values for the predictors in the model.

Following this, the effects of treatment, sex and their interaction on the time of emergence of flies were analysed. This analysis showed that female flies reared in conditioned vials exhibited a slower emergence time compared to those in unconditioned vials. Females in conditioned vials emerged on average at 391 hours [95% CI, 387 - 395], which was approximately 29 hours later [95 % CI, 27.8 – 30.9] than those in unconditioned vials, which emerged at 362 hours [95% CI, 356 - 367] (Poisson GLMM: $n = 3560$, $z = 30.4$, $P < 0.0001$). Sex-specific differences were also observed. In conditioned vials, males emerged on average at 383 hours [95 % CI, 374 – 393], approximately 7.82 hours earlier [95 % CI, 2.33 – 13.3] than females, which emerged at 391 hours [95% CI, 387 - 395] (Poisson GLMM: $n = 3560$, $z = 2.8$, $P = 0.006$). However, smaller sex-specific differences were observed between the unconditioned treatments, where males emerged on average at 361 hours [95% CI, 357-364] compared to 362 hours [95% CI 356 – 367] for females. The results show that sex differences were evident in unconditioned but not conditioned vials (Figure 3.2).

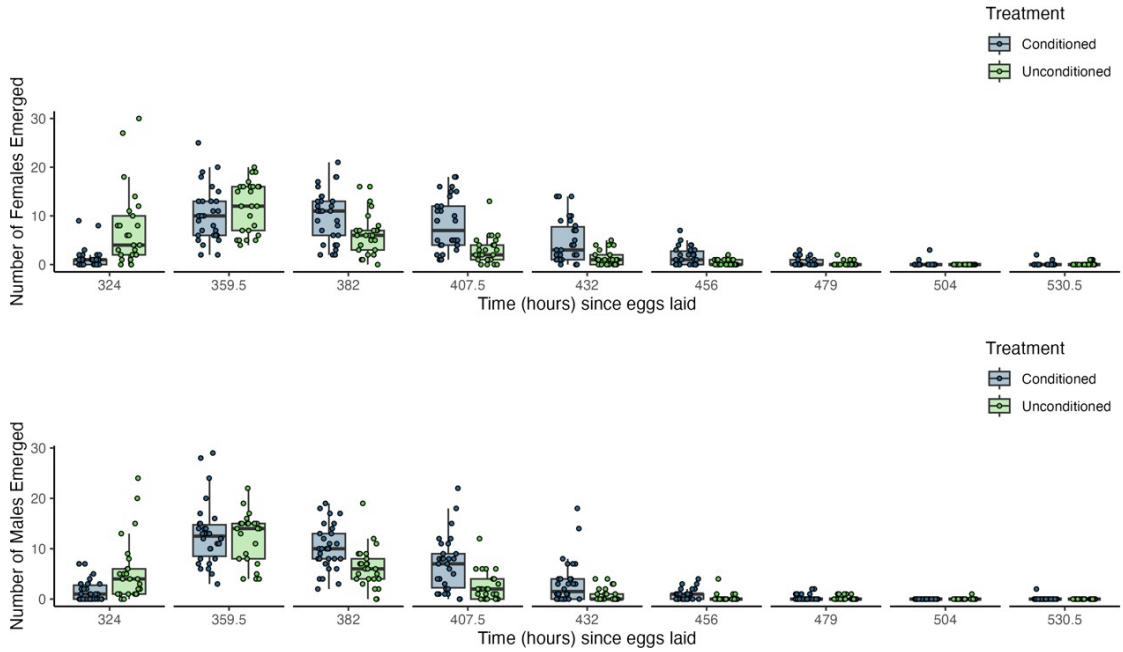


Figure 3.2. Box plot showing the number of adult flies that emerged over time in conditioned and unconditioned treatments (uncontrolled density). Time (in hours) since eggs were laid, and the number of female flies (top) and male flies (bottom) emerging, in two different diet treatments; conditioned (blue) and unconditioned (green). Individual points represent each fly count.

3.4.1.3 The effects of conditioned and unconditioned developmental diets on pupal survival:

To assess the differences in the number of pupae that formed, I analysed the total number of pupae that emerged in both conditioned and unconditioned treatments.

	total_count		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	97.10	92.35 – 102.09	**<0.001**
treatment [unconditioned]	0.99	0.92 – 1.06	0.723
Observations	55		
R^2 Nagelkerke	0.004		

Table 3.3. Results of total pupal emergence from a Negative Binomial Generalised Linear Model. Summary of the analysis of the total pupae that emerged in conditioned and unconditioned treatments (uncontrolled density). The intercept represents the total pupae emerging in conditioned treatments, per vial. The table presents the incident rate ratios, the confidence intervals and the p-values for the predictors in the model.

There were no significant differences in the total number of pupae emerging between the conditioned and unconditioned treatments (Negative Binomial GLM: *n = 55, z = 0.354, P = 0.723*). In conditioned vials, on average, 97.1 pupae [95 % CI 92.3 - 102] per vial formed compared to 95.8 pupae [95 % CI 90.7 – 101] in unconditioned vials, showing a non-significant difference of only 17.3 [95 % CI 8 – 26.9] fewer pupae per vial.

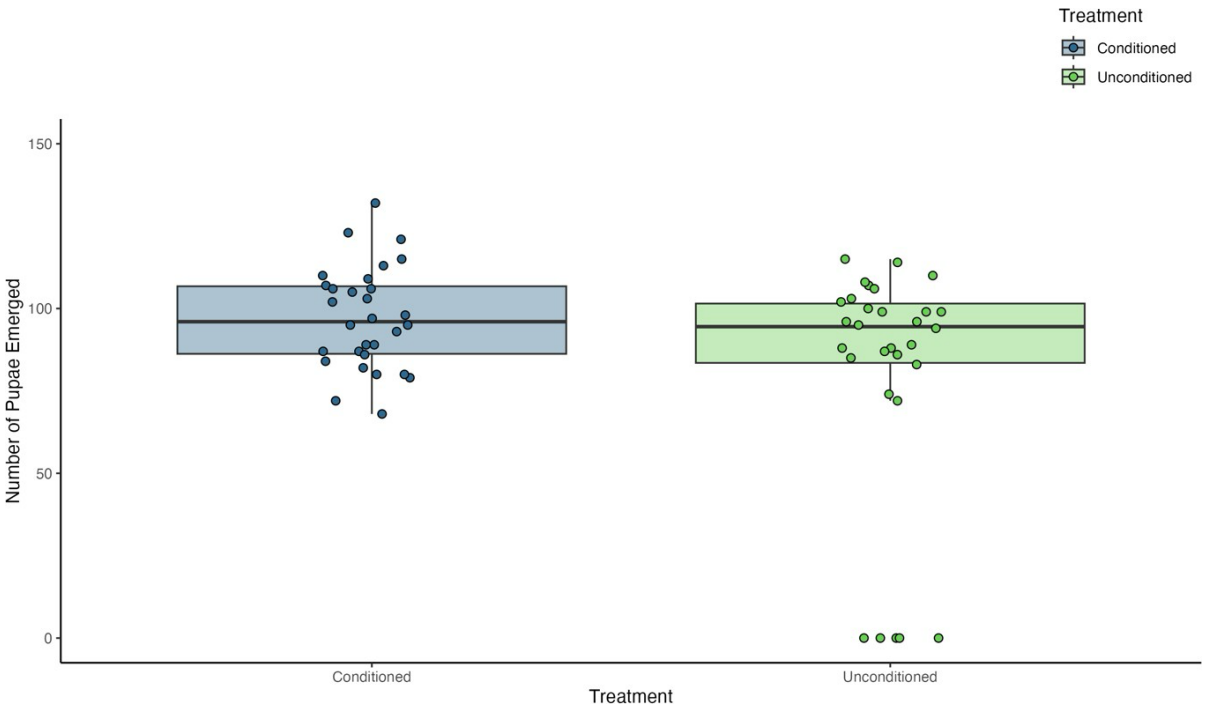


Figure 3.3. Box plot showing the overall emergence of pupae in conditioned and unconditioned treatments. The number of pupae emerging (y-axis), after being reared in two different treatments: conditioned (blue) and unconditioned (green) (x-axis). Individual points represent each vial.

3.4.1.4 The effects of conditioned and unconditioned developmental diets on fly survival

Next, I investigated potential differences in the overall emergence of male and female flies in conditioned and unconditioned treatments. There was no 2-way interaction between sex and treatment (Negative Binomial GLM: *n = 110, χ^2 = 0.36, P = 0.548*) so the interaction term was dropped (summary model presented in Table 3.4).

	total_count		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	36.61	33.54 – 39.99	**<0.001**
sex [males]	0.94	0.84 – 1.04	0.228
treatment [unconditioned]	0.81	0.73 – 0.90	**<0.001**
Observations	110		
R^2 Nagelkerke	0.204		

Table 3.4. Results of total adult fly emergence from a Negative Binomial Generalised Linear Model. Summary of the analysis of the total number of adult flies emerging across conditioned and unconditioned treatments (uncontrolled density). The intercept represents females in a conditioned treatment. The table presents the incident rate ratios, the confidence intervals and the p-values for the predictors in the model.

A model testing for responses of sex and treatment on fly emergence revealed a significant difference between conditioned and unconditioned vials for both male and female flies (Negative Binomial GLM: $n = 110$, $z = 3.92$, $P < 0.0001$). In conditioned vials, there was an average emergence of 36.6 [95% CI, 33.5 – 40] females and 34.3 [95% CI, 31.4 – 37.5] males. In unconditioned vials, an average of 29.6 [95% CI 26.9 - 32.6] females and 27.7 [95% CI 25.4 - 30.5] males emerged. This showed a decrease of 7 [95% CI 6.6 – 7.4] females and 6.6 [95% CI, 6.2 – 7] males per vial in the unconditioned treatment compared to the conditioned treatment. Although there were some differences in the total number of females or males emerging within either the conditioned or unconditioned vials, this difference in flies emerging between the two treatments was not statistically significant (Negative Binomial GLM: $n = 110$, $z = 1.21$, $P = 0.228$) (Figure 3.4).

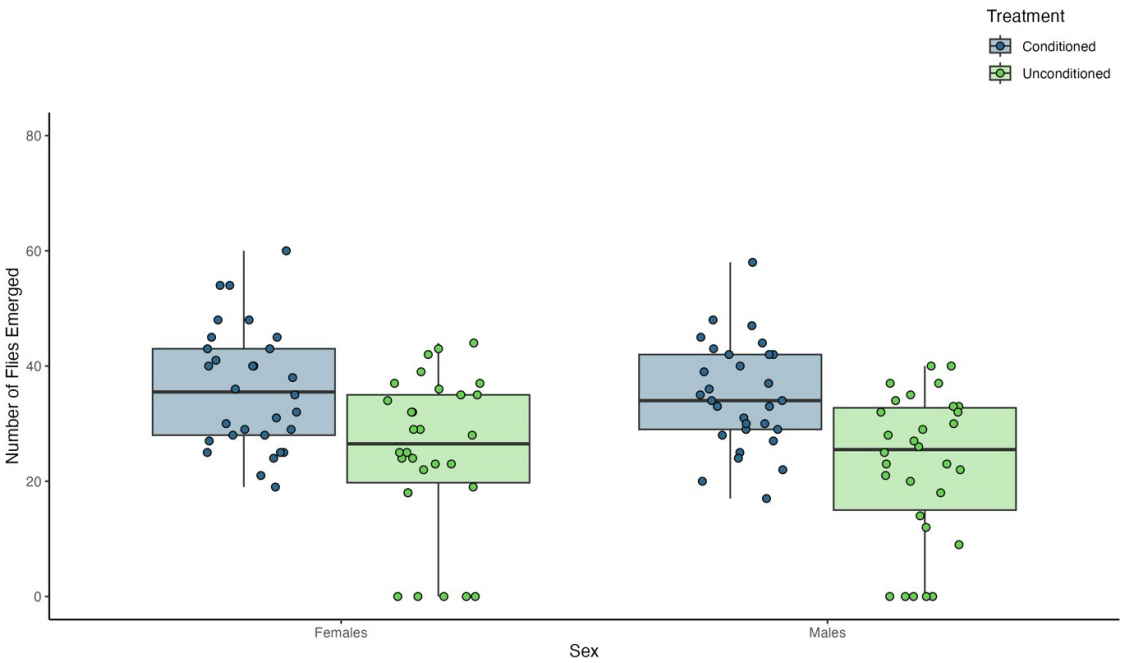


Figure 3.4. Box plot showing the overall emergence of adult flies in conditioned and unconditioned treatments. The total number of females and males having emerged (y-axis), after being reared in two different treatments: conditioned (blue) and unconditioned (green) (x-axis). Individual points represent each vial.

3.4.1.5 The effects of conditioned and unconditioned developmental diets on fly body weight

To further understand the fitness and developmental effects of rearing on a conditioned diet, the body weights of adult flies were measured. The results showed there were no significant differences in body weight between flies reared on different treatments. However, as expected, there were significant body weight differences between male and female flies (Table 3.5).

	weight_mg		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	527.38	499.63 – 557.06	**<0.001**
treatment [unconditioned]	0.96	0.90 – 1.02	0.176
sex [male]	0.75	0.71 – 0.80	**<0.001**
Observations		117	
R^2 Nagelkerke		0.597	

Table 3.5. Results of fly body weights from a Negative Binomial Generalised Linear Model. Summary of analysis of the body weight of flies across conditioned and unconditioned treatments (uncontrolled density). The intercept represents the weight of

females, in a conditioned treatment. The table presents the incident rate ratios, the confidence intervals and the p-values for the predictors in the model.

For both sexes, there were no significant differences in the body weights between flies reared in conditioned and unconditioned vials (Negative Binomial GLM, *n = 117, z = 1.354, P = 0.176*). In females, the average body weight of flies reared in conditioned vials was 525 µg [95% CI 493 - 558], and 400 µg [95% CI, 376 – 426] in males, which was higher, but not significantly so, than the 505 µg [95% CI 490 - 521] in females and the 379 µg [95% CI, 356 – 404] in males, of individuals reared in unconditioned vials. With the estimated difference in weight between treatments being 17 µg [95% CI 16 - 18] in females, and 21 µg [95% CI 20 – 22] in males. However, there were significant differences between sexes for both treatments (Negative Binomial GLM, *n = 117, z = 8.674, P < 0.0001*) - as expected, males weighed significantly less than females in both treatments, showing a 125 µg [95% CI, 117 – 132] difference in conditioned vials, and a 129 µg [95% CI,119 – 138] difference in unconditioned vials (Figure 3.5).

