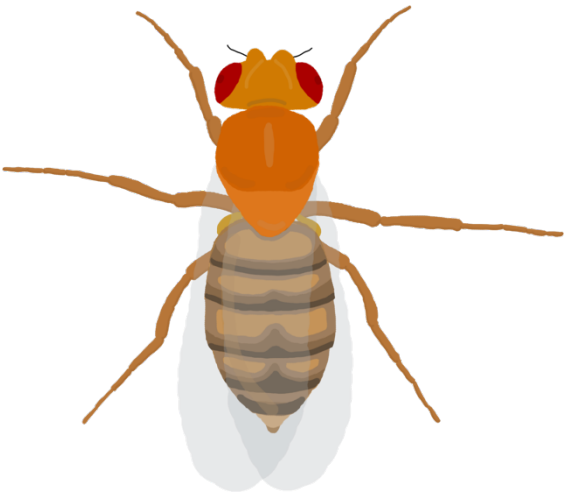
Male and female diet choice in response to the socio-sexual environment



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Abstract

Animals choose the diets that they eat, and the balance of macronutrients chosen can determine the expression of key life history traits. For example, lifespan is typically maximised on low protein diets and reproduction on high protein diets. The optimal balance of protein and carbohydrate can vary with age, sex and environment. One key example occurs in females of many invertebrate species, where a single mating increases preference for dietary protein. The research in this thesis aimed to investigate how diet choice changes under ecologically relevant social and sexual environments, using the *Drosophila melanogaster* fruit fly model. Mating multiply in succession did not cause males to increase protein consumption. Despite observed fertility costs of mating multiply, male diet choice was consistent among virgin, once mated and five times mated males. Contrary to our predictions, female condition had little impact on macronutrient intake, despite the potentially reduced reproductive capacity of low-quality females. Additionally, an increase in protein consumption and egg laying was observed after remating, which tested for a dose-dependency of the post-mating feeding switch (a potential avenue for sexual conflict over diet). Foraging and oviposition choice of females was affected by both male sexual harassment and sensory cues of males. However, contrary to the predictions, females did not avoid potential costs of interacting with males and instead preferred diet patches on which males were present. Adaptation to long-term, distinct macronutrient ratios altered survival and expression of nutrient sensing genes differentially between males and females. However, offspring production was not affected by the evolutionary diet regimes. My research applied different methods for measuring diet choice and behaviour in *D. melanogaster*, allowing assessment of the costs and benefits of preference assays using liquid (capillary feeding) versus solid diets. Overall, the results contribute to our understanding of how the socio-sexual environment alters diet choice and illustrates how macronutrient ratio choice is dynamic and tightly interwoven with reproduction.

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Author contributions

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Mabel Sydney and Jennifer Perry conceived and designed the study; Mabel Sydney conducted the experiments with assistance from Jennifer Perry during mating assays. Mabel Sydney performed data analysis and visualisation with advice and supervision from Tracey Chapman and Jennifer Perry. All authors wrote the original draft, edited and approved the final draft for publication.

Chapter 3 - Post-mating switch in diet preference and reproductive behaviour in female *D. melanogaster*

Mabel Sydney, Jennifer Perry and Tracey Chapman conceived and designed the study; Mabel Sydney conducted the experiments. Mabel Sydney performed data analysis and visualisation with advice and supervision from Tracey Chapman and Jennifer Perry. Mabel Sydney wrote the original draft, with edits and assistance from Tracey Chapman and Jennifer Perry.

Chapter 4 - Female feeding and oviposition site preference is influenced by male social cues

Mabel Sydney, Jennifer Perry and Tracey Chapman conceived and designed the study; Mabel Sydney conducted the experiments. Mabel Sydney performed data analysis and visualisation

with advice and supervision from Tracey Chapman and Jennifer Perry. Mabel Sydney wrote the original draft, with edits and assistance from Tracey Chapman and Jennifer Perry.

Chapter 5 - Differences in nutrient sensing gene expression as a result of diet and sex in two experimentally evolved populations

Mabel Sydney, Jennifer Perry and Tracey Chapman conceived and designed the study. The original 1:2 and 1:8 population cages were established by Wayne Rostant and Tracey Chapman, and later maintained by Mabel Sydney. Survival and offspring data were collected as part of an undergraduate project by Tejaswi Harshavardhan and Hannah Dickinson, co-supervised by Mabel Sydney, Jennifer Perry and Tracey Chapman. Mabel Sydney collected the experimental samples for the quantitative RT-PCR analyses and processed the samples for PCR with the assistance of Suzanne Bennett-Keki, who also optimised the primer pairs. Mabel Sydney performed data analysis and visualisation with advice and supervision from Tracey Chapman and Jennifer Perry. Mabel Sydney wrote the original draft, with edits and assistance from Tracey Chapman and Jennifer Perry.

Publications

The work in Chapter 2 of this thesis has led to the following publication:

Sydney, M. C., Chapman, T., Perry, J.C. Diet choice is insensitive to mating in male fruit flies, *Animal Behaviour* (2024), https://doi.org/10.1016/j.anbehav.2024.05.010

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Chapter 1

General Introduction

The relationship between diet and animal health has been extensively studied in recent years. Nutrition research was initially tailored to improve our understanding of human diet, and the consequences of increased food processing, sugar content and fat content of human diet in the modern era (Cordain *et al.*, 2005). Meanwhile, animal related nutrition was established predominantly in agriculturally important species, aiming to improve livestock health and quality, and optimise nutrition conversion efficiency into animal products (Wilson *et al.*, 1997).

In more recent years, the field of animal nutrition has broadened to encompass the evolution and ecology of nutrition. Diets are composed of multiple varying constituents, including micronutrients, such as vitamins and minerals, and macronutrients, such as amino acids (proteins), lipids and sugars (carbohydrates)(Simpson & Raubenheimer, 2012). The balance of protein to carbohydrates (the P:C ratio) is crucial in determining animal health, reproduction and lifespan (Simpson & Raubenheimer, 2012).

The nutritional geometry framework helped to establish the impact of P:C and calories on fitness related traits (Raubenheimer & Simpson, 1993). In this framework, multiple diets of varying chemically defined P:C ratios are individually offered to animals, creating a fixed 'nutritional rail' of P:C that an animal can access (Figure 1). Animals then feed freely from the diet and life history outcomes are measured. These life history variables can then be mapped against the quantities of protein and carbohydrate eaten on each nutritional rail, allowing the

visualisation of an optimal P:C consumption response surface (Figure 1; see Lee et al., 2008). The nutritional geometry framework establishes optimal P:C diet, in both quality and quantity, for a particular trait for males and females. The impacts of P:C ratios have been studied across taxa: in insects such as crickets (Hunt *et al.*, 2004), cockroaches (Bunning *et al.*, 2015), ants (Dussutour & Simpson, 2012) and fruit flies (Lee *et al.*, 2008), and in mammals such as mice (Solon-Biet *et al.*, 2014), rats (Blouet *et al.*, 2006) and humans (Fontana *et al.*, 2008).

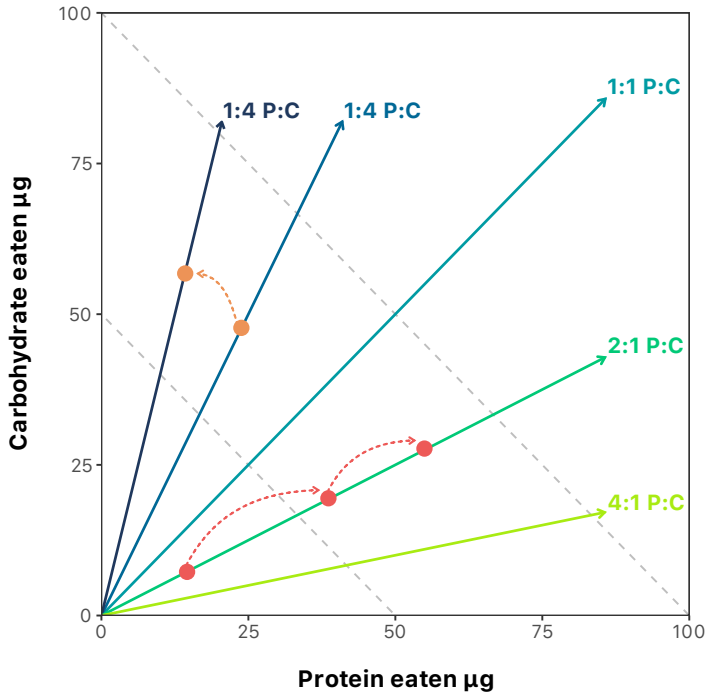


Figure 1

An example of two feeding regimes within a protein: carbohydrate nutrient space, as part of the nutritional geometry framework. Grey dashed lines indication iso-caloric lines, where quantities of protein and carbohydrate consumption are the same. Coloured lines indicate distinct P:C diets (nutritional rails), varying from low P:C (1:4) to high P:C (4:1). An individual can alter their P:C consumption can by consuming different quantities of a single P:C diet, feeding along their nutritional rail to vary caloric content only (signified by the red points), or can vary their P:C ratio by feeding from different nutritional rails (signified by the orange points).

It is important for health that animals are able to sense their nutritional requirements and make suitable choices about which foods to eat and how much to eat, to achieve an optimal balance of protein and carbohydrate intake. Such decisions about diet consumption are complex, as optimal diets can change based on factors including age, sex, environment and mating status. Despite this, studies testing animal dietary choice are less frequent than those testing fitness-related outcomes under varying P:C ratios in which there is no opportunity for choice. In the introduction to this thesis, I provide an overview of life history responses to diets of varying P:C ratios under choice and no-choice assay scenarios. This introduction considers both the impact of diet on key life history traits (lifespan and reproduction), and the impact of socio-sexual factors (i.e. sexual selection, sexual and parent-offspring conflict, social environment) on diet optima and diet choice. In addition, I will introduce the methods used in this thesis to investigate dietary choice of the model species *Drosophila melanogaster*.

Effects of diet on lifespan

The impact of P:C content on animal lifespan has been widely studied, largely in the context of dietary restriction (DR), where access to key nutrients are limited, usually in no-choice assays (Kapahi *et al*., 2017). DR research has been used to investigate the effects of dietary variation on health and ageing, with DR reported to extend lifespan through dilution of yeast concentration in *D. melanogaster* diets (Mair *et al*., 2003), reduction of *E .coli* food sources in *Caenorhabditis elegans* nematode worms (Greer & Brunet, 2009), and reduction of glucose supplied as a food source to *Saccharomyces cerevisiae* yeast (Longo *et al*., 2012). The lifespan extensions observed in these studies was believed to arise predominantly from the consumption of less food (termed caloric restriction) (Simpson & Raubenheimer, 2009).

Dietary restriction studies have since then also established the role of restricting specific nutrients, rather that reductions in overall consumption. Lifespan was maximised on lower

protein, higher carbohydrate diets in studies using the nutritional geometry framework in *D. melanogaster* (Lee *et al*., 2008; Carey *et al*., 2022), in Queensland fruit flies *Bactrocera tryoni* (Fanson *et al*., 2009; Fanson & Taylor, 2012), in ants *Lasius niger* (Dussutour & Simpson, 2012), and in crickets *Teleogryllus commodus* and *T. oceanicus* (Maklakov *et al*., 2008; Ng *et al*., 2019). These studies established the specific role of protein restriction in lifespan extension.

Despite optimal P:C ratios for lifespan within nutritional geometry frameworks, animals do not always choose these diets when tested in choice assays. Instead, animals in dietary preference studies tend to divert their P:C consumption away from the dietary ratios that would maximise their lifespan, towards diets that would maximise reproduction (Simpson & Raubenheimer, 2009) and arguably their overall fitness. Longevity-reproduction trade-offs in diet have been found in *D. melanogaster* (Lee *et al*., 2008), *T. commodus* (Maklakov *et al*., 2008) and *B. tryoni* (Fanson *et al*., 2009). Such responses suggest that interacting factors contribute to feeding decisions, resulting in trade-offs between fitness related traits.

Effects of diet on reproduction

Reproductive success is closely linked with nutrition due to the nutrient demands of associated physiological and energetic processes (Droney, 2002; Perry *et al*., 2013; Mirth *et al*., 2019), and diets that maximise reproduction also differ between the sexes. This is perhaps unsurprising considering the mismatch in costs and benefits of mating, and subsequent divergence in mating strategies between the sexes (Arnqvist & Rowe, 2005; Parker, 2006). For example, male reproductive success is often maximised by gaining access to, and mating with the most females, and production of male gametes is often fast and low-cost. Conversely, female reproductive success may be maximised by the production of high-quality viable offspring, requiring costly production of large, nutritious gametes. The high nutrient load of female gametes to sustain a developing embryo is reflected in data on the optimal P:C ratio for

female reproduction. For example, high fecundity in female insects is often maximised on diets containing more protein (Lee *et al.*, 2008; Maklakov *et al.*, 2008; Fanson *et al.*, 2009; Jensen *et al.*, 2015; Ng *et al.*, 2019).

Sexual selection can also act on dietary intake during pre-copulatory interactions such as courtship. Since males maximise their reproductive success by mating with as many females as possible, courtship strategies help males to gain additional mates and persuade potentially resistant females to mate with them. However, the success and timing of such strategies may depend on dietary intake. Energetic demands of courtship, such as the energy intensive singing and courtship behaviour of male *Drosophila*, may require an increased carbohydrate consumption. Consistent with this, male courtship song (Hunt *et al.*, 2004; Maklakov *et al.*, 2008; Ng *et al.*, 2018) and pheromone production (Ng *et al.*, 2018) in crickets, and male attractiveness in *D. melanogaster* (Morimoto & Wigby, 2016) depended on short-term P:C intake ratios. Multiple studies have also tested the indirect effects of diet on male courtship, by using different dietary regimes during development to alter body size and quality of males to test condition dependence of sexually selected traits. Reduced body condition resulting from low-quality diets is reported to reduce courtship activity rates in *D. grimshawi* (Droney, 2002) and wolf spiders *Pardosa prativaga* (Lomborg & Toft, 2009) and to alter visual and auditory mating cues in *Rabidosa rabida* (Wilgers & Hebets, 2011). Nutrition-determined condition reduced attraction calling but not pheromone production in the lesser wax moth *Achroia grisella* Fabricius (Cordes *et al.*, 2015).

Despite the view that male gametes are relatively cheap to produce, the production of sperm and ejaculate components can be costly (Dewsbury, 1982; Olsson *et al.*, 1997; Reinhardt *et al.*, 2011; Perry *et al.*, 2013; Perry & Tse, 2013; Macartney *et al.*, 2019; Simmons *et al.*, 2022). For example, nutritional costs of sperm production within the nutritional geometry framework

were observed in *N. cinerea* when fed varying ratios of P:C – sperm number, and subsequent fertility, were maximised on P:C diets of 1:2 (Bunning *et al.*, 2015), while protein, but not carbohydrate, had a negative impact on sperm viability in *T. oceanicus* (Ng *et al.*, 2018). However, perhaps surprisingly males often do not maximise reproductive outputs when given a choice of diets (Bunning *et al.*, 2015; Ng *et al.*, 2018).

Much of the variation in diet choice between the sexes may also be underpinned by the cellular signalling cascades that respond to nutrient levels. In *Drosophila*, multiple genes involved in the insulin/insulin like growth factor (IIS) and target of rapamycin (TOR) pathways were found to be either female- or male-biased in 5-day old adults (Bennett-Keki *et al.*, 2023). However, this pattern was less pronounced in larvae and older flies (Bennett-Keki *et al.*, 2023), which is consistent with other findings of changing dietary needs with age and developmental stage (Rodrigues *et al.*, 2015).

Effects of diet on evolutionary conflicts of interest

Optimal diets which maximise lifetime reproductive success differ between the sexes. Hence, sexual conflict may arise whereby the diet that one individual eats, affects their mating partner's fitness. For example, female diet can influence offspring output and, as such, female diet may influence their male partner's fitness. This example of potential conflict over diet concerns the differing effects of a diet on the fitness of the individual eating it, versus their mating partner and offspring, and may occur even while the mate partner is unable to influence it. It is also possible for the sexes to have different optimal diets, without the influence of any sexual conflict, i.e. one diet is best for males, while a different diet is best for females, but neither affects the other's fitness.

One potential mechanism by which one sex can influence the diet of the other, is the post-mating diet switch in female *Drosophila* activated by the receipt of the sex peptide within the seminal fluid, transferred along with sperm by males. Sex peptide transfer induces a range of varied responses in females, including increased fecundity, reduced sexual receptivity and increased efficiency and storage of sperm (Hopkins & Perry, 2022). The full details of the wide influence of the *Drosophila* sex peptide on females is beyond the scope of this thesis (Chapman *et al.*, 2003; Hopkins & Perry, 2022). However, relevant to this thesis is the response to sex peptide receipt that results in a dramatic switch in feeding behaviour in mated females, when compared to their virgin counterparts (Carvalho *et al.*, 2006; Hopkins & Perry, 2022). Virgin females tend to eat a similar low P:C diet comparable to that of male conspecifics (Lee *et al.*, 2013; Camus *et al.*, 2018). However, after a single mating, females dramatically increase consumption of protein and increase total diet intake as a whole (Carvalho *et al.*, 2006; Lee *et al.*, 2013; Camus *et al.*, 2018). This response might occur to support an uptick in costly egg production after mating; eggs carry a heavy protein load to support embryos with necessary nutrients, such as vitellogenin, that are required for successful development.

Conflict between dietary optima may also exist in a parent-offspring relationship. For example, female insects may choose to feed and oviposit on diets that benefit them, despite subsequent costs to offspring (Rodrigues *et al.*, 2015; Lihoreau *et al.*, 2016b). Oviposition choices made by a female may also impact upon larval growth and development: *D. melanogaster* larval development is increased at a low P:C ratio (Schwarz *et al.*, 2014; Rodrigues *et al.*, 2015), but conversely, body mass, female ovariole number and survival of emerging adults is reduced on low P:C diets (Schwarz *et al.*, 2014; Rodrigues *et al.*, 2015). Larvae offered the choice between P:C ratios prioritised development time over fitness-related traits such as body size and ovariole number, selecting to feed from low P:C diets (Schwarz *et al.*, 2014; Rodrigues *et al.*, 2015). However, females chose to lay their eggs consistently on even higher carbohydrate

diets (Rodrigues *et al.*, 2015; Lihoreau *et al.*, 2016b). Oviposition on such low P:C diets would appear to be suboptimal for both larval development time and fitness-related larval traits.

Despite this, low P:C diets may reflect natural scenarios, in which P:C of rotting fruit will increase as the fruit decays and is colonised by yeasts (Schwarz *et al.*, 2014; Rodrigues *et al.*, 2015; Lihoreau *et al.*, 2016b). If this is the case, females would be selecting the correct diet and would suggest limited parent-offspring conflict here. More research is needed into the behaviours of *Drosophila* in nature to investigate the role of parent-offspring conflict over oviposition site and diet. These findings also highlight the potential for conflict of interest over optimal nutrition at different life stages.

Interactions between dietary optima, choice and the social environment

Animals gain important social information from their conspecifics, which may alter their diet choices (Lihoreau *et al.*, 2015). This has been investigated in insects in the study of 'social nutrition', whereby social groups, whether in social or non-eusocial species, make collective foraging decisions (Lihoreau *et al.*, 2018). For example, in *D. melanogaster*, once an initial group had made a foraging choice on a diet patch, new adult females were then more likely to join that diet patch than other diet patches available (Lihoreau *et al.*, 2016a). This effect increased as group size increased (Lihoreau *et al.*, 2016a). Similar social aggregation patterns were seen in *D. melanogaster* larvae, but not adult males (Lihoreau *et al.*, 2016a). Collective decisions for foraging choice have also been observed in decisions of German cockroaches, *Blattella germanica* (Lihoreau *et al.*, 2010) and desert locusts, *Schistocerca gregaria* (Günzel *et al.*, 2023).

Effects of mating status on diet choice

Differences in diet choice exist between the sexes with regard to their mating status. For example, virgin females and males eat similar ratios of macronutrients (Lee *et al.*, 2013). Upon

mating, females increase both their macronutrient consumption, and the proportion of protein in their diet (Carvalho *et al.*, 2006; Lee *et al.*, 2013; Camus *et al.*, 2018). This response is triggered by transferral of the sex peptide in the male ejaculate, as discussed above (Carvalho *et al.*, 2006). An increase in female diet consumption after mating is also seen in other insect species, for example in two-spotted crickets *Gryllus bimaculatus* (Tsukamoto et al., 2014), *A. bipunctata* (Perry, 2011), and tephritid fruit flies (Pérez-Staples and Abraham, 2023). There is evidence that the change in feeding behaviour could be strengthened by additional matings in *A. bipunctata* and *D. melanogaster* (Perry, 2011; Bowman & Tatar, 2016). Overall, the increased protein preference in female post-mating diet is thought to account for increased production of large, proteinaceous eggs (Barnes *et al.*, 2008).

Despite this variation in female feeding upon mating, there is a lack of evidence to support an impact of mating on male diet choice. In species that produce resource-costly nuptial gifts, an increase in protein preference and diet intake has been reported after mating (Perry & Tse, 2013; Jensen & Silverman, 2018). For example in male *B. germanica*, individuals that had been frequently provided with receptive female partners consumed more protein than those offered less frequent or no mating opportunities (Jensen & Silverman, 2018).

In species without nuptial gifts, males show little change in diet intake after a single mating (Lee *et al.*, 2013; Camus *et al.*, 2018). However, males typically mate at high mating rates, such as up to 7 times in 15 min in *Tribolium castaneum* red flour beetles (Lewis, 2004) and 11 times in a day in *D. melanogaster* (Douglas *et al.*, 2020). In addition, production of sperm and non-sperm components of the ejaculate can be costly to males (Dewsbury, 1982; Olsson *et al.*, 1997; Reinhardt *et al.*, 2011; Perry *et al.*, 2013; Perry & Tse, 2013; Macartney *et al.*, 2019; Simmons *et al.*, 2022), limiting offspring production (Lewis, 2004; Kant *et al.*, 2012; Douglas *et al.*, 2020) and

remating (Reinhardt *et al*., 2011). Plasticity in male diet choice in response to mating may therefore only be observed following ecologically realistic, multiple matings.