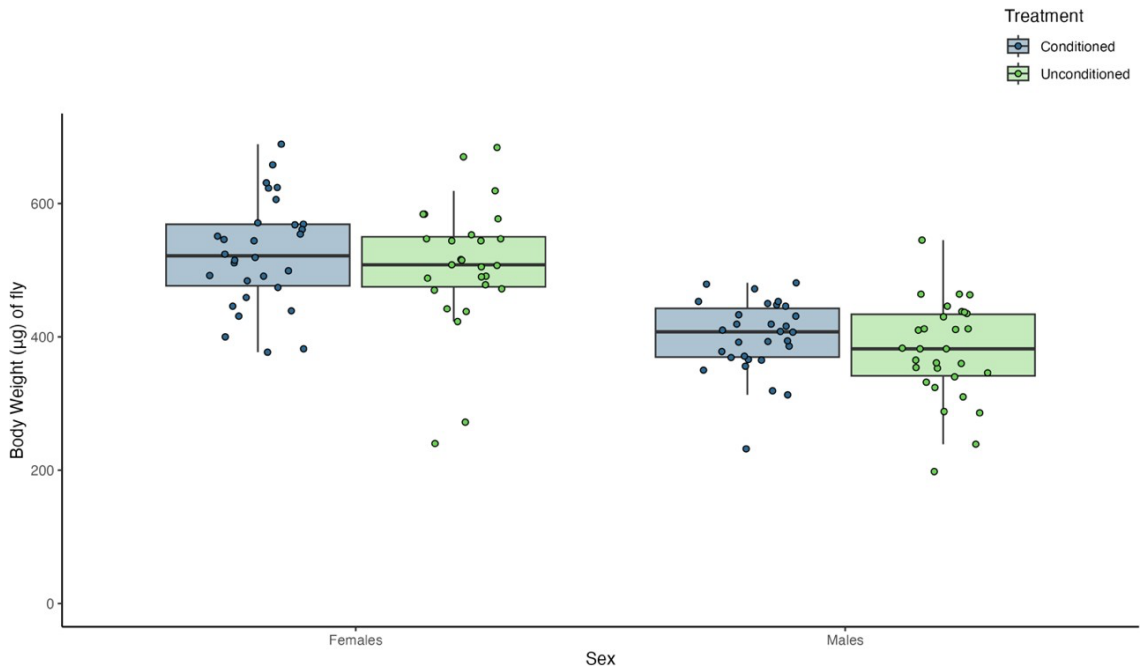


Figure 3.5 Box plot showing the body weight of flies in conditioned and unconditioned treatments. The adult body weight of females and males (y-axis), after being reared in two different treatments: conditioned (blue) and unconditioned (green) (x-axis). Individual points represent the weight of each fly.

3.4.2 Controlled density experiment

The first experiment described above did not control the density of larvae in the conditioned versus unconditioned vials. Therefore, any effects of diet could have been confounded by density differences. I removed this potential confound in the second experiment by placing specific numbers of first-instar larvae (n = 63) equally into conditioned and unconditioned vials.

3.4.2.1 The effects of conditioned and unconditioned developmental diets on pupal developmental speed

I first investigated if there were any effects of conditioned and unconditioned treatments on the time of pupal emergence (summary, Table 3.6).

	time_hours		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	251.07	247.70 – 254.51	**<0.001**
treatment [unconditioned]	0.98	0.96 – 1.00	**0.037**
Observations		1138	
R^2 Nagelkerke		0.006	

Table 3.6. Results of pupal developmental speed from a Negative Binomial Generalised Linear Model. Summary of analysis of the time of emergence of pupae across conditioned and unconditioned treatments (controlled density). The intercept represents the average time of emergence of pupa in conditioned treatments. The table presents the incident rate ratios, the confidence intervals and the p-values for the predictors in the model.

Pupae in the conditioned treatments formed significantly slower, at an average of 251 hours [95% CI, 248 - 255], while those in the unconditioned treatment formed at an average of 246 hours [95% CI, 238 - 254]. Therefore, pupae reared in the unconditioned treatment formed approximately 4.9 hours earlier [95% CI, 0.3 - 9.47] than those in the conditioned treatment (Negative Binomial GLM: $n = 1138$, $z = 2.087$, $P = 0.037$), a statistically significant difference (Figure 3.6).

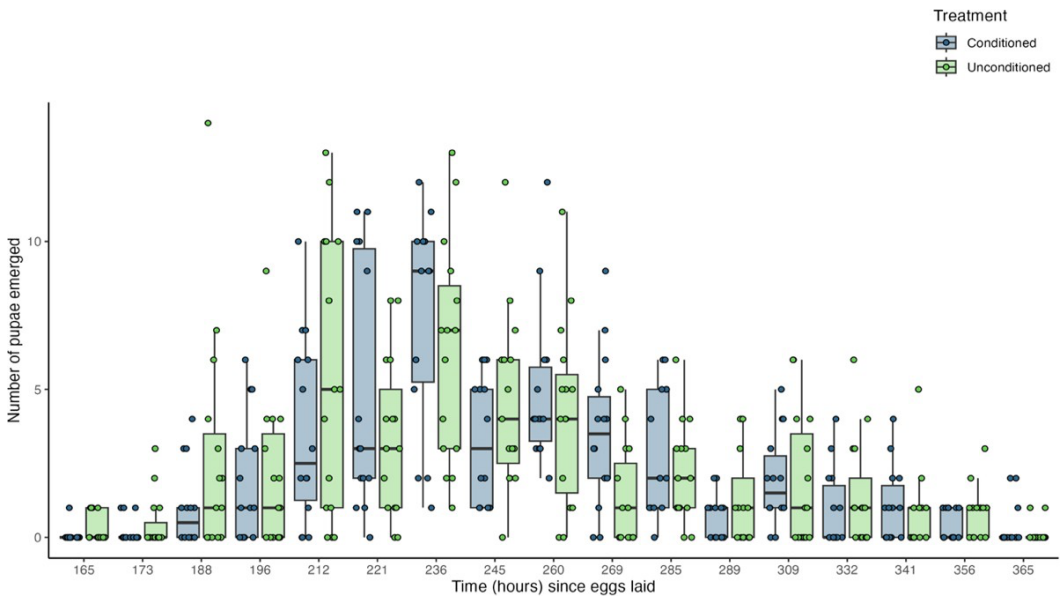


Figure 3.6. Box plot showing the number of pupae emerging over time in conditioned and unconditioned treatments. Time (in hours) since eggs were laid, and the number of pupa emerging, in two different treatments; conditioned (blue) and unconditioned (green). Individual points represent each pupa count.

3.4.2.2 The effects of conditioned and unconditioned developmental diets on fly developmental speed

There was no significant 2-way interaction between treatment and sex (Negative Binomial GLM: $n = 675$, $\chi^2 = 0.51$, $P = 0.48$) so this was removed from the model (summary, Table 3.7).

	time_hours		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	361.21	357.37 – 365.09	**<0.001**
treatment [unconditioned]	0.96	0.95 – 0.97	**<0.001**
sex [males]	0.96	0.95 – 0.97	**<0.001**
Observations		675	
R^2 Nagelkerke		0.162	

Table 3.7. Results of fly developmental speed from a Poisson Generalised Linear Mixed Model. Summary of analysis of the time of emergence of flies across conditioned and unconditioned treatments, and females and males (controlled density). The intercept represents the average time of emergence of females, in conditioned treatments. The table presents the incident rate ratios, the confidence intervals and the p-values for the predictors in the model.

There was a significant difference in emergence times between both female and male flies when reared on conditioned versus unconditioned diets (Negative Binomial GLM: *n = 675, z = 6.196, P < 0.0001*). Females from conditioned vials emerged on average at 361 hours [95% CI, 357 - 365], while males emerged at 347 hours [95% CI, 343 - 351]. In the unconditioned treatments, females emerged on average at 347 hours [95% CI 339 - 355] and males at 334 hours [95% CI, 330 - 337]. This showed significant treatment differences of 14 hours [95% CI, 9.72 - 18.1] in females and 13 hours [95% CI, 12 - 14] in males between the conditioned and unconditioned diets. These results also showed that males emerged significantly earlier than females in both the conditioned and unconditioned treatments (Negative Binomial GLM: *n = 675, z = 6.258, P < 0.0001*) (Figure 3.7).

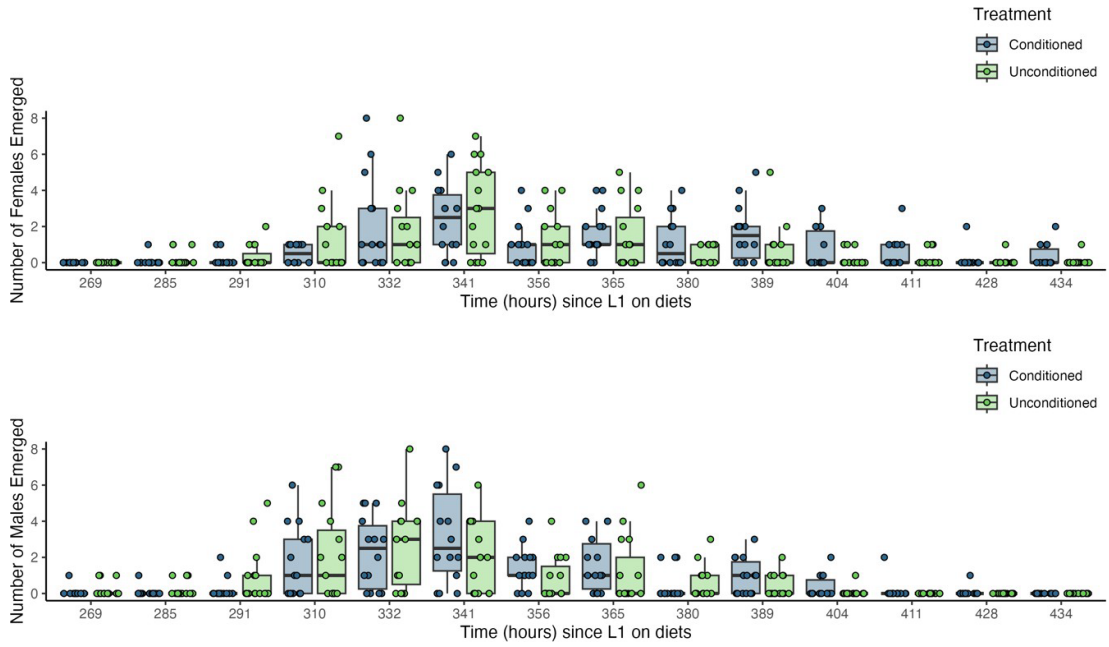


Figure 3.7. Box plot showing the number of flies emerging over time in conditioned and unconditioned treatments. Time (in hours) since eggs were laid, and the number of female flies (top) and male flies (bottom) emerging (y-axis), in two different treatments; conditioned (blue) and unconditioned (green) (x-axis). Individual points represent each fly count.

3.4.2.3 The effects of conditioned and unconditioned developmental diets on pupae emergence and survivability

After finding that development to pupal formation was faster on conditioned diets, I analysed the overall emergence and the survival rate from the larval to the pupae stage across conditioned and unconditioned treatments (Table 3.8a, 3.8b)

(a)

	total_pupae		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	38.55	33.88 – 43.87	**<0.001**
treatment [unconditioned]	1.00	0.89 – 1.13	0.960
Random Effects			
σ^2	0.03		
$\tau_{00\ vial}$	0.02		
$\tau_{00\ id}$	0.00		
ICC	0.46		
N_{vial}	15		
N_{id}	2		
Observations	29		
Marginal R^2 / Conditional R^2	0.000 / 0.459		

(b)

	survivability		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	62.47	54.39 – 72.02	**<0.001**
treatment [unconditioned]	0.99	0.82 – 1.21	0.954
Observations	29		
R^2 Nagelkerke	0.000		

Table 3.8. (a) Results of the overall emergence of pupae from a Poisson Generalised Linear Model (controlled density). The intercept represents overall pupae counts per conditioned vial. The table presents the incident rate ratios, the confidence intervals and the p-values for the predictors in the model. **(b) Results of the survivability percentages of pupae from a Negative Binomial Generalised Linear Model (controlled density)**. The intercept represents the survival percentages of conditioned pupae that emerged from n = 63 larvae. The table presents the incident rate ratios, the confidence intervals and the p-values for the predictors in the model.

My analysis found no evidence for significant differences in the total number of pupae forming from conditioned vials and unconditioned vials. In conditioned vials, an average of 38.6 pupae [95% CI 34 - 44] emerged, while an average of 38.72 pupae [95% CI 30.2 - 49] emerged in unconditioned vials, with a non-significant difference of only 0.12 [95% CI 0.1 - 3.65] more pupae in unconditioned vials (Poisson GLMM: *n = 29, z = 0.1, P = 0.923*) (Table 8a).

To assess survivability, I calculated the survival ratios to the pupal stage, by dividing counts by the initial number of individuals placed into each vial (n = 63). The analysis revealed no significant differences. Unconditioned vials had a survival rate of 62.1% [95% CI 44 - 87], compared to 62.5% [95 % CI 54 - 72] in conditioned vials, resulting in a difference of 0.4% [95% CI 0.15 - 9.9], which was not statistically significant (Negative Binomial GLM: *n = 29, z = 0.57, P = 0.954*) (Figure 3.8, analysis summary in Table 3.8b).

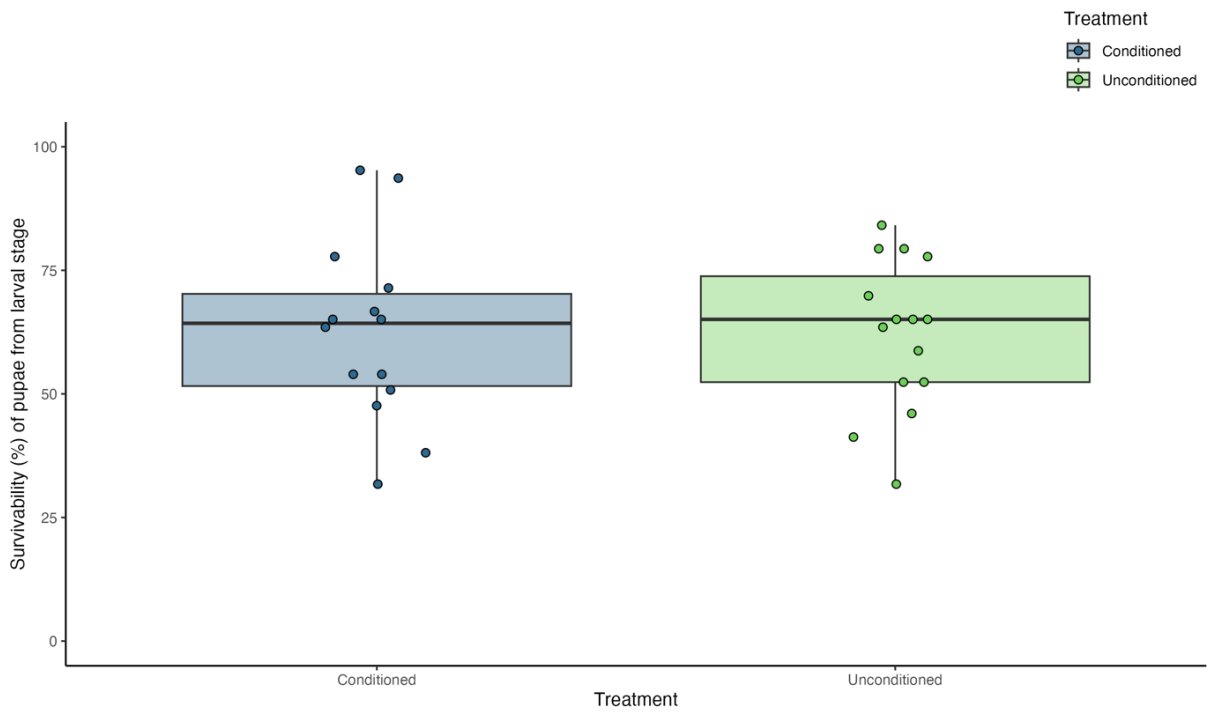


Figure 3.8. Boxplot representing the survival percentages of pupae, from conditioned (blue) and unconditioned (green) vials. Shown is the survivability percentage of pupae from the larval stage (y-axis) across conditioned (blue) and unconditioned (green) treatments (x-axis). Individual points represent the survivability (%) of pupa in each vial.