Study species

In this thesis I investigated the role of the socio-sexual environment on diet choice, in experiments using the fruit fly model *D. melanogaster*. *D. melanogaster* is an ideal animal model to investigate diet choice as there is an extensive body of existing literature on the relationships between diet, lifespan and reproduction in this species, which forms a strong, foundational basis for this research. Methods of P:C choice using the CAFE assay and solid diets (see below) are also firmly established.

D. melanogaster has a short development time (9 – 10 days at 25 °C), allowing fast, controlled rearing of experimental flies and measurement of key reproductive traits: egg laying and offspring output. For the research described in Chapter 5, these life history properties facilitated the long-term experimental evolution of populations of wildtype flies in a relatively short period of time. The nutrient signalling pathways analysed within Chapter 5 are also highly conserved across flies to humans, which increases the relevance of the results for ageing and longevity across taxa in general (Barbieri *et al*., 2003).

Methods to measure *Drosophila* dietary choice

Numerous methods to measure animal diet choice have been reported in the literature. In larger insects, the dry weight of solid diets can be easily weighed pre- and post-feeding for an accurate measurement of consumption and application to a nutritional geometry framework, such as has been conducted in cockroaches (Bunning *et al*., 2015; Jensen & Silverman, 2018) and crickets (Maklakov *et al*., 2008). However, difficulties arise in smaller species where food consumption per individual is considerably smaller and harder to measure accurately.

In *D. melanogaster*, multiple dietary measures have been established to address the challenges of small volume dietary consumption in this species. For example, reported methods include proboscis extension rate (Barnes *et al.*, 2008; Wong *et al.*, 2009), time spent on solid food (Lihoreau *et al.*, 2016b, 2016a), radioactive tracers (Thompson & Reeder, 1987), liquid feeding (Ja *et al.*, 2007), and dye markers used in adults (Carvalho *et al.*, 2006; Wong *et al.*, 2009; Ribeiro & Dickson, 2010) and larvae (Rodrigues *et al.*, 2015). A full review of feeding methodology for *Drosophila* can be found by Deshpande and colleagues (2014). In this thesis, I provided protein and carbohydrate diets to fruit flies in choice assays using liquid diets (Chapter 2 and Chapter 3) and solid diets (Chapter 4 and Chapter 5). Therefore, brief overviews of the methodologies used are provided in this section.

The capillary feeder (CAFE) assay for measuring qualitative and quantitative consumption in fruit flies

The capillary feeder (CAFE) assay (Ja *et al.*, 2007) uses liquid diets of strict chemically defined components of essential vitamins, cholesterol, salts and amino acids, combined with either a protein source (amino acids) or a carbohydrate source (sucrose). Diets are provided in microcapillary glass tubes, from which flies are able to feed freely, and tube size can be adjusted to measure individual or group consumption (Figure 2). The distance between initial liquid level and liquid level following feeding is then used to calculate the quantity of diet consumed. Protein and carbohydrate liquids can either be combined to create a varying range of P:C ratios (Jang & Lee, 2018) or protein and carbohydrate liquids may also remain separate and used to measure precise diet choice of *Drosophila* under different experimental conditions – specifically measuring the quantity of diet *and* the P:C ratio selected (Camus *et al.*, 2018).

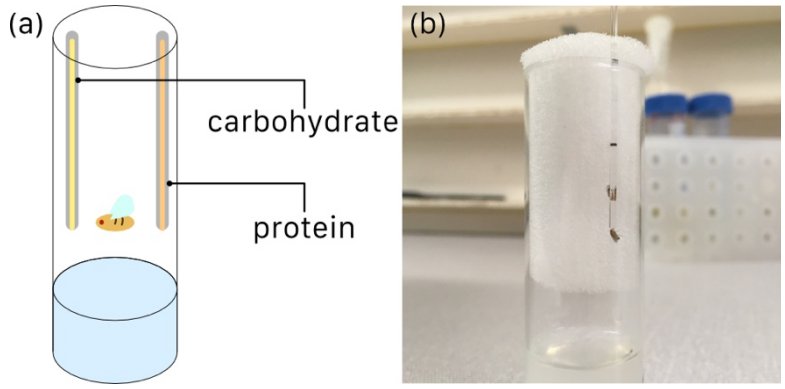


Figure 2

The capillary feeder assay (CAFE) assay for *D. melanogaster*. (a) The experimental set-up of a CAFE assay test vial: chemically defined liquid diets of protein and carbohydrate are provided within microcapillary glass tubes. Flies are able to alter both P:C ratio; by alternating feeding between the two tubes, and quantity consumed; by altering feeding rate from each tube. (b) Photo of a male fruit fly feeding from a capillary tube during a CAFE assay. Capillary tubes are held in place using a foam bung, that also prevents flies escaping, over a base of agar-water to provide moisture.

Solid food choice assays for measuring qualitative and quantitative consumption in fruit flies

The use of solid food to measure *Drosophila* consumption can avoid some logistical and technical limitations of the CAFE assay. Assays using solid diets may vary yeast and sucrose concentration of standard sugar-yeast-agar based rearing diets, to create 'high quality' and 'low quality' diets, with high yeast content and low yeast content, respectively (Duxbury & Chapman, 2020; Bath *et al.*, 2023). Such alterations to standard *Drosophila* lab diets avoid potential impacts of novel dietary components, which can be especially helpful when rearing larvae in condition dependence studies (Duxbury & Chapman, 2020). However, these diets may also have limitations in measuring dietary choice due to the complex nutrient makeup of yeast and interlinked responses to yeast odorants in *Drosophila* (Becher *et al.*, 2012).

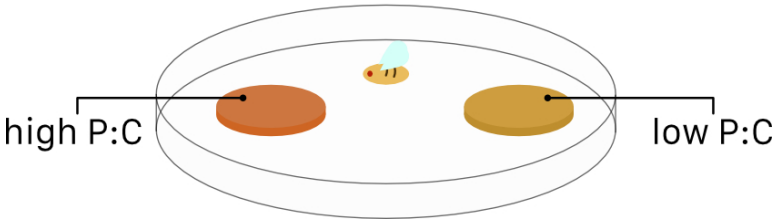


Figure 3

Example of solid food assay testing fruit fly dietary P:C preference. Patches of solid, agar-based diets of defined P:C ratios are placed into observation arenas. Location and movement of the experimental individuals can be observed over time to establish whether flies prefer one patch over the other. Quantity can be inferred by recording the number of proboscis extensions onto the food surface.

Solid diets can be made to specific P:C ratios without yeast by using a base of essential vitamins, salts, cholesterol, agar and water, combined with varying ratios of casein to sucrose (P:C) (Piper *et al.*, 2014). Various P:C ratios can then be placed as individual patches within an arena, to measure *Drosophila* choice between one ratio and another under different experimental treatments, often via behavioural observations (e.g. time spent on a diet; Lihoreau et al., 2016a, 2016b; Figure 3) or by dyeing foods and observing fly abdomen colour after periods of feeding (Carvalho *et al.*, 2006; Ribeiro & Dickson, 2010). Assays are simple to arrange and relatively low cost. However, unlike the CAFE, solid diet assays do not facilitate exact quantification of consumption. Consumption of solid diets in *Drosophila* has previously been measured via behavioural observations of proboscis extension (Barnes *et al.*, 2008) but this can vary between extensions and between species (Wong *et al.*, 2009). In addition, the softness, texture and colour of solid diets can vary between ratios due to chemical reactions during the cooking process, e.g. by the Maillard reaction (Ames, 1992) and the low-dissolvability and bulkiness of casein powder (CG de Kruif et al., 2015), which may impact substrate preference in *Drosophila*.

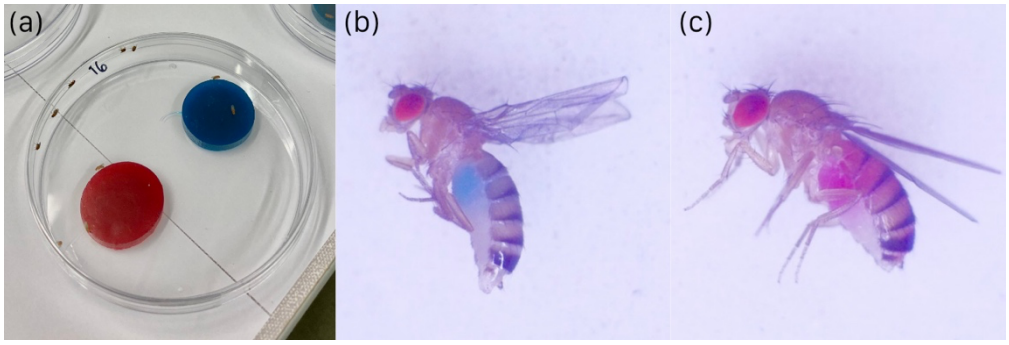


Figure 4

An example of assays using dye measure dietary preference. (a) Experimental diets of distinct P:C ratios are coloured using dyes, typically red and blue, and provided to test individuals at equal distances within observation arenas. Fly location is recorded over time to establish behavioural preference for either diet. Behavioural preference can be complemented with the visualisation of dyed diet within the flies' abdomens, i.e. to record strong feeding preference for either (b) blue or (c) red diets. Colorimetry techniques may also be used to calculate quantity of dyed food ingested.

Multiple methods of measuring animal diet choice exist due to the range of positives and negatives of each assay. Assays must therefore be chosen with consideration of the study species (method of diet delivery and consumption rates) and experimental question (choice versus no-choice, diet quality versus diet quantity). Simpler assays using solid casein-sucrose diets may provide the same level of information as CAFE assays and may be more logistically feasible, but only if precise consumption measures are not required. The quantity and quality of P:C consumption by *Drosophila* is currently most accurately measured by the CAFE assay.

Thesis aims

The main aims of this thesis were to investigate how the social and sexual environment may influence diet choice, behaviour and life history traits of male and female *D. melanogaster*. The individual aims of each chapter are listed below.

Chapter 2 – Diet choice is insensitive to mating in male fruit flies

Animals are able to make dynamic choices about their diet, and decisions on P:C consumption can be influenced by mating. For example, females switch their diet preference following a single mating, to consume more food overall, such as in the two-spot ladybird, *A. bipunctata* (Perry, 2011) and tephritid fruit flies (Pérez-Staples & Abraham, 2023). Specifically, *D. melanogaster* and *B. tryoni* mated female flies also increase the proportion of protein in their diet after mating (Meats & Leighton, 2004; Barnes *et al.*, 2008; Lee *et al.*, 2013; Bowman & Tatar, 2016; Mori *et al.*, 2017; Camus *et al.*, 2018). In contrast, there is little evidence for a shift in P:C choice after a single mating in males. For example, in *D. melanogaster*, dietary preference remained the same in unmated vs mated flies after a single mating (Camus *et al.*, 2018). This finding is consistent with the widely accepted idea that male sperm and seminal fluid are 'cheap' to produce (Bateman, 1948; Trivers, 1972).