3.4.2.4 The effects of conditioned and unconditioned developmental diets on larval-fly emergence

Next, I analysed the effects of being reared on conditioned diets against a control unconditioned diet on the total emergence of flies, from the larval stage. There was no interaction effect between sex and treatment (Zero-Inflated Poisson: $n = 58$, $\chi^2 = 0.017$, $P = 0.896$) so this was dropped from the model (Table 3.9).

	total_count		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
Count Model			
(Intercept)	13.15	9.10 – 19.00	**<0.001**
sex [males]	1.06	0.91 – 1.23	0.452
treatment [unconditioned]	1.12	0.94 – 1.33	0.211
Zero-Inflated Model			
(Intercept)	0.15	0.04 – 0.53	**0.003**
sex [males]	1.25	0.33 – 4.73	0.739
treatment [unconditioned]	1.83	0.47 – 7.09	0.385
Random Effects			
σ^2	0.25		
$\tau_{00\ vial}$	0.15		
$\tau_{00\ id}$	0.04		
ICC	0.44		
$N_{\ vial}$	15		
$N_{\ id}$	2		
Observations	58		
Marginal R^2 / Conditional R^2	0.009 / 0.442		

Table 3.9. Results of larval-fly emergence from a Zero-Inflated Poisson Model. Summary of analysis of the overall emergence of flies across conditioned and unconditioned treatments, and females and males (controlled density). The intercept represents the total emergence of female flies in the conditioned treatment. The table presents the incident rate ratios, the confidence intervals and the p-values for the predictors in the model.

There was no significant difference between the emergence rates of male and female flies from conditioned and unconditioned flies (Zero-Inflated Poisson: *n = 58, z = 1.250, P = 0.211*). In conditioned vials, females had an average emergence of 13.2 [95% CI, 9.10 – 19] flies per vial, while males averaged 13.9 [95% CI, 9.64 – 20.2]. In unconditioned vials, females emerged at an average of 14.7 [95% CI, 10.18 – 21.2] and males at 9.9 [95% CI, 6.72 – 14.6] per vial. This showed a difference of only 1.5 [95% CI 1.08 – 2.2] more females and 1.7 [95% CI, 1.15 – 2] more males had emerging from unconditioned compared to conditioned vials. In addition, these results showed no significant differences between sexes in the number of flies emerging from conditioned vials (Zero-Inflated Poisson: *n = 58, z = 0.5, P = 0.452*) (Figure 3.9).

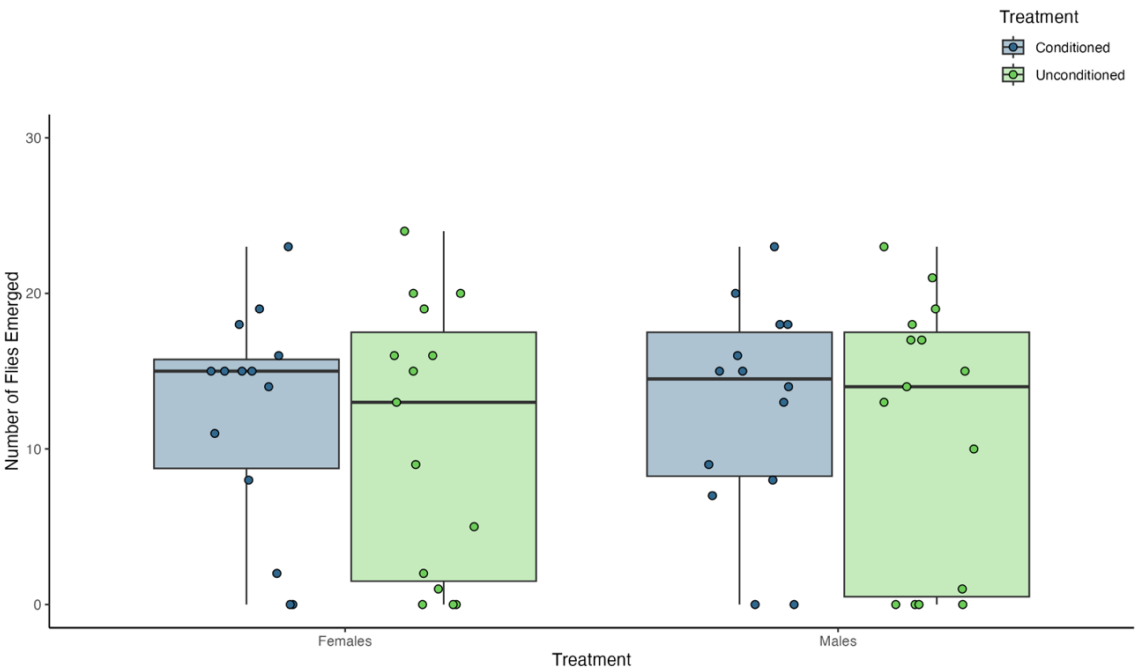


Figure 3.9. Box plot demonstrating overall fly emergence. Shown is the total number of flies (y-axis) of males and females emerged in conditioned and unconditioned treatments (x-axis). Individual points represent each vial.

3.4.2.5 The effects of conditioned and unconditioned developmental diets on larval-fly survivability

The survivability of overall fly emergence, compared to the initial number of larvae at the beginning of the experiment was then analysed (Table 3.10).

	survivability		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
Count Model			
(Intercept)	39.99	25.55 – 62.60	**<0.001**
treatment [unconditioned]	1.15	1.00 – 1.33	**0.048**
Zero-Inflated Model			
(Intercept)	0.17	0.04 – 0.74	**0.019**
treatment [unconditioned]	1.50	0.21 – 10.65	0.685
Random Effects			
σ^2	0.24		
$\tau_{00\ vial}$	0.34		
$\tau_{00\ id}$	0.05		
ICC	0.62		
N_{vial}	15		
N_{id}	2		
Observations	29		
Marginal R^2 / Conditional R^2	0.008 / 0.623		

Table 3.10. Results of larval-fly survivability from a Zero-Inflated Poisson Model. Summary of analysis of the survivability of flies from the larval stage, across conditioned and unconditioned treatments (controlled density). The intercept represents the average survivability percentage of flies in conditioned treatments, from the larval stage. The table presents the incident rate ratios, the confidence intervals and the p-values for the predictors in the model.

There was a significant difference in percentage survival observed from larvae to adulthood. Flies reared in unconditioned vials had an estimated marginal means survival rate of 24.1% [95% CI 16.2 – 35.6], compared to 21% [95% CI 14.2 – 31.1] (Zero-Inflated Poisson Model: *n = 58, z = 1.975, P = 0.0483*) (Figure 3.10).

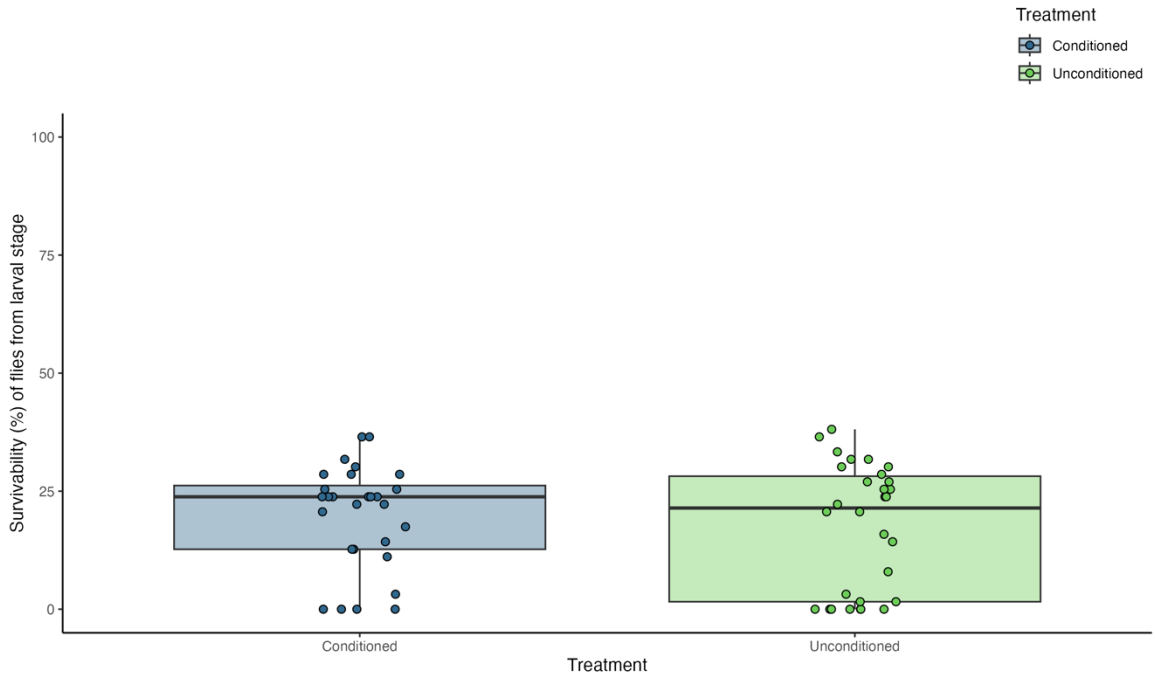


Figure 3.10. Box plot showing the survivability percentage of flies. (y-axis), after being reared in conditioned (blue) and unconditioned (green) (x-axis). Individual points represent the survivability (%) of flies per vial.

3.4.2.6 The effects of conditioned and unconditioned developmental diets on pupa-fly survivability

To understand how development differed between different stages of the life cycle, I also analysed the effects of survival percentage rates, from the pupal to adult stage (Table 3.11).

	survivability		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
Count Model			
(Intercept)	68.95	52.69 – 90.23	**<0.001**
treatment [unconditioned]	0.93	0.64 – 1.37	0.723
(Intercept)	113.21	12.76 – 6530.05	
Zero-Inflated Model			
(Intercept)	0.17	0.04 – 0.74	**0.019**
treatment [unconditioned]	1.50	0.21 – 10.65	0.685
Random Effects			
σ^2	0.33		
$\tau_{00\ vial}$	0.00		
ICC	0.00		
N_{vial}	15		
Observations	29		
Marginal R^2 / Conditional R^2	0.004 / 0.004		

Table 3.11. Results of pupa-fly survivability from a Zero-Inflated Negative Binomial Model. Summary of analysis of the survivability of flies from the pupal stage, across conditioned and unconditioned treatments (controlled density). The intercept represents the average survivability percentage of flies in conditioned treatments, from the pupal stage. The table presents the incident rate ratios, the confidence intervals and the p-values for the predictors in the model.

Larvae reared in conditioned vials had an estimated marginal means survival rate of 34.2% [95% CI 28.3 – 41.2] of flies surviving from the pupal stage, whereas those in unconditioned had a survival rate of 32.7% [95% CI 26.7 – 40.1]. However, this difference was not statistically significant (Zero-Inflated Negative Binomial Model: *n = 29, z = 0.406, P = 0.723*).

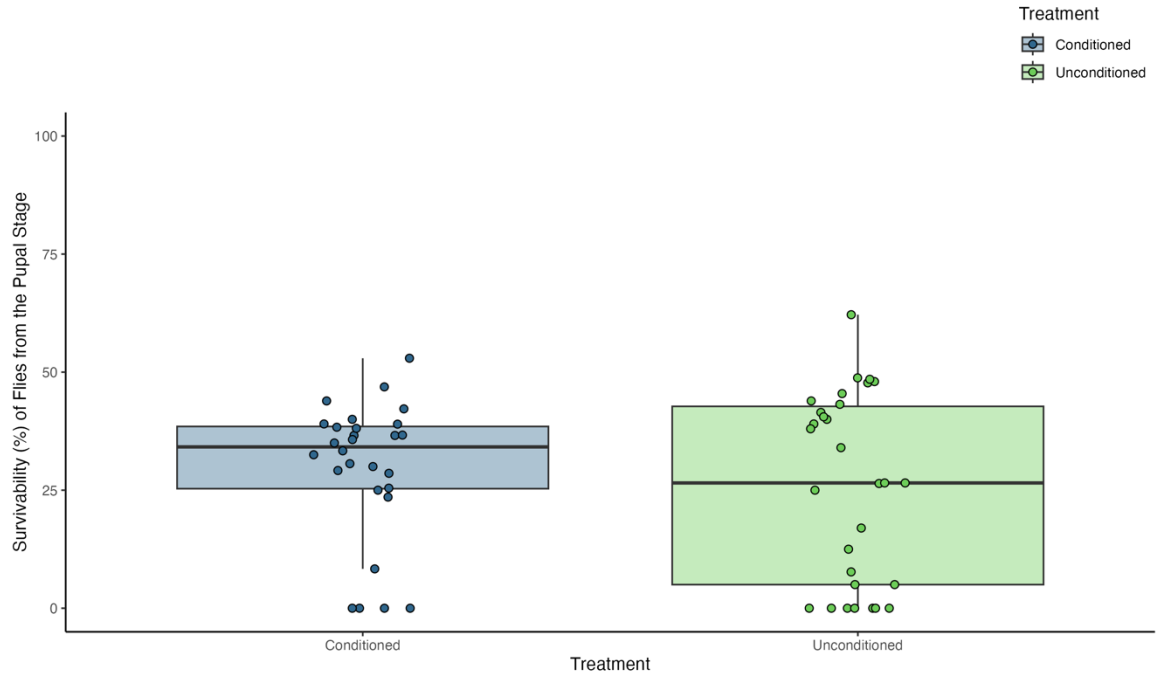


Figure 3.11. Box plot representing the survivability percentages of flies from the pupal stage. (y-axis) across conditioned and unconditioned treatments (x-axis). Individual points represent the survivability (%) of each fly from the pupal stage.