However, in realistic scenarios males are able to mate multiply and in quick succession (Lewis, 2004; Douglas *et al.*, 2020), such that the costs of mating may only be apparent after multiple matings. Recent work suggests ejaculate production may be more costly than previously thought (Reinhardt *et al.*, 2011; Perry *et al.*, 2013; Simmons *et al.*, 2022), resulting in depletion of ejaculate components (Hihara, 1981; Linklater *et al.*, 2007) and subsequent fertility (Kant *et al.*, 2012; Douglas *et al.*, 2020) across consecutive matings.

In Chapter 2, I tested the diet choice of male *D. melanogaster* under ecologically realistic rates of mating to investigate whether males were able to adjust their P:C intake to account for ejaculate depletion. I assigned male *D. melanogaster* to remain unmated, or mate once or five times in a single day, as this is nearing the daily maximum matings for this species (Lefevre & Jonsson, 1962; Douglas *et al.*, 2020). The number of viable offspring produced from each mating event was measured to confirm ejaculate depletion with successive matings, and P:C intake of male flies was measured using the CAFE assay (Ja *et al.*, 2007). This chapter tested whether males were able to respond to ejaculate and energetic depletion resulting from extended mating bouts by regulating their intake of P:C in a dietary choice assay.

Chapter 3 – Post-mating switch in diet preference and reproductive behaviour in female *D. melanogaster*

Much of the difference in P:C intake between males and females arises after mating. Females experience a diet 'switch' following a single mating, and subsequently increase their consumption of protein and total food (e.g. Camus et al., 2018; Lee et al., 2013).

However, this diet switching response in females has not yet been entirely characterised. It is not yet fully understood whether the post-mating female diet switch is fully induced from a single mating or can be amplified in a dose-dependent manner with additional matings. A dose-dependent feeding response would provide an avenue for sexual conflict to occur as males could influence female feeding away from optimum for female reproduction. This idea was previously tested in *A. bipunctata* and *D. melanogaster*, with some evidence for an increase in female consumption after a second mating (Perry, 2011; Bowman & Tatar, 2016).

Additional gaps in knowledge exist when considering the relationship between egg laying and female P:C requirements. Post-mating female diet choice in *D. melanogaster* is thought to be

closely interwoven with the production of resource-costly, protein-rich eggs (Mirth *et al.*, 2019). However, previous studies may have underestimated the protein debt of mated *D. melanogaster* females due to ecologically unrealistic egg laying scenarios in the lab conditions tested. Many studies have used the CAFE assay, in which fruit flies are held on a non-food substrate such as agar-water, to prevent desiccation. Female *Drosophila* normally lay their eggs into a food surface, with yeast volatile cues (Becher *et al.*, 2012; Gorter *et al.*, 2016) combined with a soft texture (Zhang *et al.*, 2020) to mimic rotting fruit. Without these cues, female flies within CAFE assays tend to reduce their egg laying output considerably versus females on standard food media, potentially reducing the nutrient load of egg production.

Egg laying rates are also closely linked to female condition. Female *D. melanogaster* raised on low quality or low yeast diets developed fewer ovarioles (Rodrigues *et al.*, 2015; Mirth *et al.*, 2019; Klepsatel *et al.*, 2020; Bath *et al.*, 2023) and displayed lower reproduction (Duxbury & Chapman, 2020; Klepsatel *et al.*, 2020), though not all studies found this effect (May *et al.*, 2015). Therefore, low quality females producing fewer eggs may require a lower P:C diet, but this has not been fully explored.

In Chapter 3, I tested the interactions between ecologically relevant levels of condition, egg laying and mating on the trajectory of diet preference in *D. melanogaster* females. Female *D. melanogaster* raised as larvae on diets of low or high quality were assigned to remain unmated or mate once. Halfway through the assay, one cohort of females was remated to investigate the role of a second mating on the female diet switch. Dietary intake was then tracked using the CAFE assay (Ja *et al.*, 2007), to measure consumption of chemically defined protein or carbohydrate over a two-week period. In between each CAFE measurement, females were placed onto standard food media to provide a suitable egg laying substrate and egg production was tracked alongside P:C consumption.

Chapter 4 – Female feeding and oviposition site preference is influenced by male social cues

Sexual harassment exists in species with a mismatch between mating rate optima between the sexes, where males may attempt to court and coerce resistant females into mating. Females may be reluctant to mate in an effort to avoid costly interactions such as physical damage (Stutt & Siva-Jothy, 2001) and reduced longevity (Fowler & Partridge, 1989). In contrast, male reproduction is maximised by mating with as many females as possible. Previous studies investigating female feeding in the presence of males have found disturbance of nutrient acquisition under sexual harassment. For example, females experience reduced foraging time while dodging males (Rowe, 1994; Griffiths, 1996; Magurran & Seghers, 1997; Pilastro *et al*., 2003; Teseo *et al*., 2016).

However, it is unknown whether females dynamically offset their P:C preference (spatially and temporally) in order to avoid feeding from food sources already occupied by males. Previous work in the solitary bee *Anthophora plumipes* found that females avoided feeding from high quality flowers at times of day when males were likely to be present, instead feeding from more sheltered, less nutritious flowers further within the food plant (Stone, 1995). Therefore, females may balance the cost-benefits of feeding from quality diets against male interactions.

In Chapter 4, I tested whether female fruit flies experiencing differing levels of harassment would dynamically alter their P:C preference in the presence of males. Groups of females were placed into arenas with one of two diet patches, representing a non-preferred (low P:C) versus preferred (high P:C) diet (Jang & Lee, 2018). In the first treatment, females remained in single-sex groups. In the second treatment, males were added to the centre of the arena and able to freely interact with females. To disentangle physical displacement as a result of courtship, from female avoidance of males in the 3rd and 4th treatments, males were placed into small

enclosures on either the preferred or non-preferred diet patches. Foraging site selection of females, and instances of courtship were then measured during behavioural observations.

Chapter 5 – The impact of long-term dietary evolution on nutrient sensing and life history phenotypes

Sex differences in dietary optima between the sexes have recently been underpinned by gene expression patterns in the insulin/insulin like growth factor (IIS) and target of rapamycin (TOR) nutrient sensing pathways in adult *D. melanogaster* (Bennett-Keki *et al*., 2023), including sex-biased expression in genes effecting lifespan.

Despite short-term plasticity in response to macronutrient content, little is known about how males and females evolve in response to local variation in food resources. To our knowledge, it is unknown whether males and females differentially adapt to long-term diets over multiple generations, such that each sex's performance on an initially sub-optimal diet improves over generations.

In Chapter 5, I measured gene expression patterns and life history phenotypes of populations of *D. melanogaster*, which had been evolving on diets of 1:2 P:C (high protein) or 1:8 P:C (low protein) over 3 years. Diets were made using casein as the protein source and sucrose as the carbohydrate source. The ratios of female-preferred (high P:C, 1:2) versus male-preferred (low P:C, 1:8) diets were chosen to assess differential evolution of the sexes on sex-specific beneficial or adverse diets. I measured the relative expression of *dilp2*, *dfoxo* and *dTOR* genes, involved in lifespan extension within the IIS/TOR nutrient signalling pathways, using quantitative reverse transcription PCR. Lifespan, offspring production and activity levels of the 1:2 and 1:8 populations were measured and compared against wildtype flies following rearing on a common garden substrate.

References

Ames, J.M. (1992) The Maillard Reaction. In *Biochemistry of Food Proteins* (ed. by Hudson, B.J.F.). Springer US, Boston, MA, pp. 99–153.

Arnqvist, G. & Rowe, L. (2005) *Sexual Conflict*.

Barbieri, M., Bonafè, M., Franceschi, C. & Paolisso, G. (2003) Insulin/IGF-I-signaling pathway: an evolutionarily conserved mechanism of longevity from yeast to humans. *American Journal of Physiology-Endocrinology and Metabolism*, **285**, E1064–E1071.

Barnes, A.I., Wigby, S., Boone, J.M., Partridge, L. & Chapman, T. (2008) Feeding, fecundity and lifespan in female *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences*, **275**, 1675–1683.

Bateman, A.J. (1948) Intra-sexual selection in *Drosophila*. *Heredity*, **2**, 349–368.

Bath, E., Rostant, W., Ostridge, H.J., Smith, S., Mason, J.S., Rafaluk-Mohr, T., *et al.* (2023) Sexual selection and the evolution of condition-dependence: an experimental test at two resource levels. *Evolution*, **77**, 776–788.

Becher, P.G., Flick, G., Rozpędowska, E., Schmidt, A., Hagman, A., Lebreton, S., *et al.* (2012) Yeast, not fruit volatiles mediate *Drosophila melanogaster* attraction, oviposition and development. *Functional Ecology*, **26**, 822–828.

Bennett-Keki, S., Fowler, E.K., Folkes, L., Moxon, S. & Chapman, T. (2023) Sex-biased gene expression in nutrient-sensing pathways. *Proceedings of the Royal Society B: Biological Sciences*, **290**, 20222086.

Blouet, C., Mariotti, F., Azzout-Marniche, D., Bos, C., Mathé, V., Tomé, D., *et al.* (2006) The reduced energy intake of rats fed a high-protein low-carbohydrate diet explains the lower fat deposition, but macronutrient substitution accounts for the improved glycemic control. *The Journal of Nutrition*, **136**, 1849–1854.

Bowman, E. & Tatar, M. (2016) Reproduction regulates *Drosophila* nutrient intake through independent effects of egg production and sex peptide: Implications for aging. *Nutrition and Healthy Aging*, **4**, 55–61.

Bunning, H., Rapkin, J., Belcher, L., Archer, C.R., Jensen, K. & Hunt, J. (2015) Protein and carbohydrate intake influence sperm number and fertility in male cockroaches, but not sperm viability. *Proceedings of the Royal Society B: Biological Sciences*, **282**, 20142144.

Camus, M.F., Huang, C.-C., Reuter, M. & Fowler, K. (2018) Dietary choices are influenced by genotype, mating status, and sex in *Drosophila melanogaster*. *Ecology and Evolution*, **8**, 5385–5393.

Carey, M.R., Archer, C.R., Rapkin, J., Castledine, M., Jensen, K., House, C.M., *et al.* (2022) Mapping sex differences in the effects of protein and carbohydrates on lifespan and reproduction in *Drosophila melanogaster*: is measuring nutrient intake essential? *Biogerontology*, **23**, 129–144.

Carvalho, G.B., Kapahi, P., Anderson, D.J. & Benzer, S. (2006) Allocrine modulation of feeding behavior by the Sex Peptide of *Drosophila*. *Current Biology*, **16**, 692–696.