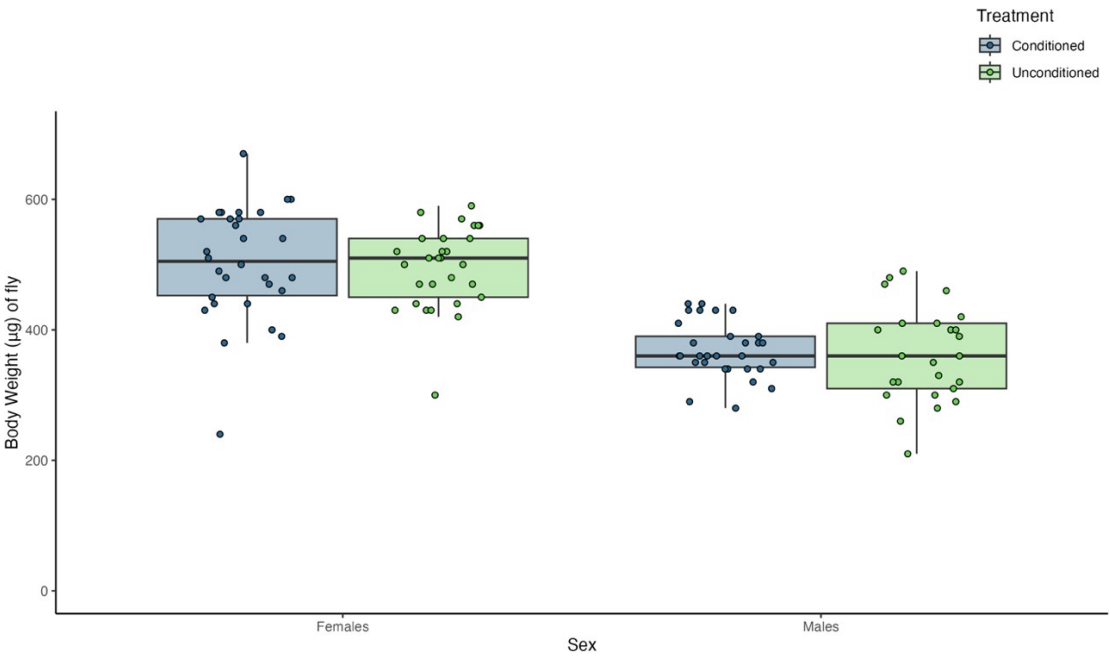
3.4.2.7 The effects of conditioned and unconditioned developmental diets on fly body weight

The body weight of adults reared in conditioned and unconditioned treatments at a controlled density showed no interaction between treatment and sex (Negative Binomial GLM: $n = 114$, $\chi^2 = 0.259, P = 0.611$) so this term was dropped from the model (Table 3.12).

	weight_mg		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	503.90	479.68 – 529.62	**<0.001**
treatment [unconditioned]	0.98	0.93 – 1.04	0.533
sex [male]	0.73	0.69 – 0.78	**<0.001**
Observations		114	
R^2 Nagelkerke		0.715	

Table 3.12. Results of fly body weight from a Negative Binomial Generalised Linear Model. Summary of analysis of the body weight of male and female flies after being reared in conditioned and unconditioned treatments (controlled density). The intercept represents the weight of a female fly in a conditioned treatment. The table presents the incident rate ratios, the confidence intervals and the p-values for the predictors in the model.

There was no significant difference in the body weight of either female or male flies reared in conditioned treatments versus unconditioned treatments (Negative Binomial GLM, $n = 114, z = 0.624, P = 0.533$). In conditioned treatments, female flies weighed on average, 504 µg [95% CI 480 - 529], while males weighed 369 µg [95% CI, 351 - 388]. In unconditioned treatments, females weighed an average rate of 495 µg [95% CI 470 - 520], and males weighed 362 µg [95% CI 343 - 382]. This resulted in a non-significant difference of 9 µg [95% CI 9 – 10] for females and 7 µg [95% CI 6 – 8] for males between treatments. However, as expected, significant differences were observed between the sexes, with females weighing significantly more than males (Negative Binomial GLM, $n = 114, z = 10.447, P < 0.0001$)



3.4.3 Results Summary

Overall, the results demonstrate that being reared in a conditioned treatment significantly slowed developmental speed in both the uncontrolled density and the controlled density experiments. However, at controlled density, although overall survivability between the larval and fly stage was marginally significantly higher in the unconditioned treatment, faster development did not lead to any significant differences in overall emergence of pupae or adult flies. In addition, no differences in body weight were observed between adult flies from the different treatments. These findings suggest that while an early-life fly-conditioned dietary environment impacted developmental speed, there were no clear effects on pre-adult survivability.

3.5 Discussion

In Chapter 2, I demonstrated that *D. melanogaster* females favour feeding and laying their eggs on diets which have been conditioned by previous exposure to flies, particularly if the conditioning is by male flies. The fitness effects of this choice are not known and were tested here. I conducted experiments of offspring developing on conditioned versus unconditioned diets to investigate any developmental and fitness impacts. The aim was to explore potential reasons for the observed dietary preferences.

3.5.1 Uncontrolled density experiment

I first conducted an experiment in which females were allowed to lay an uncontrolled number of eggs, in both male-conditioned and unconditioned (control) vials. The results indicated that speed to pupariation was faster on unconditioned than conditioned diets. This is consistent with Ormerod et al. (2017), who found that varying dietary compositions affected pupariation time. Eggs reared in unconditioned vials also emerged into adults faster than those from conditioned vials, which aligns with findings where developmental times to the adult stage were faster on suboptimal diets (Klepsatel et al., 2020b). However, faster pre-adult development can sometimes lead to negative post-adult consequences (Sharma and Shakarad, 2021), so these results could therefore explain the findings from the dietary choice experiment (Chapter 2), where mated females both feed and prefer to lay their eggs on a conditioned diet. Females may be intentionally choosing these diets, knowing they slow development, with the knowledge that slower development may result in more benefits. This has been shown with a study by Lihoreau et al. (2016) where larvae showed faster development on high protein foods, but survival was higher on nutritionally balanced foods.

Despite the faster development in unconditioned vials, there was no significant difference in pupal survival between conditioned diets and unconditioned diets. However, positive trade-offs at the post-adult stages may still exist (Martelli et al., 2024). A contrasting finding in my experiment, possibly in line with the faster development seen from the egg-fly stage, was that significantly more flies emerged from vials where eggs had been reared under a fly-conditioned treatment. This suggests either that more eggs were placed in the conditioned vials, or that dietary conditioning promotes developmental survival. Hence, it is possible that the faster development in unconditioned treatments was influenced by a population density effect (lower density in the unconditioned vials which are a non-preferred oviposition substrate). This is similar to studies where developmental differences were seen in mice reared in smaller versus larger litters (Faust et al., 1980). The second experiment at controlled density was done to distinguish these possibilities.

Following these results, I analysed the effects of developmental diets on adult body weight. There were no significant differences in the adult body between conditioned and unconditioned treatments, but significant differences between males and females were found, as expected. This indicates that in this uncontrolled density experiment, there was no evidence for trade-offs between e.g. slower developmental speed and larger adult body weight from developing in the conditioned treatment vials.

3.5.2 Controlled density experiment

Similar to the uncontrolled density experiment, significant differences were observed in the speed of pupal emergence between conditioned and unconditioned treatments when first-instar larvae were placed into vials at the same density, with those developing in unconditioned vials emerging faster. This suggests that development is influenced by the rearing environment within fly-conditioned diets. The consistent finding of slower development time in conditioned diets in both experiments suggests additional investigations of the underlying reasons why females specifically choose to feed and rear their offspring on a fly-conditioned diet (Chapter 2) would be useful. A study on pre-adult development in *D. melanogaster* showed that selecting for faster development resulted in reduced viability in later life (Prasad et al., 2000). Therefore, females may potentially be choosing conditioned diets to optimise fitness at the adult stage, even if it incurs developmental trade-offs. Similarly, developmental time from larval to adulthood was consistent with pupal development speed and also revealed significant differences across conditioned and unconditioned treatments, with flies emerging sooner when reared on unconditioned diets. This is consistent with previous studies showing that larvae reared on either poor or rich diets showed a delay in development compared to a control diet with ideal amounts of yeast and sugar (May et al., 2015).

The reason for the slower development at both the larva-pupa stage and the larva-adult stage seen with conditioned diets in these experiments remains unclear. As mentioned above, exactly why females choose to feed and lay on these diets is also not well understood. Many studies have demonstrated that slower development at pre-adult stages may not necessarily negatively impact later life stages and there could be important physiological or behavioural trade-offs (Klepsatel et al., 2020b). A greater understanding of the mechanisms of fly conditioning would be useful here. One mechanistic hypothesis of fly conditioning is that it promotes gut microbe deposition, and thus the presence of microbes on the diet may provide nutritional benefits, by potentially acting as an additional protein component (Lesperance and Broderick, 2020). However, other studies have shown protein

restriction increases the pupation time (Krittika et al., 2019), implying that if gut microbe deposition is a mechanism, and is acting as a protein component, larvae raised in conditioned vials would pupate faster. The contradicting results found through this experiment could suggest that there may be additional mechanisms of fly conditioning that contribute to the slower pupation time observed. Further studies are needed to investigate both the mechanisms behind why a conditioned diet results in slower development and why flies choose a diet that results in slower development.

Although a marginally significant difference was observed with higher survivability in the unconditioned treatment over the conditioned treatments from the larval to fly stage, there were no apparent differences observed between the treatments in the overall emergence of both pupae and adults. This is despite both the faster pupation and faster adult emergence observed in unconditioned treatments. This finding contrasts with some studies that have linked faster development with survivability trade-offs. For example, (Chippindale et al., 1997) demonstrated that faster development was balanced by fitness costs, such as reduced pre-adult survivorship, meaning fewer individuals reached the adult stage. This could imply that faster development rates could lead to lower survivability from larval stages. However, as mentioned above, it is possible that the consequences of faster or slower developmental times may not be apparent until the adult stage for offspring, which were not recorded in my experiments. For example, one study showed that the consequences of variation in a defined pre-adult diet were not seen until adulthood (Martelli et al., 2024) and another showed larvae reared on poor diets, which resulted in negative developmental effects, showed an increase in overall lifespan (May et al., 2015) again suggesting that poor pre-adult development might not be detrimental to adult life-history traits. These studies suggest that the advantages or disadvantages of developmental timing could become evident later in the adult stage, underlining the need for further investigation into the long-term effects of an early-rearing environment.

In addition, there was no significant difference in the overall number of pupae that successfully reached the adult stage between conditioned and unconditioned diets. This showed that, despite differences in developmental speed or diet conditions, the transition from pupa to adult was successfully achieved across both treatments. Previous studies indicate that pupal mortality can be a significant factor in overall survival for *D. melanogaster* raised in varying environments, such as under high crowding (Moya and Botella, 1985), suggesting that diet rearing effects on development may occur at the pupa-fly stage.

Finally, flies reared in conditioned treatments were heavier, although this difference was not statistically significant. This finding contrasts with a study examining the impact of diet on body weight, where protein-restricted diets resulted in significantly lower body weights for flies (Krittika et al., 2019). While no significant direct effects on adult body weight were apparent in this experiment, early dietary conditions might impact other aspects of life-history traits, such as reproductive success (Ruchitha et al., 2024a) or lifespan (Stefana et al., 2017).

3.5.3 Conclusion

Overall, this chapter demonstrates that rearing *Drosophila melanogaster* on a conditioned diet significantly slows their development to both pupal and adult stages, yet does not show any significant differences between pre-adult emergence or body weight. While no immediate pre-adult effects of emergence were observed, this may have implications at post-adult stages, such as through reproductive success or lifespan, suggesting a need for further investigation into any potential long-term effects.

The results reveal important findings of how early-life diet impacts health and physiology, highlighting developmental speed as a significant factor. Developmental speed can fluctuate with early-life environmental conditions, such as diet, revealing species-specific responses (Monaghan, 2007). In humans, faster developmental speed in early life has been linked to lower body fat percentage in later life (Karaolis-Danckert et al., 2007), suggesting that the relationship between early-life diet and adult outcomes could be mediated through intermediary factors such as developmental speed. For example, slower developmental speed in fire salamanders (*Salamandra salamandra*) in scarce diet conditions will lead to compensatory growth in later life (Krause and Caspers, 2016). Thus, the slower development associated with pre-conditioned diets observed in this chapter might have broader implications at post-adult stages that I did not experiment with, reflecting the potential long-term effects of early-life dietary conditions.

In addition to the developmental speed effects I observed, early-life exposure to various fly-conditioning factors, such as the deposition of gut microbes and pheromones, may play a role in shaping life-history outcomes. Studies have shown that early-life interactions with gut microbes can influence cognitive function and overall physiology in later life (Hunter et al., 2023). Early exposure to pheromones can also have significant effects; for example, female mate preference in butterflies (*Bicyclus anynana*) are influenced by early pheromone exposure, a trait which can be inherited by their offspring (Dion et al., 2020). Similarly, early

exposure to queen mandibular pheromone in honey bees can affect gene expression related to chemosensory perception and ageing (Peng et al., 2024).

Another possible factor of fly conditioning is digestate, where the diet is pre-digested, which may influence developmental factors. In humans, a study showed that variations in food chewing and digestibility altered gut microbiome composition without changing the nutrient content of the diet (H.-J. Kim et al., 2022). However, a contrasting study by Cherta-Murillo et al (2023) showed that neither pre-digested mycoproteins nor non-digested mycoproteins will alter the composition of the gut microbiome. Suggesting that the impact of digestate on gut microbe composition may vary depending on the specific conditions or factors involved in the study. In addition to these studies, a study in rats showed that when they were fed natural nutrients and partially digested nutrients, there were no differences in body weight between these two treatments (Zafra et al., 2007). These studies suggest that although early-life diet can sometimes influence gut microbiome composition, this isn't consistent, and the impact on other health factors, such as body weight, is minimal. This may explain why no body weight differences between conditioned and unconditioned diets were observed in my experiments.

One additional observation was that males were sometimes observed emerging faster than females, this was seen in the conditioned treatment in the uncontrolled density treatment, and in both the conditioned and unconditioned treatment in the controlled density experiment Usually, in normal laboratory diets, many studies have shown that females will emerge first (Seong and Kang, 2022). This finding may be something that requires further investigation, specifically whether this is due to conditioning effects, or the effect of a high carbohydrate, 1:4 diet.

Overall, this chapter demonstrates that rearing *D. melanogaster* on a conditioned diet affects developmental speed but does not impact pre-adult survivability or adult body weight. Future research should investigate the post-adult effects of different developmental speeds and investigate the long-term consequence of early exposure to fly conditioning factors, such as gut microbes, pheromones, and pre-digested diets.

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Chapter 4 - General Discussion

4.1 Introduction

This thesis has explored how fly environmental cues can affect dietary choices in the model organism *Drosophila melanogaster* and has tested the subsequent fitness implications this may have. In this chapter, I summarise the key findings of the results and propose wider applications for future research. I discuss the potential of studying the mechanisms of fly conditioning and explore why mated females may exhibit distinct dietary preferences. Through this, I explain how a preliminary experiment I completed, investigating the effects of different doses of fly conditioning on dietary choice, could be used to further investigate the mechanisms underlying conditioning effects. I also discuss the further implications of preferences for conditioned diets on fly fitness throughout the adult stages and explore the possible effects of having alternative perspectives of dietary choices. This includes the potential for divergent outcomes if males, rather than mated females, or if flies of different mating statuses feeding behaviours were tested in the same dietary choice assays.

4.2 Key findings

4.2.1 The effect of fly-conditioned diets on dietary choice in *Drosophila melanogaster* (Chapter 2)

In Chapter 2, I investigated how diets pre-conditioned by other flies, onto which various cues of occupation or utilisation have been deposited, affect mated female dietary choices. I conducted three experiments in which diets were conditioned by males, virgin females, and by Ovo^{D1} females that do not lay eggs. I tested the effects of conditioning versus non conditioning on dietary substrates containing two different Protein: Carbohydrate ratios; (i) 4:1 (known from previous work to be preferred by mated females for feeding) and (ii) 1:4, (favoured for egg laying) (Almeida de Carvalho and Mirth, 2017). I conducted two types of assays to test preferences: (i) an ‘absolute’ preference assay (two-way choice) which tested the direct effects of conditioning vs non conditioning within each diet, and (ii) a ‘relative’ preference assay (four-way choice) which tested the effects of conditioning vs non conditioning across both diets.

I first investigated how diets conditioned by males affect female dietary choices and found significant preferences for feeding on conditioned diets, especially when they were high in protein. Preference for conditioned diets was less marked in the non-preferred high carbohydrate diets. For oviposition behaviour, conditioning preferences were less

pronounced but still evident on 4:1 diets, which was unexpected, as high protein diets are generally avoided for egg-laying. Next, I investigated the impacts of diets conditioned by virgin females to determine any sex-specific effects and the influence of the presence of eggs on dietary choices. Conditioning effects were less pronounced in diets conditioned by virgin females as opposed to those conditioned by males or Ovo^{D1} females. Nevertheless, significant preferences were still observed for feeding on 4:1 diets in both the two-way and four-way choice assays. Conditioning preferences were again present but less marked on the 1:4 diets. An intriguing finding was that conditioning by virgin females led to an opposite oviposition preference for unconditioned 4:1 diets in the two-choice assays. This could suggest that mated females may avoid laying eggs on a diet already containing eggs. The most marked conditioning effects were seen when analysing the effects of diets conditioned by Ovo^{D1} (eggless) females. Strong conditioning preferences were evident for the 1:4 diet in both the two-way and four-way choice assays and for the 1:4 diet in the two-choice assays for feeding. However, the expected preference for conditioned diets was absent for the 4:1 diet in the two-choice assays, which requires further investigation. The most substantial oviposition effects were also observed in the experiments in which conditioning was by Ovo^{D1} females, with most diet ratios and assay environments showing significant preferences for egg laying on conditioned diets. This suggests that the absence of eggs on a substrate magnifies the preference for egg laying on conditioned diets.

4.2.2 The impact of fly conditioning on development in *Drosophila melanogaster* (Chapter 3)

In Chapter 3, I investigated the developmental and fitness effects of being reared on a fly-conditioned diet. To explore this, I used a no-choice experimental set-up, conditioning vials by using male flies and a P: C 1:4 meridic diet, which was typically favoured by females for egg laying (Almeida de Carvalho and Mirth, 2017). I then allowed females to either naturally lay eggs, or placed known numbers of larvae, in vials containing conditioned or non-conditioned media (uncontrolled and controlled density experiments, respectively). I then monitored developmental speed and survival for both experiments, to assess any pre-adult fitness consequences associated with being reared on a conditioned diet.

In the initial uncontrolled density experiment, development was faster for eggs laid in unconditioned vials, though there was no significant difference in the total pupae that had emerged between treatments. However, significantly more adult flies emerged from the conditioned vials, suggesting that the faster developmental speed in the lower density unconditioned diet vials could be confounded with density differences. This prompted the

second, controlled density experiment in which density was equalised across conditioned and non conditioned vials. This revealed that larvae in unconditioned vials again developed significantly faster to both pupal and adult stages than those in conditioned vials, suggesting an effect of conditioning treatment on developmental speed. There were no significant differences in survivability for the larvae-to-pupa, pupa-to-fly stages, and minimal differences in larvae-to-fly stages, suggesting no immediate survivability trade-offs with faster development at pre-adult stages. There were also no significant differences in adult body weight between treatments. Further experiments on the fitness of adults are now needed to investigate any additional potential consequences of developmental diet and developmental speed.

4.3 Implications, importance and conclusions

Across many studies using *Drosophila melanogaster*, flies are often kept in shared dietary environments. This means they feed on diets that have previously been consumed or occupied by other flies (Klepsatel et al., 2020). It is therefore crucial to understand the effects of “conditioned” diets. In Chapter 2, I demonstrated that when given a choice, flies prefer to feed and lay their eggs on diets conditioned by other individuals, and in Chapter 3 I found that a conditioned diet will result in slower development, but found minimal pre-adult survivability consequences, or effects on adult body weight at emergence. These findings, along with those from other studies testing dietary choice, suggest that dietary variation significantly impacts the preference behaviours of mated females. Furthermore, many studies confirm that the diet chosen and consumed can lead to different life-history outcomes. This is important in understanding how environmental cues, such as the social environment, influence dietary choices and can enhance our knowledge of behaviours in both humans and other animals.

Dietary variation and choices are important factors that influence behaviour and health across different species. In humans, although food consumption is significantly influenced by factors such food availability, like access to nutritional foods (Mela, 1999). Attributes such as colour, smell and temperature can affect food intake and selection. Understanding these factors is important, as the diet consumed plays a crucial role in overall health and quality of life (Stroebele and De Castro, 2004; Yeung et al., 2021). In this thesis, I demonstrated that in *D. melanogaster*, dietary choice is influenced by both nutritional factors and the conditioning effects (previous occupancy) of the dietary environment. I hypothesise that the cues to which mated females might be responding may include (i) gut microbe deposition, (ii) pheromonal

deposition and (iii) detection of pre-digested diets. I discuss these hypotheses, and the potential benefits associated with responding to each cue, in more detail below.

Firstly, the consumption of beneficial microbes has been associated with positive health outcomes in humans, such as resulting in reduced systolic blood pressure (Hill et al., 2023). Gut bacteria are also known to play an important role in providing essential nutrients (Gerritsen et al., 2011), and synthesising vitamins (Mueller and Macpherson, 2006). Therefore, if gut microbe deposition is the cue to which females respond in showing a preference for conditioned diets, then dietary choices could have potential health impacts. Pheromonal deposition could also be a cue associated with fly conditioning. Pheromones are known to induce different behaviours, both directly and indirectly influencing life-history traits. For instance, in the cane toad (*Bufo marinus*), exposure to pheromones has been shown to reduce developmental time, and decrease size at metamorphosis, with larvae altering their developmental trajectories and behaviours. This can have lasting effects into post-metamorphic life (Hagman et al., 2009). In the experiments shown through this thesis, larvae reared in the same conditioned dietary environments may exhibit consequential life-history effects arising from pheromone exposure. In addition, a diet previously consumed by flies may contain dietary components that have been pre-digested, which could also be a benefit of preference for fly conditioned media. Flies feeding on different nutritional diets may excrete active digestive enzymes (Strilbytska et al., 2022), hence pre-digested diets may contain enzymes that subsequent flies can utilise and potentially benefit from. Alternatively, pre-digested diets may simply be easier to digest.

4.4 The mechanisms underlying dietary choices; the effects of microbial and pheromone deposition:

In Chapter 2, I demonstrated that mated females exhibit a preference for feeding and laying their eggs on diets which have previously been conditioned by other flies. However, the underlying mechanisms driving this preference remain unclear and warrant further investigation. A potential explanation for this behaviour, which I have discussed throughout this thesis, involves responses to the deposition of gut microbes or pheromones onto diets, potentially making these diets richer in microbial and pheromonal content. Benefits could include the acquisition of beneficial microbes or specific pheromones, which may influence the overall health of flies that consume or offspring that have been reared on these conditioned diets. To investigate these hypotheses, I developed an experimental approach (which there was insufficient time to complete) which through microbial filter washes would create dietary choice assays which would test (i) environmental microbes, fly microbes and

fly pheromones against (ii) environmental microbes and (iii) fly pheromones. By doing this, it would be possible to determine whether these fly cues present on the diet play a key role in shaping dietary preferences.

As a preliminary study, I conducted a conditioning dose-response experiment (Chapter 4 Supplementary Material). This demonstrated a clear dose-dependent response of conditioning, consistent with the existence of cues and responses of a quantitative nature. This then allowed me to develop a refined experiment protocol using specific doses of conditioning, to allow an assessment of the effects of removing both environmental and gut-associated microbes from a diet against other fly and environmental cues.

Planned (but not yet completed) protocol: For the planned microbial wash experiment, I designed a protocol using 35 mm Petri dishes filled with meridic diets with Protein: Carbohydrate (P: C) ratios of either 4:1 or 1:4. Unlike the previous dietary choice experiments in Chapter 2, the microbial wash experiment would have full media-filled 35 mm Petri dishes for easier washing of the diet surface to collect cues, and based on findings from my initial dose-response experiment (Chapter 4 Supplementary Material). For each 35 mm Petri dish, containing either P: C 4:1 or 1:4 media, I proposed to add n = 10 male flies to n = 60 dishes, and leave n = 30 Petri dishes unconditioned, doing this for both 4:1 and 1:4 media petri dishes. All dishes would then be placed in a 25°C CT room for 24 hours. After the 24 hour conditioning period, the dishes would be removed and the surfaces of all the dishes washed, using n = 30 of the conditioned Petri dishes to eventually remove microbes and other cues present. Based on the Petri dish size, I calculated the required volume of sterile water needed for each dish to be 0.62 ml (based on the protocol in (Sato et al., 2021)). I proposed to pipette this onto the diet 10 times, thoroughly mixing each time, then drawing the wash back up, and putting the wash solution into a 0.45 µL centrifuge tube filter (Figure 4.1).

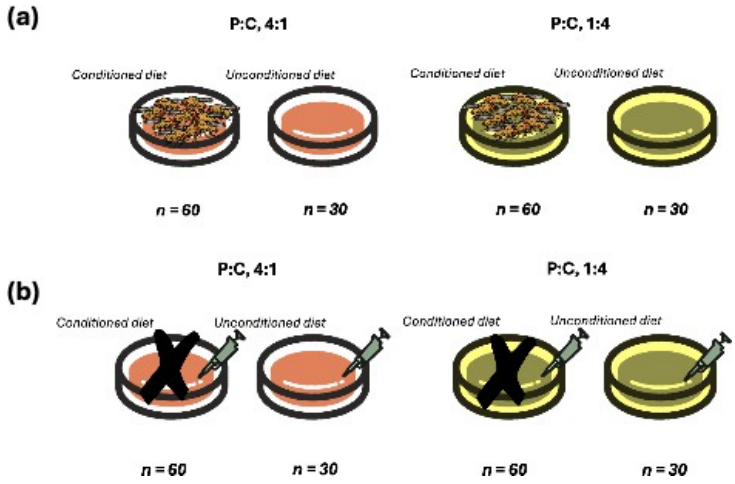

Figure 4.1. Illustration of the conditioning, non-conditioned and surface wash process. (a). 35 mm Petri dishes filled with meridic diets of P: C 4:1 and 1:4, with n = 10 male flies (conditioned) in n = 60 plates or no flies (unconditioned) in n = 30 plates are left in the 25°C CT room for 24 hours. (B). Post-conditioning and unconditioned, a pipette with sterile water is used to wash the surface of the diet.

Following the washing process, n = 30 of the wash solutions from both the 4:1 and 1:4 conditioned diets would be placed in a centrifuge tube to separate the microbes from the solution. This step would ensure that half of the conditioned diet washes contained only fly pheromones, while the other half would contain both fly and environmental microbes, as well as fly pheromones, and the unconditioned diet would only contain environmental microbes (Figure 4.2).

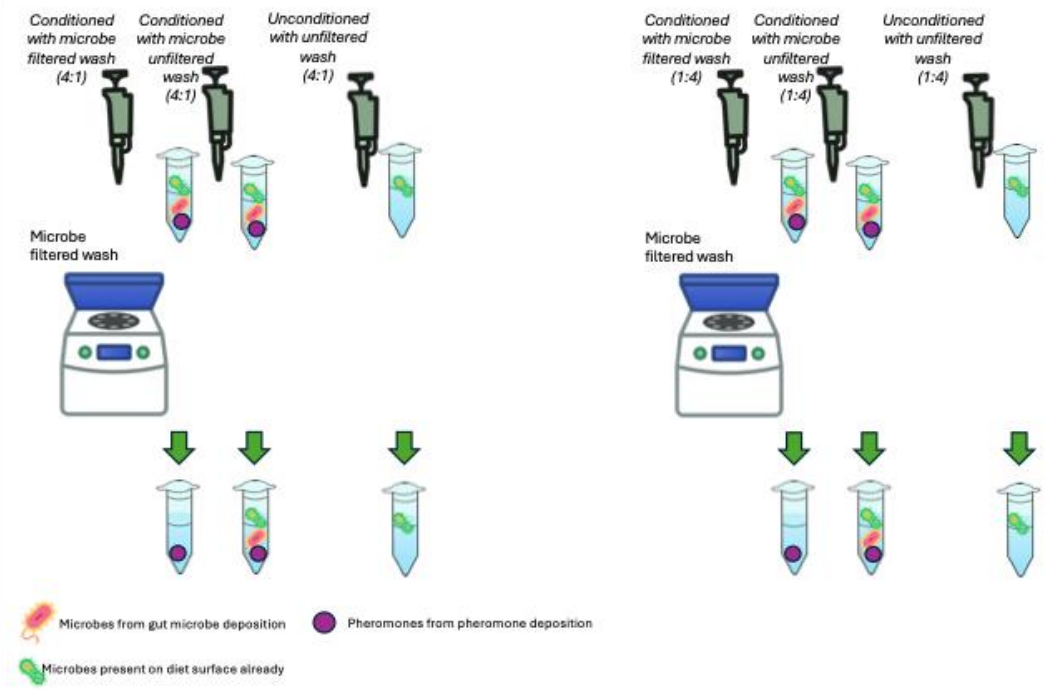

Figure 4.2. A schematic of the microbe filtering process. 0.45 µL centrifuge tube filters containing wash solutions from n = 30 P: C 4:1 and 1:4 conditioned diets are centrifuged to filter out microbes, while n = 30 4:1 and 1:4 conditioned diets, and n = 30 4:1 and 1:4 unconditioned diets are left unfiltered. Leaving solutions that contain (i) fly microbes, environmental microbes and fly pheromones, (ii) environmental microbes and (iii) pheromones.

The filtered wash solutions were then proposed to be applied to new, sterile made diets under controlled conditions, to ensure no additional environmental microbes were introduced. This would allow me to test fly preference based on the components in the wash solutions only.

Figure 4.3. A schematic showing the application of wash solutions onto new sterile diets using a micropipette.

These sterile diets, now containing washes of (i) pheromones + environmental microbial + fly microbes , (ii) environmental microbes and (iii) pheromones, would then be used in a new

dietary choice assay. I planned to use three-choice assays (directly testing 4:1 and 1:4 individually) and a six-choice assay (testing both 1:4 and 4:1 simultaneously) (Figure 4.4).

Figure 4.4. Schematic showing dietary choice assays. Three different dietary choice assay designs will be used: three-choice to test the direct effect of one nutrient composition, and six-choice assays to evaluate preferences with varying nutrient compositions.

This experiment was designed to investigate how different components of conditioned media affect female fly preferences. The aim is to better understand the mechanisms underlying fly conditioning by isolating and analysing various factors present in the media.

4.5 The fitness effects of female preferences for conditioned media

In Chapter 3, I investigated some of the potential developmental fitness consequences of *D. melanogaster* being reared on either a conditioned or unconditioned diet. I found there were significant differences in developmental speed, with flies reared on an unconditioned diet developing faster. Although significant differences in developmental speed were observed between the conditioned and unconditioned diets, these differences did not result in immediate effects on pre-adult survival or body weight when flies first emerged. However, the impact of developmental speed may occur in post-adult stages, influencing factors such as lifespan and reproductive success.