C.G. (Kees) de Kruif, Skelte G. Anema, Changjun Zhu, & Palatasa Havea. (2015) Water holding capacity and swelling of casein hydrogels. *Food Hydrocolloids*, **44**, 372–379.

Chapman, T., Bangham, J., Vinti, G., Seifried, B., Lung, O., Wolfner, M.F., *et al.* (2003) The sex peptide of *Drosophila melanogaster*: Female post-mating responses analyzed by using RNA interference. *Proceedings of the National Academy of Sciences*, **100**, 9923–9928.

Cordain, L., Eaton, S.B., Sebastian, A., Mann, N., Lindeberg, S., Watkins, B.A., *et al.* (2005) Origins and evolution of the Western diet: health implications for the 21st century1,2. *The American Journal of Clinical Nutrition*, **81**, 341–354.

Cordes, N., Albrecht, F., Engqvist, L., Schmoll, T., Baier, M., Müller, C., *et al.* (2015) Larval food composition affects courtship song and sperm expenditure in a lekking moth. *Ecological Entomology*, **40**, 34–41.

Deshpande, S.A., Carvalho, G.B., Amador, A., Phillips, A.M., Hoxha, S., Lizotte, K.J., *et al.* (2014) Quantifying *Drosophila* food intake: comparative analysis of current methodology. *Nature Methods*, **11**, 535–540.

Dewsbury, D.A. (1982) Ejaculate Cost and Male Choice. *The American Naturalist*, **119**, 601–610.

Douglas, T., Anderson, R. & Saltz, J.B. (2020) Limits to male reproductive potential across mating bouts in *Drosophila melanogaster*. *Animal Behaviour*, **160**, 25–33.

Droney, D.C. (2002) The influence of the nutritional content of the adult male diet on testis mass, body condition and courtship vigour in a Hawaiian *Drosophila*. *Functional Ecology*, **12**, 920–928.

Dussutour, A. & Simpson, S.J. (2012) Ant workers die young and colonies collapse when fed a high-protein diet. *Proceedings of the Royal Society B: Biological Sciences*, **279**, 2402–2408.

Duxbury, E.M.L. & Chapman, T. (2020) Sex-Specific Responses of Life Span and Fitness to Variation in Developmental Versus Adult Diets in *Drosophila melanogaster*. *The Journals of Gerontology: Series A*, **75**, 1431–1438.

Fanson, B.G. & Taylor, P.W. (2012) Protein:carbohydrate ratios explain life span patterns found in Queensland fruit fly on diets varying in yeast:sugar ratios. *AGE*, **34**, 1361–1368.

Fanson, B.G., Weldon, C.W., Pérez-Staples, D., Simpson, S.J. & Taylor, P.W. (2009) Nutrients, not caloric restriction, extend lifespan in Queensland fruit flies (*Bactrocera tryoni*). *Aging Cell*, **8**, 514–523.

Fontana, L., Weiss, E.P., Villareal, D.T., Klein, S. & Holloszy, J.O. (2008) Long-term effects of calorie or protein restriction on serum IGF-1 and IGFBP-3 concentration in humans. *Aging cell*, **7**, 681–687.

Fowler, K. & Partridge, L. (1989) A cost of mating in female fruitflies. *Nature*, **338**, 760–761.

Gorter, J.A., Jagadeesh, S., Gahr, C., Boonekamp, J.J., Levine, J.D. & Billeter, J.-C. (2016) The nutritional and hedonic value of food modulate sexual receptivity in *Drosophila melanogaster* females. *Scientific Reports*, **6**, 19441.

Greer, E.L. & Brunet, A. (2009) Different dietary restriction regimens extend lifespan by both independent and overlapping genetic pathways in C. elegans. *Aging Cell*, **8**, 113–127.

Griffiths, S.W. (1996) Sex differences in the trade-off between feeding and mating in the guppy. *Journal of Fish Biology*, **48**, 891–898.

Günzel, Y., Oberhauser, F.B. & Couzin-Fuchs, E. (2023) Information integration for decision-making in desert locusts. *iScience*, **26**.

Hihara, F. (1981) Effects of the Male Accessory Gland Secretion on Oviposition and Remating in Females of *Drosophila melanogaster*. *動物学雑誌 Zoological Magazine*, **90**, 307–316.

Hopkins, B.R. & Perry, J.C. (2022) The evolution of sex peptide: sexual conflict, cooperation, and coevolution. *Biological Reviews*, **97**, 1426–1448.

Hunt, J., Brooks, R., Jennions, M.D., Smith, M.J., Bentsen, C.L. & Bussière, L.F. (2004) High-quality male field crickets invest heavily in sexual display but die young. *Nature*, **432**, 1024–1027.

Ja, W.W., Carvalho, G.B., Mak, E.M., Rosa, N.N. de la, Fang, A.Y., Liong, J.C., *et al.* (2007) Prandiology of *Drosophila* and the CAFE assay. *Proceedings of the National Academy of Sciences*, **104**, 8253–8256.

Jang, T. & Lee, K.P. (2018) Comparing the impacts of macronutrients on life-history traits in larval and adult *Drosophila melanogaster*: the use of nutritional geometry and chemically defined diets. *Journal of Experimental Biology*, **221**, jeb181115.

Jensen, K., McClure, C., Priest, N.K. & Hunt, J. (2015) Sex-specific effects of protein and carbohydrate intake on reproduction but not lifespan in *Drosophila melanogaster*. *Aging Cell*, **14**, 605–615.

Jensen, K. & Silverman, J. (2018) Frequently mated males have higher protein preference in German cockroaches. *Behavioral Ecology*, **29**, 1453–1461.

Kant, R., Trewick, S. a., Sandanayaka, W. r. m., Godfrey, A. j. r. & Minor, M. a. (2012) Effects of multiple matings on reproductive fitness of male and female *Diaeretiella rapae*. *Entomologia Experimentalis et Applicata*, **145**, 215–221.

Kapahi, P., Kaeberlein, M. & Hansen, M. (2017) Dietary restriction and lifespan: Lessons from invertebrate models. *Ageing Research Reviews*, Nutritional interventions modulating aging and age-associated diseases, **39**, 3–14.

Klepsatel, P., Knoblochová, D., Girish, T.N., Dircksen, H. & Gáliková, M. (2020) The influence of developmental diet on reproduction and metabolism in *Drosophila*. *BMC Evolutionary Biology*, **20**, 93.

Lee, K.P., Kim, J.-S. & Min, K.-J. (2013) Sexual dimorphism in nutrient intake and life span is mediated by mating in *Drosophila melanogaster*. *Animal Behaviour*, **86**, 987–992.

Lee, K.P., Simpson, S.J., Clissold, F.J., Brooks, R., Ballard, J.W.O., Taylor, P.W., *et al.* (2008) Lifespan and reproduction in *Drosophila*: New insights from nutritional geometry. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 2498–2503.

Lefevre, G. & Jonsson, U.B. (1962) Sperm Transfer, Storage, Displacement, and Utilization in *Drosophila melanogaster*. *Genetics*, **47**, 1719–1736.

Lewis, S.M. (2004) Multiple mating and repeated copulations: effects on male reproductive success in red flour beetles. *Animal Behaviour*, **67**, 799–804.

Lihoreau, M., Buhl, J., Charleston, M.A., Sword, G.A., Raubenheimer, D. & Simpson, S.J. (2015) Nutritional ecology beyond the individual: a conceptual framework for integrating nutrition and social interactions. *Ecology Letters*, **18**, 273–286.

Lihoreau, M., Clarke, I.M., Buhl, J., Sumpter, D.J.T. & Simpson, S.J. (2016a) Collective selection of food patches in *Drosophila*. *Journal of Experimental Biology*, **219**, 668–675.

Lihoreau, M., Deneubourg, J.-L. & Rivault, C. (2010) Collective foraging decision in a gregarious insect. *Behavioral Ecology and Sociobiology*, **64**, 1577–1587.

Lihoreau, M., Gómez-Moracho, T., Pasquaretta, C., Costa, J.T. & Buhl, C. (2018) Social nutrition: an emerging field in insect science. *Current Opinion in Insect Science*, Vectors and medical and veterinary entomology * Social insects, **28**, 73–80.

Lihoreau, M., Poissonnier, L.-A., Isabel, G. & Dussutour, A. (2016b) *Drosophila* females trade off good nutrition with high-quality oviposition sites when choosing foods. *Journal of Experimental Biology*, **219**, 2514–2524.

Linklater, J.R., Wertheim, B., Wigby, S. & Chapman, T. (2007) Ejaculate depletion patterns evolve in response to experimental manipulation of sex ratio in *Drosophila melanogaster*. *Evolution*, **61**, 2027–2034.

Lomborg, J.P. & Toft, S. (2009) Nutritional enrichment increases courtship intensity and improves mating success in male spiders. *Behavioral Ecology*, **20**, 700–708.

Longo, V.D., Shadel, G.S., Kaeberlein, M. & Kennedy, B. (2012) Replicative and Chronological Aging in *Saccharomyces cerevisiae*. *Cell Metabolism*, **16**, 18–31.

Macartney, E.L., Crean, A.J., Nakagawa, S. & Bonduriansky, R. (2019) Effects of nutrient limitation on sperm and seminal fluid: a systematic review and meta-analysis. *Biological Reviews*, **94**, 1722–1739.

Magurran, A.E. & Seghers, B.H. (1997) A cost of sexual harassment in the guppy, *Poecilia reticulata*. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **258**, 89–92.

Mair, W., Goymer, P., Pletcher, S.D. & Partridge, L. (2003) Demography of Dietary Restriction and Death in *Drosophila*. *Science*, **301**, 1731–1733.

Maklakov, A.A., Simpson, S.J., Zajitschek, F., Hall, M.D., Dessmann, J., Clissold, F., *et al.* (2008) Sex-Specific Fitness Effects of Nutrient Intake on Reproduction and Lifespan. *Current Biology*, **18**, 1062–1066.

May, C.M., Doroszuk, A. & Zwaan, B.J. (2015) The effect of developmental nutrition on life span and fecundity depends on the adult reproductive environment in *Drosophila melanogaster*. *Ecology and Evolution*, **5**, 1156–1168.

Meats, A. & Leighton, S.M. (2004) Protein consumption by mated, unmated, sterile and fertile adults of the Queensland fruit fly, *Bactrocera tryoni* and its relation to egg production. *Physiological Entomology*, **29**, 176–182.

Mirth, C.K., Nogueira Alves, A. & Piper, M.D. (2019) Turning food into eggs: insights from nutritional biology and developmental physiology of *Drosophila melanogaster*. *Current Opinion in Insect Science*, Insect genomics: Development and regulation, **31**, 49–57.

Mori, B.A., Whitener, A.B., Leinweber, Y., Revadi, S., Beers, E.H., Witzgall, P., *et al.* (2017) Enhanced yeast feeding following mating facilitates control of the invasive fruit pest *Drosophila suzukii*. *Journal of Applied Ecology*, **54**, 170–177.

Morimoto, J. & Wigby, S. (2016) Differential effects of male nutrient balance on pre- and post-copulatory traits, and consequences for female reproduction in *Drosophila melanogaster*. *Scientific Reports*, **6**, 27673.