Previous studies have demonstrated that *D. melanogaster* larvae can make dietary choices that minimise developmental time, with the shortest developmental time being observed at equal P: C ratios (Rodrigues et al., 2015). Different development rates in *D. melanogaster* have been linked to variations in post-adult characteristics. For example, flies from a faster-developing population tend to be significantly smaller in adult size, a finding supported by

multiple studies (Chauhan et al., 2020). It has frequently been shown that dietary conditions during development are crucial, as shown by studies indicating that factors such as population density can significantly impact adult lifespans (Klepsatel et al., 2018). In addition, developmental diet and developmental speed affect reproductive outcomes in adult life (Ruchitha et al., 2024b).

These studies provide valuable insights into the potential consequences of rearing offspring on a conditioned diet, suggesting that dietary choices in early life could significantly impact the adult life of *D. melanogaster*. To fully understand these effects, further investigation into how a conditioned diet which will result in slower development, influences adult fitness traits such as lifespan and reproductive success, would be needed.

4.6 The impact of diet on divergent dietary choices

Throughout this thesis, I focused on the dietary choices of mated females, aiming to understand how their diet is influenced by the dietary conditions of their social environment and the subsequent effects on their offspring after laying eggs on these diets. However, an intriguing angle to explore would be the dietary choices of males or virgin females to test for the existence of sex or mating status specific responses to conditioned diets. There are known sex and mating status specific differences in dietary choice in *D. melanogaster*. For example, one study found that males tend to choose to consume more carbohydrates than females, regardless of whether they are mated or virgin. In contrast, the mating status of females influences their dietary choices, with virgins consuming more carbohydrates (Camus et al., 2018b). In addition, the diet consumed can have different life-history effects depending on the sex. Research has shown that protein and carbohydrate intake can have sex-specific impacts on reproduction, with males and females experiencing different life-history outcomes when consuming the same diets (Jensen et al., 2015). These findings suggest that exploring the dietary choices of males and flies of different mating statuses and their responses to conditioned versus unconditioned diets, could be valuable in order to provide a more comprehensive understanding of the effects of the fly social environment.

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Chapter 4 Supplementary Material

S4.1 Dose-dependency of conditioning on mated female preferences

S4.1.1 Introduction

Drosophila melanogaster may be attracted to diets which have previously been exposed to other flies. This attraction could be due to several factors, such as the introduction of additional microbes from the flies' microbiome, the deposition of fly pheromones, or the diet being partially pre-digested. However, the extent to which these diets are conditioned, and the "dose" or level of conditioning might have varying effects on fly behaviour, potentially influencing dietary choices. For example, it is known that the quantity of microbes can significantly impact *Drosophila* development, and in some cases, microbial quantity may be more influential than microbial quality (Keebaugh et al., 2018). My aim in this experiment was to investigate whether the extent of conditioning affects the dietary preferences of *Drosophila melanogaster,* and thus whether conditioning effects show dose-dependency.

S4.1.2 Materials and Methods:

S4.1.2.1 Fly stocks and rearing:

All flies were reared, and experiments took place, in a controlled temperature room of 25 °C, 50% RH on a 12:12 light: dark regime. Fly rearing was conducted in vials of sugar yeast agar medium (SYA; **Appendix 2**). Eggs were collected from Dahomey wild type population cages using purple agar plates (**Appendix 2)** with yeast paste in the centre to attract ovipositing females. These plates were placed in the population cages from the start of when the lights were on for 3 hours. The purple agar plates were then put in a pillowcase to incubate for 24 hours. After this, first instar larvae were picked and placed into SYA vials (50 larvae/vial, to standardise density and minimise any environmentally driven differences in body size). The vials were left in the 25 °C CT room for rearing, and after 9 days, the flies started to eclose.

S4.1.2.2 4:1 and 1:4 Meridic Diet Treatments:

To investigate the effect of the Protein: Carbohydrate (P: C) diet ratio with and without conditioning on dietary preference, two different P: C diets were used (4:1 and 1:4). These allowed an investigation of the effect of nutrient composition for both high carbohydrate / low protein and high protein / low carbohydrate. Fly food media was prepared using an autoclave, to ensure consistency across batches. Different Protein and Carbohydrate levels were controlled using differing levels of Casein and Sucrose (Table S4.1), as well as 0.3 g of

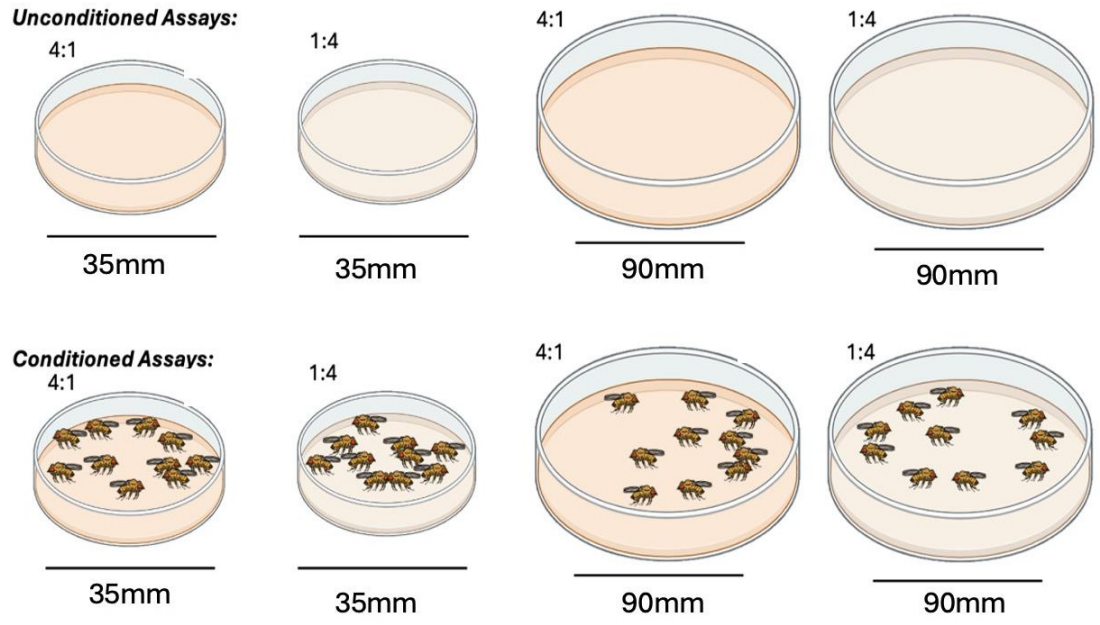
Cholesterol, 4 g of Lecithin and 20 g of Agar. After dry ingredients were added, liquid salt solutions (100 ml of KH_2PO_4, K_2HPO_4, $MgSO_4$, $NaHCO_3$, nucleic acid and 200 ml of distilled water) were added. The diets were then autoclaved, and after the diets had cooled to 60 °C, 10 ml of nipagin solution, 3 ml of propionic acid and 150 ml of a vitamin mix were added. These diets were then poured into Petri dishes to be used for dietary choice assays.

P:C diet	Casein	Sucrose
1:4	24 g	96 g
4:1	96 g	24 g

Table S.4.1. Protein: Carbohydrate ratios. The table shows the amount of Casein and Sucrose (g) added for the appropriate Protein: Carbohydrate ratio diets used.

S4.1.2.3. Conditioning of Diets by males:

I used both Protein: Carbohydrate 4:1, and 1:4 media for the dietary choice experiments. I used two different sized Petri dishes; 35 mm, and 90 mm, and allowed n = 10 males to roam around each dish filled with media, then cut squares of media out. I cut 1 square from the 35 mm dish, and 5 squares from the 90 mm dish (Figure S4.1).



Whole diet plates, being left unconditioned or being conditioned by males.

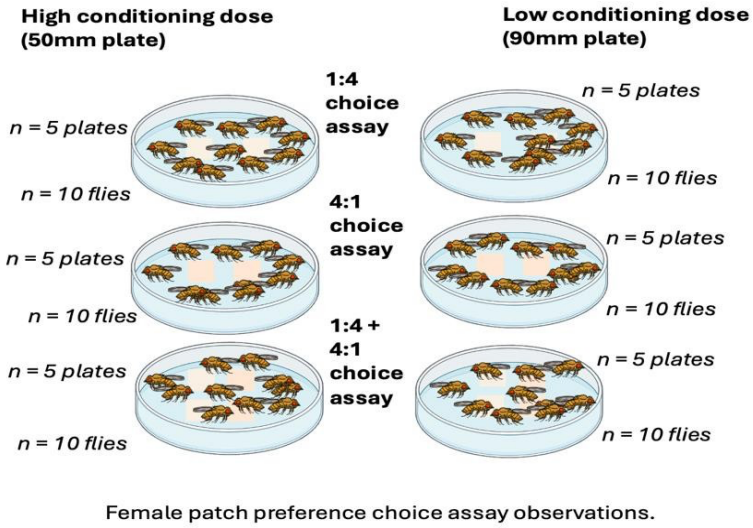


Figure S4.1. Schematic showing the dose-density conditioning process. Petri dishes of 35 mm and 90 mm were filled with P: C 1:4 and 4:1 media, n = 10 male flies were left to condition the diets for 24 hours.

S4.1.2.4 Dietary Choice Observations:

Based on the results in a power analysis I conducted (**link to code for power analysis in Appendix 1**), I found that increasing the observations would increase power for the dietary choice observations. Thus, I increased observations from every 30 minutes to every 20 minutes. To collect feeding preference data, every 20 minutes for 5 hours, I took a picture from approximately 1 metre away. I used the patch at which a fly was spending their time as the patch of dietary preference.

S4.1.3 Results:

S4.1.3.1 Feeding Preference:

I initially investigated how the ratio of protein to carbohydrate in the diet and the conditioning density influenced a mated female preference for feeding on a conditioned or unconditioned diet. To look at these effects, I first tested the effects in a two-choice assay, an absolute environment. I initially tested for a two-way interaction between the diet ratio and conditioning density, and a significant interaction was found (Binomial GLMM, $n = 427$, $X^2 = 24.197$, $P < 0.0001$), so this interaction was kept in the model (Table S4.2).

	cbind(Conditioned, Unconditioned)		
Predictors	*Odds Ratios*	*CI*	*p*
(Intercept)	3.92	2.69 – 5.70	**<0.001**
ratio1 × 4	0.47	0.33 – 0.66	**<0.001**
density [90]	0.26	0.19 – 0.37	**<0.001**
(ratio1 × 4) × density [90]	3.60	2.16 – 6.01	**<0.001**
Random Effects			
σ^2		3.29	
$\tau_{00\ observation}$		0.00	
$\tau_{00\ plate}$		0.17	
ICC		0.05	
N_{plate}		7	
$N_{observation}$		16	
Observations		427	
Marginal R^2 / Conditional R^2		0.060 / 0.107	

Table S4.2. Results of fly feeding preference of conditioned versus unconditioned diets from a Binomial Generalised Linear Mixed Model. The intercept represents a fly's preference for a conditioned diet in the 4:1 ratio, in a 35 mm conditioning density. The table includes the odd ratios, confidence intervals, and the p-values for all factors considered in the model.

Using this model, I investigated the effects of diet ratio and conditioning density, as well as their interaction on whether a fly chooses to feed on a conditioned or an unconditioned diet. There was a significant preference for a conditioned diet shown with the 4:1 diets at a 35 mm conditioning density (Binomial GLMM, $n = 427$, $z = 7.122$, $P < 0.0001$), with the

marginal means probability of choosing a conditioned diet over an unconditioned diet in this scenario being 0.797 [95% CI, 0.729 – 0.851]. However, this preference significantly decreased with the 1:4 diet ratios (Binomial GLMM, *n = 427, z = z4.357, P < 0.0001*), where the marginal means probability of preferring a conditioned diet dropped to 0.648 [95% CI, 0.55 – 0.735]. In addition, when the density of conditioning was 90 mm, meaning less dense conditioning, there was a further significant decrease in the preference for a conditioned diet (Binomial GLMM, *n = 427, z = 7.798, P < 0.0001*). In this scenario, the marginal means probability decreased to 0.506 [95% CI, 0.405 – 0.606] (Figure S4.2), showing conditioning preferences were much less prominent. In addition, there was an interaction found between a 1:4 diet, and conditioning density (Binomial GLMM, *n = 427, z = 4.897, P < 0.0001*), indicating that the preference for a conditioned diet at this ratio was also affected by the density.

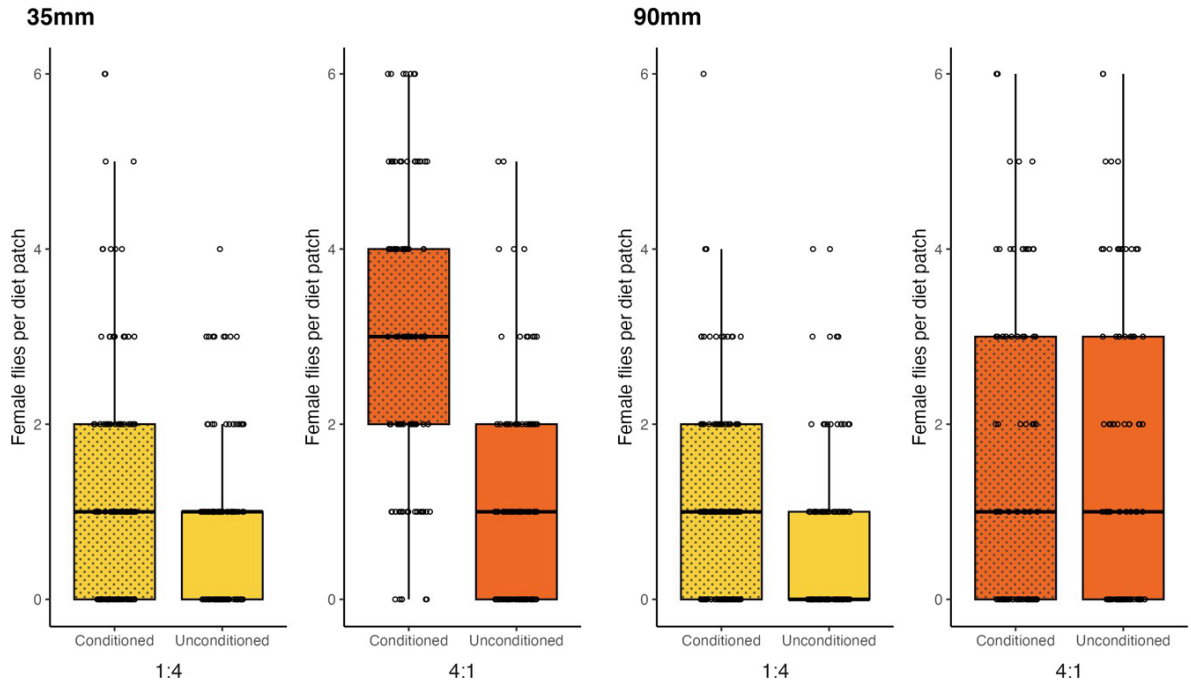


Figure S4.2. Box plot of two-choice tests, showing effects of conditioning density and diet ratio on female flies' feeding preference. The plot shows how the flies' preference for conditioned (dots) and unconditioned (blank) diets in 1:4 (yellow) and 4:1 (orange) ratios vary under 35 mm and 90 mm conditioning densities (x-axis), shown with the number of flies per diet patch (y-axis).