Ng, S.H., Simpson, S.J. & Simmons, L.W. (2018) Macronutrients and micronutrients drive trade-offs between male pre- and postmating sexual traits. *Functional Ecology*, **32**, 2380–2394.

Ng, S.H., Simpson, S.J. & Simmons, L.W. (2019) Sex differences in nutrient intake can reduce the potential for sexual conflict over fitness maximization by female and male crickets. *Journal of Evolutionary Biology*, **32**, 1106–1116.

Olsson, M., Madsen, T. & Shine, R. (1997) Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **264**, 455–459.

Parker, G. a. (2006) Sexual conflict over mating and fertilization: an overview. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **361**, 235–259.

Pérez-Staples, D. & Abraham, S. (2023) Postcopulatory Behavior of Tephritid Flies. *Annual Review of Entomology*, **68**, 89–108.

Perry, J.C. (2011) Mating stimulates female feeding: testing the implications for the evolution of nuptial gifts. *Journal of Evolutionary Biology*, **24**, 1727–1736.

Perry, J.C., Sirot, L. & Wigby, S. (2013) The seminal symphony: how to compose an ejaculate. *Trends in Ecology & Evolution*, **28**, 414–422.

Perry, J.C. & Tse, C.T. (2013) Extreme Costs of Mating for Male Two-Spot Ladybird Beetles. *PLOS ONE*, **8**, e81934.

Pilastro, A., Benetton, S. & Bisazza, A. (2003) Female aggregation and male competition reduce costs of sexual harassment in the mosquitofish *Gambusia holbrooki*. *Animal Behaviour*, **65**, 1161–1167.

Piper, M.D.W., Blanc, E., Leitão-Gonçalves, R., Yang, M., He, X., Linford, N.J., *et al.* (2014) A holidic medium for *Drosophila melanogaster*. *Nature Methods*, **11**, 100–105.

Raubenheimer, D. & Simpson, S.J. (1993) The geometry of compensatory feeding in the locust. *Animal Behaviour*, **45**, 953–964.

Reinhardt, K., Naylor, R. & Siva-Jothy, M.T. (2011) Male Mating Rate Is Constrained by Seminal Fluid Availability in Bedbugs, *Cimex lectularius*. *PLOS ONE*, **6**, e22082.

Ribeiro, C. & Dickson, B.J. (2010) Sex Peptide Receptor and Neuronal TOR/S6K Signaling Modulate Nutrient Balancing in *Drosophila*. *Current Biology*, **20**, 1000–1005.

Rodrigues, M.A., Martins, N.E., Balancé, L.F., Broom, L.N., Dias, A.J.S., Fernandes, A.S.D., *et al.* (2015) *Drosophila melanogaster* larvae make nutritional choices that minimize developmental time. *Journal of Insect Physiology*, **81**, 69–80.

Rowe, L. (1994) The costs of mating and mate choice in water striders. *Animal Behaviour*, **48**, 1049–1056.

Schwarz, S., Durisko, Z. & Dukas, R. (2014) Food selection in larval fruit flies: dynamics and effects on larval development. *Naturwissenschaften*, **101**, 61–68.

Simmons, L.W., Ng, S.H. & Lovegrove, M. (2022) Condition-dependent seminal fluid gene expression and intergenerational paternal effects on ejaculate quality. *Functional Ecology*, **36**, 798–811.

Simpson, S.J. & Raubenheimer, D. (2009) Macronutrient balance and lifespan. *Aging (Albany NY)*, **1**, 875–880.

Simpson, S.J. & Raubenheimer, D. (2012) The Nature of Nutrition: A Unifying Framework from Animal Adaptation to Human Obesity. Princeton University Press.

Solon-Biet, S.M., McMahon, A.C., Ballard, J.W.O., Ruohonen, K., Wu, L.E., Cogger, V.C., *et al.* (2014) The ratio of macronutrients, not caloric intake, dictates cardiometabolic health, aging, and longevity in ad libitum-fed mice. *Cell Metabolism*, **19**, 418–430.

Stone, G.N. (1995) Female foraging responses to sexual harassment in the solitary bee *Anthophora plumipes*. *Animal Behaviour*, **50**, 405–412.

Stutt, A.D. & Siva-Jothy, M.T. (2001) Traumatic insemination and sexual conflict in the bed bug *Cimex lectularius*. *Proceedings of the National Academy of Sciences*, **98**, 5683–5687.

Teseo, S., Veerus, L., Moreno, C. & Mery, F. (2016) Sexual harassment induces a temporary fitness cost but does not constrain the acquisition of environmental information in fruit flies. *Biology Letters*, **12**, 20150917.

Thompson, E.D. & Reeder, B.A. (1987) Method for selecting exposure levels for the Drosophila sex-linked recessive lethal assay. *Environmental and Molecular Mutagenesis*, **10**, 357–365.

Trivers, R.L. (1972) Parental Investment and Sexual Selection. In *Sexual Selection and the Descent of Man: The Darwinian Pivot* (ed. by Campbell, B.). Taylor & Francis Group, Somerset, United States, p. 137.

Wilgers, D.J. & Hebets, E.A. (2011) Complex courtship displays facilitate male reproductive success and plasticity in signaling across variable environments. *Current Zoology*, **57**, 175–186.

Wilson, P.N., Bowman, J.C., Bell, G.D.H., Moss, C.J. & Williams, O.G. (1997) Livestock physiology and nutrition. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, **267**, 101–112.

Wong, R., Piper, M.D.W., Wertheim, B. & Partridge, L. (2009) Quantification of Food Intake in *Drosophila*. *PLOS ONE*, **4**, e6063.

Zhang, L., Yu, J., Guo, X., Wei, J., Liu, T. & Zhang, W. (2020) Parallel Mechanosensory Pathways Direct Oviposition Decision-Making in *Drosophila*. *Current Biology*, **30**, 3075-3088.e4.

Chapter 2

Diet choice is insensitive to mating in male fruit flies

Abstract

Animals can adjust their consumption of different nutrients to adaptively match their current or expected physiological state. Changes in diet preference can arise from social and sexual experience. For example, in female *Drosophila melanogaster* fruit flies, a single mating triggers a behavioural switch in diet choice towards increased protein intake and total food consumption, which supports offspring production. In contrast, male diet choice appears to be unaffected by a single mating. However, one mating may not fully capture the impact of mating on male feeding behaviour. Males can often mate multiply in natural settings, and the costs of ejaculate production and energetic courtship may be cumulative, such that males might experience increased nutritional demands only after multiple matings. In this study we tested this prediction by measuring the effect of multiple matings on the diet choice of male *D. melanogaster* fruit flies. Males were assigned to one of three mating treatments, unmated, mated once or mated five times consecutively, and then allowed to feed freely on chemically defined diets of protein and carbohydrate. In contrast to the prediction, we found that males that mated five times did not alter the amount of food, or the proportion of protein and carbohydrate consumed, when compared with unmated or once-mated males. This absence of a feeding response occurred despite substantial ejaculate depletion from multiple matings: males sired fewer offspring in each consecutive mating. These results reveal a lack of plasticity

in male feeding behaviour according to mating status, despite substantial potential physiological costs, and highlight the remarkably distinct nutritional ecologies of males versus females.

Introduction

Animals have complex nutritional needs, with optimal diets varying with age, sex, metabolic rate and environment (Simpson and Raubenheimer, 2012). Previous studies have demonstrated that, in many species, individuals are able to sense their own physiological state and adjust feeding to match nutritional demand, by fine-tuning consumption of micronutrients, such as vitamins and minerals, and macronutrients, including carbohydrate, protein (amino acids) and lipids (Ribeiro and Dickson, 2010; Simpson et al., 2015). The balance of macronutrients eaten strongly affects an individual's fitness, with the balance of protein and carbohydrate (P:C ratio) being particularly important across animals of many different taxa. The impacts of P:C consumption have been studied in both vertebrate systems (e.g. mice, *Mus musculus*, Solon-Biet et al., 2015; rainbow trout, *Oncorhynchus mykiss*, Suárez et al., 2002) and extensively in invertebrate systems (e.g. locusts, *Locusta migratoria*, Raubenheimer and Simpson, 1993; field crickets, *Teleogryllus commodus*, Reifer et al., 2018; German cockroaches, *Blattella germanica*, McPherson et al., 2021; tephritid fruit flies, such as the Queensland fruit fly, *Bactrocera tryoni*, Prabhu et al., 2008). Experiments carried out in *Drosophila melanogaster* fruit flies show that low P:C diets can maximize the longevity of both sexes, while optimal P:C diets for reproductive success differ between males and females (Lee et al., 2008; Jensen et al., 2015; Camus et al., 2017; Carey et al., 2022; but see Reddiex et al., 2013). When male and female insects are able to choose, they generally favour diets that maximize reproductive success; for males this is generally a lower P:C diet than for females (Lee et al., 2008; Maklakov et al., 2008; Fanson et al., 2009; but see Jensen et al., 2015).

Mating, and the initiation of reproductive processes that stem from it, have important implications for nutrient demands in females. Females of many species are observed to increase food intake after mating, e.g. in the two-spot ladybird, *Adalia bipunctata* (Perry, 2011), tephritid fruit flies (see Pérez-Staples and Abraham, 2023) and *D. melanogaster* (Camus et al.,

2018; Carvalho et al., 2006; Lee et al., 2013). Upon mating, female *D. melanogaster* also markedly increase the proportion of protein and yeast intake in their diet, in comparison with unmated females (Barnes et al., 2008; Camus et al., 2018; Corrales-Carvajal et al., 2016; Jensen et al., 2015; Lee et al., 2013; Newell et al., 2020; Ribeiro and Dickson, 2010). Macronutrient preference is similarly altered postmating in the two-spotted cricket, *Gryllus bimaculatus* (Tsukamoto et al., 2014) and during pregnancy in *Rattus norvegicus domestica* rats (Leshner et al., 1972; Richter and Barelare, 1938; Simpson and Raubenheimer, 1997). In *D. melanogaster*, the increase in females' preference for protein after mating is thought to occur to meet the demands of elevated egg production (Bownes and Blair, 1986; Drummond-Barbosa and Spradling, 2001; Lee, Kim and Min, 2013; but see Ribeiro and Dickson, 2010).

In contrast, data on the effect of mating on male dietary preference are scant. The few studies have been conducted in species that produce resource-costly nuptial gifts such as *B. germanica* (Jensen and Silverman, 2018) and *A. bipunctata* (Perry and Tse, 2013). In both species, males exhibit some dietary compensation after their first mating. However, there is little evidence for a shift in male dietary preference after a single, first mating in non-nuptial gift-giving species. For example, in *D. melanogaster*, a single mating had no significant effect on male diet preference (P:C ratio) or the overall quantity of food consumed (Camus et al., 2018). This result is in accord with the view that male ejaculates may be relatively 'cheap' to produce (Bateman, 1948; Trivers, 1972) and hence that males might not need to increase protein intake to replenish reserves depleted by mating.

It is possible that males incur cumulative costs of ejaculate production, such that costs that are minimal after a single mating increase significantly with multiple matings. In fact, males of many species are able to mate multiply in quick succession. For example, males of the red flour beetle, *Tribolium castaneum*, can mate with up to seven females in 15 min (Lewis, 2004) and *D.*

melanogaster males can mate up to 11 times in a day (Douglas et al., 2020). Moreover, contrary to the 'cheap' male ejaculate idea, it is now understood that the production of sperm and other ejaculate components can be costly and limiting for males (Dewsbury, 1982; Macartney et al., 2019; Olsson et al., 1997; Perry et al., 2013; Perry and Tse, 2013; Reinhardt et al., 2011; Simmons et al., 2022). For example, offspring production decreases with each consecutive mating by males of a parasitoid wasp, *Diaeretiella rapae* (Kant et al., 2012), *T. castaneum* (Lewis, 2004) and *D. melanogaster* (Douglas et al., 2020). Ejaculate components start to become depleted in male *D. melanogaster* after three sequential matings (Lefevre and Jonsson, 1962) and continue to decrease with additional matings (Hihara, 1981; Linklater et al., 2007; Loyau et al., 2012; Macartney et al., 2021). Males can be rendered infertile due to depletion of nonsperm ejaculate components (Hihara, 1981). Therefore, we predicted that a significant impact of mating on male diet preference would occur after multiple matings.

To test this prediction and improve understanding of how mating impacts male diet choice, we manipulated the mating rate of male *D. melanogaster* fruit flies and then measured male diet preferences and intake. We used *D. melanogaster* because protocols for measuring its dietary preferences are well established, it is an important model for nutritional preference studies and males can mate multiply. Separate treatment groups of male flies were assigned at random to remain unmated, to mate once, or to mate five times consecutively. The unmated and single mating treatments were included to investigate whether a feeding switch is triggered when males mate for the first time, as occurs in females. The five sequential mating treatment was chosen as this approaches the average maximal daily mating rate for this species (Douglas et al., 2020; Lefevre and Jonsson, 1962). We tracked the number of viable offspring produced from each mating to confirm that the highest sequential mating rate treatment did result in ejaculate depletion. Following the matings, male diet preference for all treatments was measured by offering male flies carbohydrate and protein solutions simultaneously using the

capillary feeder (CAFE) assay (Ja et al., 2007). The CAFE method allows the quantification of food intake and macronutrient preference using synthetic diets with known nutritional content. We predicted that males from the highest sequential mating rate treatments would (1) suffer energy and ejaculate depletion, evident as cumulative reductions in their siring success with each mating bout, (2) increase total food consumption to recoup energy expenditure during courtship and mating, and (3) increase the proportion of protein eaten after mating to restore proteinaceous sperm and nonsperm components of the ejaculate.

Methods

Fly stocks

Fly rearing and experiments were conducted in a 25 °C humidified room under a 12:12 h light:dark cycle. Experimental flies were collected from a large stock population of outbred wildtype Dahomey flies (Chapman, Trevitt and Partridge, 1994) maintained on a standard sugar – yeast–agar (SYA) diet (50 g sucrose, 100 g brewer's yeast, 15 g agar, 30 ml Nipagin (10% solution), 3 ml propionic acid, 970 ml water). To generate experimental flies, eggs from the stock population were collected on grape juice–agar plates with live yeast paste. First-instar larvae were transferred into glass vials containing SYA medium at a controlled density of 50 larvae per vial. Experimental adults were collected as virgins using ice anaesthesia within 4–6 h of eclosion. Flies were housed in single-sex groups of 15 males or 10 females in glass vials containing 7 ml SYA medium supplemented with live yeast granules for 3 days before mating assays.

Experiments were carried out in four experimental blocks. In blocks 1 and 2 an additional mating treatment of three sequential matings was included. Offspring data were collected

from blocks 1 and 2, dietary data were collected from blocks 3 and 4 and mating data were collected from all four blocks (Appendix, Figure A1).

Sequential mating treatments

Experimental males were randomly assigned to one of three mating treatments: zero matings, one mating or five matings in a single day (*N*=528, all blocks). Males were gently aspirated into individual mating vials containing 7 ml of 0.75% agar - water. This nutrient-lacking medium was used to remove any potentially confounding influence of a nutritional substrate on mating behaviour, while providing moisture. One virgin female was introduced to each vial for males assigned to the one and five mating treatments and latency to mate and mating duration were recorded by scan sampling across all the vials approximately every minute. Once mating was complete, the female was removed. For males assigned to the five matings treatment, this process was repeated until five consecutive matings were achieved. If no mating occurred within 60 min, the female was replaced with a new virgin female. Matings under 5 min were excluded (*N* = 9) as short matings may not allow complete transfer of sperm and seminal fluids (Gilchrist and Partridge, 2000; Manier et al., 2010). Matings over 40 min were also excluded (*N* = 4) because they appeared to represent cases in which males and females had become stuck during mating (Mason et al., 2016).

Males that were unable to complete their assigned number of sequential matings were dropped from the study (a total across all blocks of *N* = 56 males, all from the five matings treatment, as expected given the cumulative effects of serial matings within a day). To test whether the exclusion of these individuals from the five matings treatment might have introduced bias, we compared like-for-like mating characteristics across mating treatments. We first analysed the duration of the first mating between individuals that completed five matings and individuals that did not. Blocks 1, 2 and 3 showed no significant differences, while

in block 4 nonmaters (i.e. dropouts) had a somewhat longer first mating duration than maters retained in the final data set (P= 0.03). We next compared the duration of the first matings between the successful maters across all treatment groups. Although this showed some differences in first mating duration between males from the different mating treatments, it was not consistent across all blocks (blocks 1 and 2: both P> 0.05; block 3: P=0.02; block 4: P=0.03). Overall, neither analysis provided evidence consistent with a systematic bias. We did not find differences in nutrient intake between males that mated different numbers of times. Thus, any slight differences between the males eventually retained in the different mating treatments, even if they were present, would be expected to have a conservative effect with respect to the main result we obtained. The no mating and one mating treatment vials were set up and handled in the same way as the five matings treatment vials. After the mating assay and prior to the CAFE assays of diet intake, experimental males were all housed singly in agar–water mating vials overnight.

Diets and CAFE assay

We used the CAFE assay (Ja et al. 2007) to measure the consumption of protein and carbohydrate liquid synthetic diets (Camus et al., 2018, 2017; Piper et al., 2014). Recipes are included in Appendix, Tables A1 and A2. Liquid diets included identical volumes of synthetic components (lipids, vitamins and salts) to create a fully chemically defined diet. We added a supplement of 20% autoclaved yeast suspension to the protein diet following previous reports that *D. melanogaster* adults do not eat a pure protein solution (Camus et al., 2018, 2017). The resulting protein diet therefore contained 4% of carbohydrate due to the sugar content of the killed yeast suspension.

On the day following the mating assay, experimental males were transferred to fresh agar–water vials in groups of three, because preliminary experiments indicated that individual males

ingest too little protein to detect through the CAFE assay. Coded labelling was used to ensure that the experimental treatments were anonymized relative to the observer. Vials were each provided with two 5 µl microcapillary tubes (Ringcaps; Hirschmann Instruments, Baden-Württemberg, Germany), one containing the liquid protein diet and the other containing the liquid carbohydrate diet, held in place with a foam bung. Both microcapillary tubes were replaced every 24 h for 5 days and the loss of liquid diet was measured from each tube at the meniscus using a digital calliper. We calculated total food consumption as the length of the vector from the origin to the intake values of protein and carbohydrate, in a protein and carbohydrate nutrient space (Appendix, Figure A2; Camus et al., 2018). We calculated relative amounts of protein to carbohydrate ingested as the angle between this vector and the protein axis to give a value between 1 and 90°. Values of over 45° signify a greater proportion of carbohydrate consumed and values of less than 45° signify a greater proportion of protein consumed. During the experiment, vials were housed in a sealed 50-litre lidded box containing a saturated salt solution to create a high humidity environment (77% on average; Greenspan, 1977) to limit evaporation from the microcapillaries. Ten vials with protein- and carbohydrate-containing microcapillary tubes but without flies were interspersed amid the experimental vials for each day of the measuring period to track evaporative loss. Mean evaporation was calculated from these vials for both protein and carbohydrate and subtracted from the feeding measures for each day of the measurement period. Instances where evaporation was greater than the measured diet consumption were excluded (*N*=18) because calculations of dietary preference (angle, see Appendix, Figure A2) require positive intake values. Vials where one or more males escaped or died during the assay were also excluded.

Reproductive output

Females that mated with experimental males (*N*=387, blocks 1 and 2) were retained in individual SYA vials seeded with live yeast granules and allowed to lay eggs for approximately

4 weeks to allow us to profile offspring production over time from each mating. Females were moved to fresh vials every 3–5 days to ensure larvae would not be food limited. Vials were frozen 13 days after egg laying to allow all offspring to eclose. The number of adult offspring was counted.

Statistical analysis

Statistical analyses were carried out in R version 4.0.4 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org) and all statistical models can be found in Appendix, Table A3.

Dietary preference and mating duration

We excluded outliers for diet consumption identified by Z-score calculations (*N*=15 protein values, *N*=8 carbohydrate values). Outliers included tubes that had drained of liquid due to accidental contact with a substrate. To account for the effect of block on dietary and mating duration data, we first fitted generalized linear models with block assigned as a fixed factor for each of the response variables length of vector, angle and duration of mating. Residuals from these models were analysed as response variables in generalized linear mixed models (GLMM) using the R package glmmTMB (Brooks et al., 2017) in which treatment and day were included as fixed effects for analysing length of vector and angle, and mate number for mating duration. Individual male ID was included as a random effect. We also analysed differences between the treatment groups on day 1 only to untangle whether there was an effect of the mating treatment on feeding behaviour directly following the mating assay. Data from each block were additionally analysed separately for duration following tests for model fit. We also included angle and length of vector as response variables in a multivariate analysis of variance (MANOVA) to investigate the effects of treatment and day on the joint response of both variables (Pillai's trace). Angle and length of vector were centred around a mean of 0 for the

MANOVA. Throughout, model fit was checked using the R package DHARMa (Hartig, 2022). Post hoc pairwise comparisons were carried out on estimated marginal means using the R package emmeans (Lenth et al., 2023).

Reproductive output

To investigate male sperm depletion with multiple mating, we tested the effect of a female's mate number (i.e. whether the female was the first, second, third, fourth or fifth mate of their male partner) on her offspring output over time (e.g. per vial). For each mated female, we calculated the slope of her adult offspring production regressed against time (i.e. each of the seven sequential 24 h periods (vials)). Females without data for all seven timepoints were excluded (*N*=90, blocks 1 and 2), such as those that died or escaped during the data collection period (*N*=76), or that produced no offspring, to exclude reproductive failure events (*N*=14). To account for effects of block, we first fitted a generalized linear model with block as a fixed factor and with individual slopes as the response variable. Residuals from the initial model were entered as the response variable in a linear model against mate number (whether the female was the first, second, third, fourth or fifth mate of their male partner).