Building on the significant findings related to conditioning preferences in the two-choice assays and the preference observed when diets were conditioned in a high-density, 35 mm Petri dish, I then analysed the effects within a four-choice environment. Initially, I tested for a

3-way interaction between diet ratio, conditioning density and conditioning treatment, but no significant 3-way interaction was found (Poisson GLMM, $n = 896$, $X^2 = 0.345$, $P = 0.55$). As a result, I dropped this from the model and tested the effects of two-way interactions. A significant two-way interaction between diet ratio and conditioning treatment was found (Poisson GLMM, $n = 896$, $X^2 = 13.82$, $P = 0.0002$), so this was retained in the model (Table S4.3).

	fly_numbers		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	2.86	2.52 – 3.25	**<0.001**
ratio1 × 4	0.30	0.26 – 0.35	**<0.001**
treatment [Unconditioned]	0.31	0.27 – 0.36	**<0.001**
density [90mm]	1.16	1.03 – 1.29	**0.011**
(ratio1 × 4) × treatment [Unconditioned]	1.71	1.30 – 2.25	**<0.001**
Random Effects			
σ^2	0.55		
$\tau_{00\ plate}$	0.00		
$\tau_{00\ observation}$	0.03		
ICC	0.05		
N_{plate}	7		
$N_{observation}$	16		
Observations	896		
Marginal R^2 / Conditional R^2	0.435 / 0.462		

Table S4.3. Results of fly feeding preference from a Poisson Generalised Linear Mixed Model. The intercept represents a fly's preference for a conditioned diet in the 4:1 ratio, in a 35 mm conditioning density. The table includes the odd ratios, confidence intervals, and the p-values for all factors considered in the model.

I then tested the effects of diet ratio, conditioning treatment and conditioning density, along with the interaction between diet ratio and conditioning treatment, on the feeding preference of flies. Analysis showed there was a significant preference for the conditioned diet within the 4:1 diets, (Binomial GLMM, $n = 896$, $z = 15.041$, $P = < 0.0001$), with an average of 2.86 [95% CI, 2.519 – 3.248] flies per observation choosing this diet. Interestingly, in the four-choice assays, there was a small but significant increase in preferring a conditioned 4:1 diet when conditioned in the lower, 90 mm density (Binomial GLMM, $n = 896$, $z = 2.554$, $P = 0.01$), with an average of 3.31 [95% CI, 2.92 – 3.74] flies per observation choosing to feed here. In addition, a significant 2-way interaction between the 1:4 ratio and the conditioning treatment was found, showing that a preference for the 1:4 ratio can vary depending on the conditioning treatment (Binomial GLMM, $n = 896$, $z = 3.832$, $P < 0.001$) (Figure S4.3).

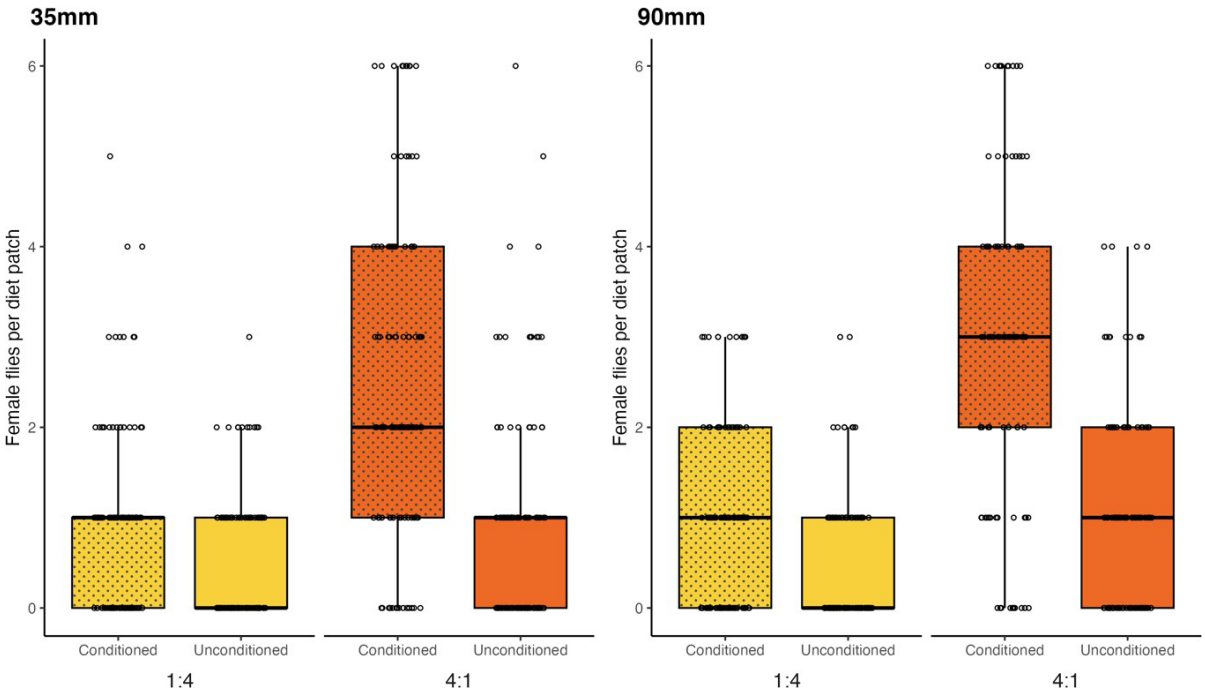


Figure S4.3. Box plot of four-choice tests, showing effects of conditioning density and diet ratio on female flies' feeding preference. The plot shows how the flies' preference for conditioned (dots) and unconditioned (blank) diets in 1:4 (yellow) and 4:1 (orange) ratios vary under 35 mm and 90 mm conditioning densities (x-axis), shown with the number of flies per diet patch (y-axis).

S4.1.3.2 Oviposition Preference:

I then investigated the effects of diet ratio and conditioning density on whether a mated female fly chooses to lay their eggs on a conditioned or an unconditioned diet. I first tested for a 2-way interaction between diet ratio and conditioning density, and a significant interaction was found (Binomial GLMM, $n = 22$, $X^2 = 18.3$, $P < 0.0001$). As a result, this interaction was retained in the model (Table S4.4).

	cbind(Conditioned, Unconditioned)		
Predictors	*Odds Ratios*	*CI*	*p*
(Intercept)	2.05	1.58 – 2.66	**<0.001**
ratio1 × 4	0.60	0.50 – 0.72	**<0.001**
density [90]	0.35	0.27 – 0.46	**<0.001**
(ratio1 × 4) × density [90]	1.95	1.43 – 2.65	**<0.001**
Random Effects			
σ^2		3.29	
$\tau_{00\ plate}$		0.08	
ICC		0.02	
$N_{\ plate}$		7	
Observations		22	
Marginal R^2 / Conditional R^2		0.041 / 0.063	

Table S.4.4. Results of fly oviposition preference of conditioned versus unconditioned diets from a Binomial Generalised Linear Mixed Model. The intercept represents the number of eggs for on a conditioned diet in the 4:1 ratio, in a 35 mm conditioning density. The table includes the odd ratios, confidence intervals, and the p-values for all factors considered in the model.

Using a model that tested the effects of diet ratio, conditioning density, as well as their interaction on whether a mated female fly lays on a conditioned or an unconditioned diet, I found a significant preference for a conditioned diet in the 4:1 diets when the diets had been conditioned in a 35 mm density (Binomial GLMM, $n = 22$, $z = 5.375$, $P < 0.0001$). However, there was a significant decrease for a conditioned diet in the 1:4 ratio 35 mm density diets (Binomial GLMM, $n = 22$, $z = 5.443$, $P < 0.0001$) with a probability of 0.672 [95% CI, 0.612 – 0.727] choosing a conditioned over an unconditioned diet. In addition, the preference for a 4:1 conditioned diet reduced when the conditioning density was 90 mm (Binomial GLMM, $n = 22$, $z = 4.261$, $P < 0.0001$) with a marginal means probability of only 0.42 [95% CI, 0.35 – 0.495] selecting a conditioned diet. Furthermore, a two-way interaction between diet ratio

and conditioning density was found, indicating that the preference for a conditioned in the 1:4 ratio also significantly decreased at the 90 mm density (Binomial GLMM, *n = 22, z = 4.261, P < 0.0001*) (Figure S4.4).

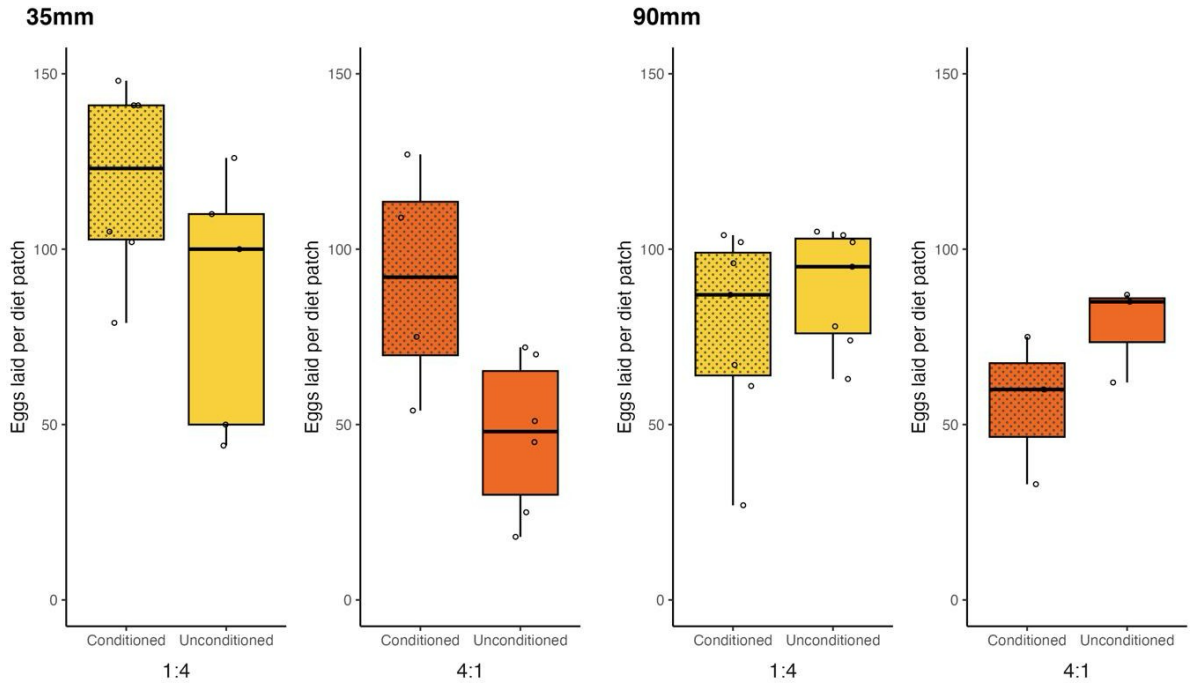


Figure S4.4. Box plot of two-choice tests, showing effects of conditioning density and diet ratio on female flies' oviposition preference. The plot shows how the flies' egg-laying preference for conditioned (dots) and unconditioned (blank) diets in 1:4 (yellow) and 4:1 (orange) ratios vary under 35 mm and 90 mm conditioning densities (x-axis), shown with the number of eggs per diet patch (y-axis).

After testing for oviposition effects in a two-choice assay, I tested effects in a four-choice assay. I first tested for a 3-way interaction between diet ratio, conditioning density and conditioning treatment, and a 3-way interaction was found (Negative Binomial GLM, *n = 56, X^2 = 7.52, P = 0.006*) and retained in the model (Table S4.5).

	egg_numbers		
Predictors	*Incidence Rate Ratios*	*CI*	*p*
(Intercept)	21.00	15.21 – 29.55	**<0.001**
ratio1 × 4	6.24	4.00 – 9.75	**<0.001**
treatment [Unconditioned]	0.13	0.07 – 0.24	**<0.001**
density [90mm]	3.32	2.12 – 5.21	**<0.001**
(ratio1 × 4) × treatment [Unconditioned]	5.06	2.40 – 10.93	**<0.001**
(ratio1 × 4) × density [90mm]	0.20	0.11 – 0.38	**<0.001**
treatment [Unconditioned] × density [90mm]	2.22	1.03 – 4.87	**0.043**
(ratio1 × 4 × treatment [Unconditioned]) × density [90mm]	0.26	0.09 – 0.68	**0.006**
Observations	56		
R^2 Nagelkerke	0.994		

Table S4.5. Results of fly oviposition preference from a Negative Binomial Generalised Linear Model. The intercept represents the number of eggs for on a conditioned diet in the 4:1 ratio, in a 35 mm conditioning density. The table includes the odd ratios, confidence intervals, and the p-values for all factors considered in the model.

Following this, I tested the effects of conditioning treatment, diet ratio and conditioning density, as well as their interaction on egg-laying preferences. In the 4:1 diets with a 35 mm density of conditioning, a significant preference for a conditioned diet was observed (Negative Binomial GLM, $n = 56$, $z = 6.378$, $P < 0.0001$). However, there was a significant increase for a conditioned diet, if the density of the conditioning patch was taken from a 90 mm Petri dish (Negative Binomial GLM, $n = 56$, $z = 5.245$, $P < 0.0001$). Showing that in this scenario in the four-choice assay, flies preferred less dense conditions (Figure S4.5).

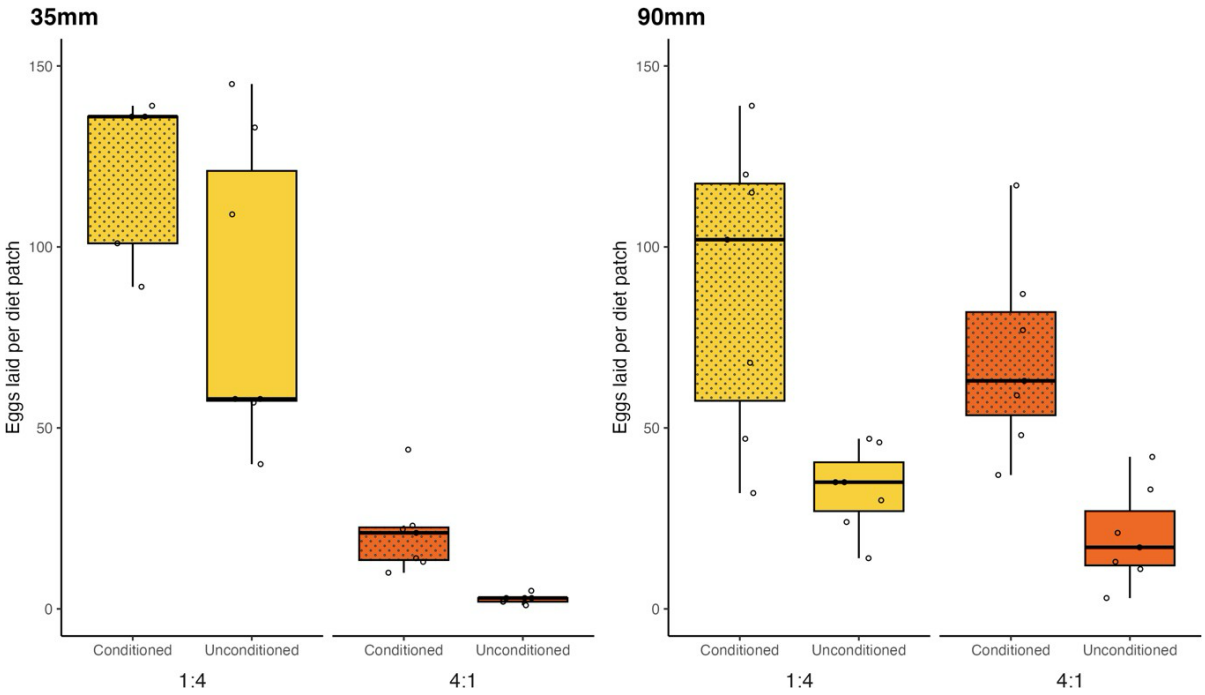


Figure S4.5. Boxplot of two-choice tests, showing effects of conditioning density and diet ratio on female flies' oviposition preference. The plot shows the flies' egg-laying preference for conditioned (dots) and unconditioned (blank) diets in 1:4 (yellow) and 4:1 (orange) ratios vary under 35 mm and 90 mm conditioning densities (x-axis), shown with the number of eggs per diet patch (y-axis).

S4.1.3.3 Summary of Results

In summary, these results show that there were significant dose-dependent effects. Although the dose-dependent results differed depending on the assay environment, I concluded that through conducting a microbial wash experiment, I would use a 35 mm dish, meaning n = 10 flies would have conditioned one diet patch, to provide a more intense conditioning treatment.

S4.1.4 Discussion

The results of this experiment demonstrated a dose-dependent response to dietary fly conditioning, with flies exhibiting different behaviours depending on the extent of diet conditioning. These behavioural responses varied not only between feeding and oviposition but also across different assay environments (absolute vs. relative). In the two-choice assays for both feeding and oviposition, there was a significant decrease in choosing a 4:1 conditioned diet when it had been conditioned in 90 mm Petri dishes (with n = 10 flies spread amongst n = 5 diet patches) compared to when it was conditioned in 35 mm Petri dishes (with n = 10 flies spread amongst n = 1 diet patch). Conversely, in the four-choice assays, there were small yet significant increases for both feeding and oviposition preferences on the 4:1 conditioned diet, when the diet patch had been conditioned in a 90 mm Petri dish, compared to a 35 mm Petri dish. Overall, while there was a marked overall preference for more densely conditioned environments, this preference was influenced by the specific dietary assay being conducted.

Some of these findings align with previous studies of dose-dependent effects on life history traits. For example, (Massie et al., 1993) found that high doses of vitamin A increase median lifespan, suggesting that flies may be able to detect higher doses of vitamins, and thus possibly in the experiment shown in this thesis, may be associating stronger conditioned diets with better life-history outcomes. Quantitative variation in exposure to heat-killed microbes also have a dose-dependent effect on fly longevity. For example, increasing the microbe dose of *Lactuca orientalis* (*L. orientalis*) can lead to a shortened fly lifespan in flies (Keebaugh et al., 2018b), indicating that there may be a threshold at which the presence of microbes becomes detrimental. Furthermore, the dose of pheromones related to population density may modulate behaviour in *D. melanogaster,* and can also affect mating and aggression behaviours in social insects (Sethi et al., 2019), which could be important for more dense conditioning factors observed in this experiment. A similar conclusion could be made regarding the extent to which the diet has been pre-digested, as a higher concentration of digestive enzymes could be present in the 35 mm Petri dishes, leading to a stronger conditioning effect, and different life-history outcomes.

In addition, results from the four-choice assays in this experiment revealed a different pattern of dose-dependent responses compared to the two-choice assays. While flies in the two-choice assays preferred to feed and lay their eggs in a denser environment, the availability of more choices in the four-choice assays altered their preferences. This difference aligns with previous observations in this thesis (as discussed in Chapter 2, the dietary choice experiments), where two-choice assays and four-choice assays resulted in distinct

behavioural outcomes. However, it is unclear why providing additional options leads to differing preferences in *D. melanogaster*, further investigation into the complexity of the decision-making processes in *D. melanogaster* would be needed to further understand these effects.

S4.1.5. References

Keebaugh, E.S., Yamada, R., Obadia, B., Ludington, W.B., Ja, W.W., 2018. Microbial quantity Impacts *Drosophila* nutrition, development, and lifespan. iScience 4, 247–259. https://doi.org/10.1016/j.isci.2018.06.004

Massie, H.R., Aiello, V.R., Williams, T.R., Baird, M.B., Hough, J.L., 1993. Effect of vitamin A on longevity. Exp. Gerontol. 28, 601–610. https://doi.org/10.1016/0531-5565(93)90049-j

Sethi, S., Lin, H.-H., Shepherd, A.K., Volkan, P.C., Su, C.-Y., Wang, J.W., 2019. Social context enhances hormonal modulation of pheromone detection in *Drosophila*. Curr. Biol. 29, 3887-3898.e4. https://doi.org/10.1016/j.cub.2019.09.045

Appendices

Appendix 1

GitHub Repositories containing code of this project and previous projects

Link to GitHub Repository containing code, data files and analysis for this project (2023-2024): https://github.com/katherinemillar02/microbial-impacts-on-dietary-choice-in-drosophila-melanogaster

Link to GitHub Repository containing code, data files and analysis (Millar, Chapman, Unpublished Data, 2023): https://github.com/katherinemillar02/dietary-choice-substrate-conditon-consequences-for-lifespan-reproductive-success-and-fitness

Link to GitHub Repository containing code, data files and analysis (Millar, Chapman, Unpublished Data, 2022): https://github.com/katherinemillar02/effects-of-dietary-choice-on-lifespan-in-drosophila-melanogaster

Appendix 2

Diet recipes

SYA diet recipe with protocol

120% SYA medium

Yeast at concentration of 120% from standard SYA - all other ingredients follow standard SYA

A5

FINAL VOLUME REQUIRED (L)	0.5	1	1.5	2	3	4	4.5	5	6	7	8	9	10
Water (ml)	480	970	1440	1860	2690	3530	3950	4360	5200	6030	6870	7700	8540
Agar (g)	7.5	15	22.5	30	45	60	67.5	75	90	105	120	135	150
Sugar (g)	25	50	75	100	150	200	225	250	300	350	400	450	500
Yeast (g)	60	120	180	240	360	480	540	600	720	840	960	1080	1200
Nipagin solution (ml)	15	30	45	60	90	120	135	150	180	210	240	270	300
Propioninc Acid (ml)	1.5	3	4.5	6	9	12	13.5	15	18	21	24	27	30

1. Add distilled, if possible, or otherwise tap water to the saucepan to the required volume
2. Add agar and bring to the boil, stirring regularly to avoid burning
3. Take off the heat and stir in all the dried ingredients thoroughly
4. Cool to ~60 degrees C
5. Add Nipagin solution and propionic acid and stir in thoroughly
6. Dispense

Protein: Carbohydrate Meridic diets with protocol

120g L^{-1}

Ingredients	1:1	1:4	4:1	1:8	8:1
P:C (g)					
Casein	60	24	96	13.34	106.6
Sucrose	60	96	24	106.6	13.34
Dry additives (g)					
Lecithin	4.00	4.00	4.00	4.00	4.00
Cholesterol	0.30	0.30	0.30	0.30	0.30
Agar	20	20	20	20	20
Liquid additives (ml)					
KH_2PO_4	100	100	100	100	100
K_2HPO_4	100	100	100	100	100
$MgSO_4$	100	100	100	100	100
$NaHCO_3$	100	100	100	100	100
Nucleic acid	100	100	100	100	100
Distilled water	200	200	200	200	200
Post-cook additives (ml)					
Nipagin	10	10	10	10	10
Proprionic acid	3	3	3	3	3
Distilled water	Top up to 1L	Top up to 1L	Top up to 1L	Top up to 1L	Top up to 1L
Vitamin mix	150	150	150	150	150

(V2. 20/10/2021) + revisions (11/02/22, 02/03/22):

1. Weigh out dry materials into Duran bottle

Casein, sucrose, lecithin, cholesterol, agar for diet bottle/s. Just agar for the load temp bottle (30g)

- One Duran bottle makes 1.5l, these are in the food prep room to the right of small fly autoclave (along with red and green boxes, stirrers, thermometer, 5l beaker)
- Use both large and small measuring beakers that can be taken from glassware room. For the larger beakers, select those with thicker glass, as these are more suitable for heavy stirring.
- Use whatman paper to measure out the cholesterol as this is very light and sticks to the measuring boat. Can rip to smaller piece.
- (use these papers to make the liquid diets too)
- Lecithin and cholesterol are stored in the freezer
- Label the bottles here with autoclave tape if making different diets

2. Measure into beaker:

the **four salt solutions** and the **one nucleic acid solution** to diet bottles (and equivalent of just water to the load temperature bottle).

- Use the automatic pipette loader – should be on charge on the bench, and lots of the pipettes in a box on trolley, one for each solution
- Once added 150ml of the 5 salts, should be 750ml

3. Measure the deinoised water into cylinder and add to dry ingredients in Duran bottle
    - Shake vigorously, ensuring no powder is stuck to bottom of glass

4. Add the salts and nucleic acids into Duran and shake again
    - Top agar bottle up with de-ionised water to the same level (or half if using smaller bottle for probe

5. Cover with the lids (tightly or will leak) and thoroughly mix by shaking.
    - Make sure to use full lid for agar bottle shaking, and then replace with one with hole in

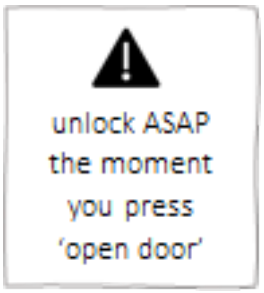
6. Switch on autoclave. Add tap water to chamber if prompted.
    - There will be some water in the bottom, but the screen will say if more is required.
    - Add this to the chamber, taking care to ensure the chamber temp probe remains out of the water
    - Add the shelf back
    - Once at the right water level, the screen will no longer ask for water automatically

7. Place bottles into chamber. Put probe into the load temp bottle.
    - Chamber will fit 4 bottles. 1x 1:2, 1x 1:8 and 1x agar is good
    - The agar bottle is used as the temp probe bottle – this is as a proxy to the temperature of the food mixes themselves, as this will be slightly different to the chamber temp
    - The wirey temp probe should be put through the lid hole into the agar bottle

8. Close door, lock and press:
   - select cycle > 5. Media (on second page) > cycle > cycle start
   - Note that the clock will start, but there's no obvious on-screen confirmation of start

9. Wait ~3.5 hours or so. Depends on number of bottles cooking, suitability of sacrifices to autoclave gods.
   - You will need to wait until the probe says the liquid has cooled to 80.0C
   - This autoclave will automatically keep at 80.0C once it has finished its cycle, so you don't need to rush back/dispense right away
   - You can also set the autoclave on a delay time – set up the bottles and ask to run early in the morning (6am), so that you can come in and dispense after then (9am)

10. While waiting measure out required vitamin mix (berocca mixture) into **measuring cylinder** and propionic acid/ nipagin mix into **small beaker**. Wear gloves.
    - Also, soften up and check dispenser is clean
    - Use flush program to run through warm water
    - Nipagin and propionic acid can go in beaker together, use cylinder for nipagin and glass



11. When cycle is complete press 'open door', unlock door and open. Retrieve Duran/s **with heatproof gloves**, shake vigorously to mix and then place on stirring hotplate.
    - **Unlock ASAP after pressing 'open door'**, as system may reboot and lock. In which case, will need to turn the autoclave off at plug
    - You can rush the cooling stage, but only if absolutely necessary:
      - Skip stage > site engineer > password 333333

- BUT it is best to avoid doing this as may get error messages and annoying warnings

12. Add magnetic stir bar to each bottle and gradually dial up rotations to about level 3 max.

- For the bottles you are not prepping first, you can also turn the hot plate function on to just above 50C
- It should reach the right temp when you are ready
- Wait for liquid temp to reach 60C

13. To expedite cooling to 60C, can instead place first Duran into a large 5L plastic beaker filled to about 2L with cool tap water and stir the diet with thermometer to monitor. Temp will drop quickly as you stir.

14. Once cooled to <60 degrees pour the diet into a 2L glass beaker (with magnetic stirrer too) and add the vitamin mix while stirring vigorously with glass rod.

- Use thick-walled beakers from glass store to avoid breakage, particularly when stirring 1:2 diet

15. Then add the propionic acid/nipagin and stir vigorously with glass rod until diet looks homogenous (no white streaks).

- Remember to stir asap when adding as proteinaceous stringy bits form instantly – pay particular attention to stirring this in the 1:2

16. 

Top up with deionised water to specified volume (1500 ml if we are making a full tray)

17. Place beaker with mixed diet onto stirring hotplate and dial up rotations to about level 3 max. Set plate temperature to about 60 degrees.

18. Prep the peristaltic pump by running hot water through on a flush cycle for about a minute, the expel all water from tubes.

19. Place inlet tube (RHS) from peristaltic pump into the mixed diet and set up the program for desired volume and doses:

- Shift(yellow arrow)+7 > Menu > Dose > Enter > Set New Program > 9.5mls > Enter > Down to interval > 0.8 sec (use Shift to get decimal point) > Enter > Down to doses > 170 (tray of tubes) or 30 (bottles) > Enter > Start > Start > Start.
- NB:
  - If any errors entering just use 'Stop' to go back a step.
  - 9.5ml for 1:2 diet tube, 60-70ml for 1:2 diet bottle)
  - Adjust volume lower for 1:8 diet (try 8 ml for tube, 55ml for bottle) as viscosity lower
  - About 60ml for bottles and 8.5 for vials of 1:5
  - Flush with hit tap water before, between and after dispensing diets. Do this straight away between diets as mixture will solidify quickly in tubes.
  - 0.3s is quickest interval

Diet	Bottle	Vial
1:2	70ml	
1:5		
1:8	60ml	

20. After dispensing:

- Put trays into pillowcases to cool and set overnight in prep room. Top with cotton balls and move to 02 cold room the next morning.
- Rinse Durans and put in red bin labelled 01.09 (Fly lab). With lids on (keep lid with hole and put back by bay). Rinse any agar into sieve, not down sink
- Rinse glassware and put into a green bin for washup and general store. Can be found in glass room
- Take full trays to the wash up room along the corridor on their trolley

- Pour out agar solution from load temperature bottle into shallow plastic tray to set (dispose of set agar in general waste bin once set/following day).
- Make sure peristaltic pump has been properly flushed with hot water.
- Store cholesterol and lecithin back in Chapman lab freezer in the molecular lab, bottom drawer

Grape juice medium with protocol

PURPLE GRAPE JIUCE MEDIUM

NUMBER OF PETRI DISHES REQUIRED	~9	~18	~36	~72
NUMBER OF SMALL LIDS REQUIRED	~20	~40	~80	~160
WATER (ml)	250	500	1000	2000
AGAR (g)	12.5	25	50	100
RED GRAPE JUICE (ml)	150	300	600	1200
EXTRA WATER (ml)	25	50	100	200
NIPAGIN SOLUTION (ml)	10.5	21	42	84

1. Stir agar and water and bring to the boil, stirring regularly

2. Add grape juice, stir well and bring back to the boil

3. Remove from heat

4. Use extra cold water to rinse measuring cylinder and add it to the mix

5. Add nipagin solution, mix well and dispense