Latency to mate

Latency to mate was analysed as a function of female mate number using the R package survival (Therneau, 2023; Therneau and Grambsch, 2000) and visualized using a Kaplan–Meier curve. Instances where the female partner was replaced were treated as censored values.

Repeatability analysis

Repeatability of male mating behaviour was analysed for both mating duration and latency for males that mated multiply (three and five times mating treatments), by using the R package rptR (Stoffel et al., 2017). Mate number was included as a fixed effect in both models to

perform an analysis of enhanced agreement repeatability of male ID. Instances where the female partner was replaced were treated as censored values for repeatability of latency behaviour.

Results

Multiple matings do not alter food intake or P:C preference

The combined total of protein and carbohydrate diet eaten by males (i.e. length of vector) over the 5 days was not significantly different between the zero, one or five mating treatment groups (χ^2_2=2.12, *P*=0.35; Figure 1a). There was no significant interaction between treatment and day (χ^2_8=5.47, *P*=0.71) suggesting little change in consumption over the 5 days of the assay. It is possible that an effect of mating on food intake might be strongest immediately after mating. However, the quantity of food consumed on day 1 was not significantly different between mating treatments (χ^2_2=1.97, *P*=0.37). There was a significant effect of day on total food consumption (χ^2_4=11.89, *P*<0.05) but no consistent effect of day between blocks.

The P:C diet composition (angle) that males ate was not significantly different between males mated zero, one or five times over the 5 days (χ^2_2=3.18, *P*=0.20). There was no discernible effect of day (χ^2_4=4.83, *P*=0.30) and no significant interaction between treatment and day (χ^2_8=8.7, *P*=0.37; Figure 1b). Males showed a consistent preference for carbohydrate over protein: all recorded P:C angles were over 45°, indicating a skew to carbohydrate (Appendix, Figure A2). We found no evidence for a stronger effect of mating on diet composition preference immediately after mating (no effect of treatment on day 1 diet composition; χ^2_2=2.56, *P*=0.28). There was no treatment effect in the raw consumption data for either protein or carbohydrate (Appendix, Figure A3).

We tested whether treatment affected the joint feeding response of both total diet intake and diet composition using MANOVA. This showed no significant effect of treatment (Pillai test statistic df_2=0.0096, approximate $F_{4,1084}$=1.31, P=0.26) or interaction between treatment and day (Pillai test statistic df_8=0.0219, approximate $F_{16,1084}$=0.75, P=0.74) in the multivariate analysis. The joint feeding response varied between days (Pillai test statistic df_4=0.0302, approximate $F_{8,1084}$=2.07, P<0.05). These results support the finding that there was no change in the nutrient intake for males as a function of their mating frequency.

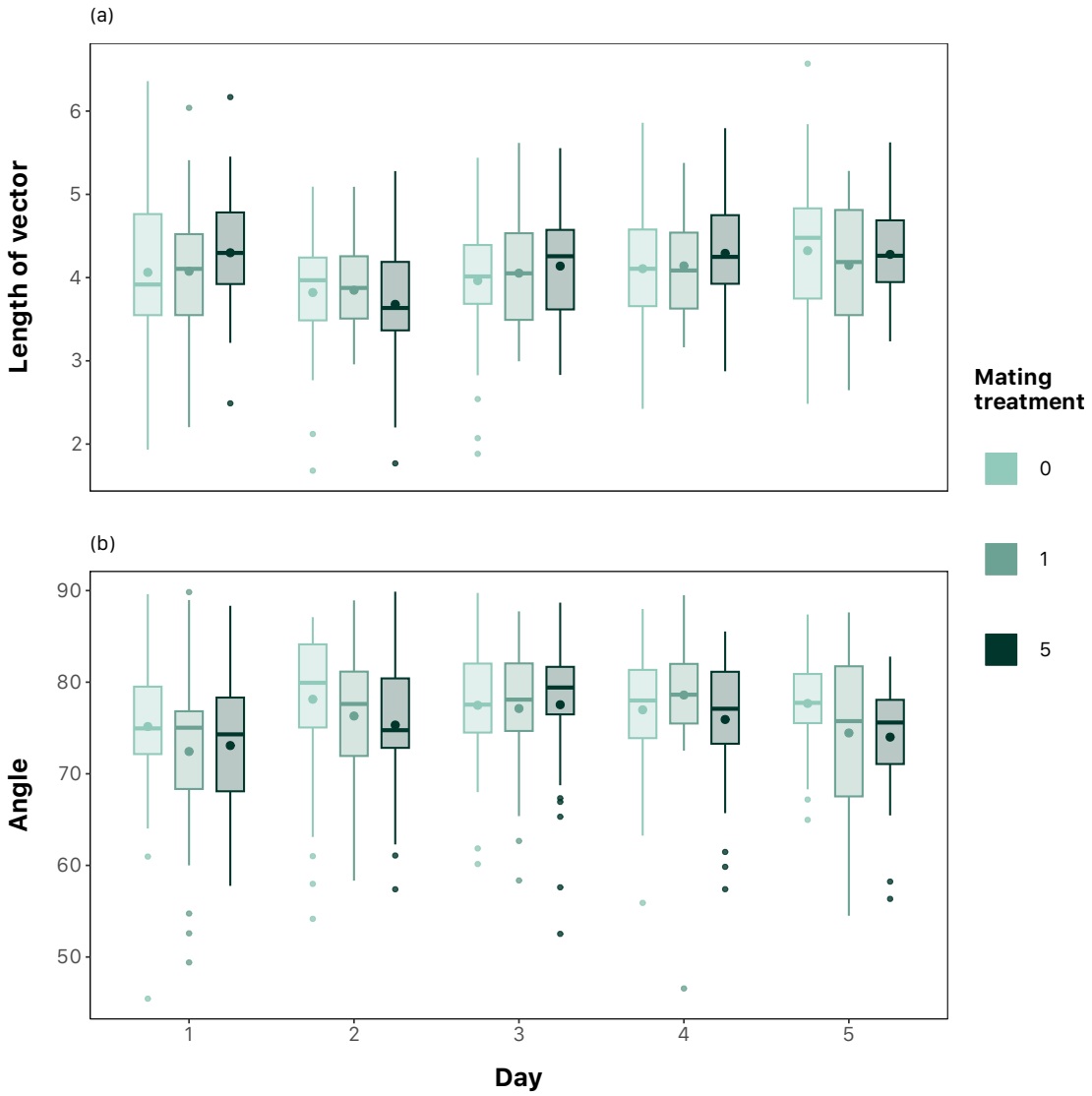


Figure 1

The (a) quantity and (b) composition of protein and carbohydrate eaten over 24 h periods for 5 days by experimental males that had mated zero, one or five times. (a) Combined consumption of protein and carbohydrate is represented by the length of the vector, calculated as the distance of an intake value from the origin in P:C nutrient space (see Appendix, Figure A2). (b) Relative composition of protein to carbohydrate eaten (μl), represented by the angle between the length of the vector and the protein axis to give a value between 0 and 90°. Angles over 45° indicate that more carbohydrate than protein was consumed, whereas angles under 45° indicate that more protein than carbohydrate was consumed. Boxes represent the interquartile range (IQR), with medians as thick horizontal lines and means as large, filled circles. Whiskers represent 1.5 x IQR, and small circles represent outliers.

Males become ejaculate limited after serial multiple matings

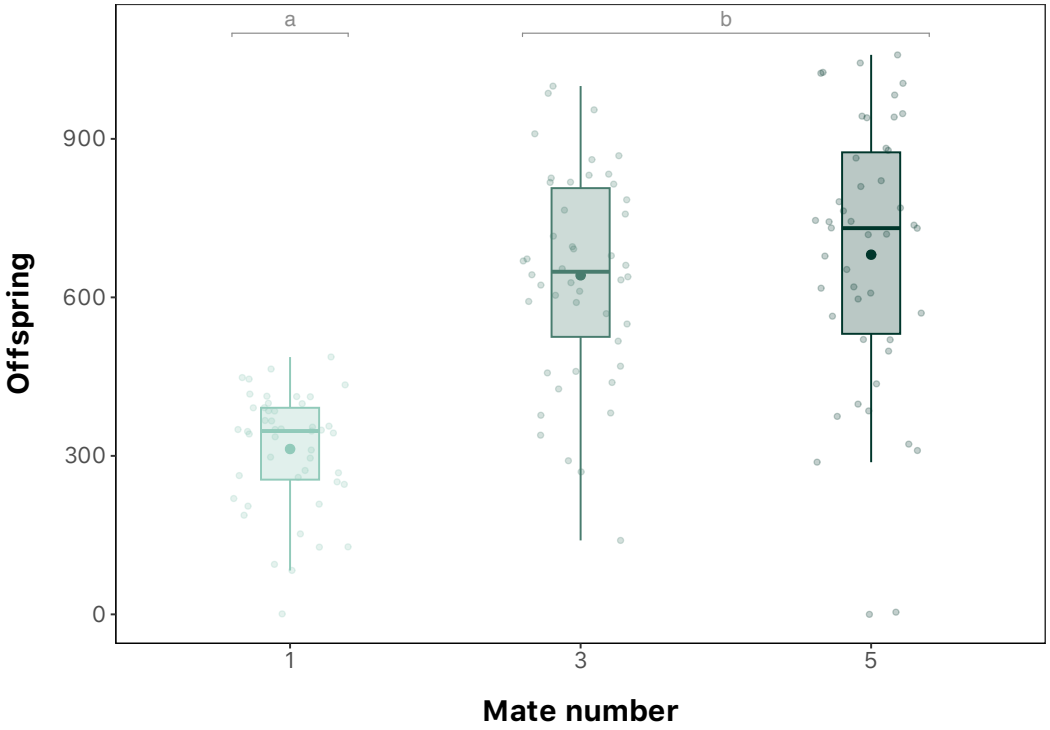


Figure 2

Total numbers of adult offspring sired by males when mated once, three or five times sequentially. Data shown are from experimental blocks 1 and 2 where an additional three sequential matings treatment was included. Boxes represent the interquartile range (IQR), with medians as thick horizontal lines and means as large, filled circles. Whiskers represent 1.5 x IQR, and small circles are raw data points overlaid on their respective box. Letters above boxes denote significant differences between treatment groups as determined by post hoc Tukey tests (P<0.001).

As expected, the total number of offspring sired by a male increased when males were mated more than once in series ($F_{2,136}$=49.44, P<0.001; Figure 2). Males that mated once produced 313 ±SE 16 (N = 47) adult offspring on average, while an average of 642 ±SE 29 (N = 46) and 681 ±SE 38 (N = 46) offspring were produced by those that serially mated three and five times, respectively. The number of offspring produced by a male was significantly higher in males that

mated serially three or five times compared to those that mated only once (post hoc Tukey tests: one versus three matings: t_{136}=-8.08, P<0.001; one versus five matings: t_{136}=-9.04, P<0.001). However, reproductive output did not differ between those mated three and five times (t_{136}=-0.96, P=0.61) despite an additional two matings with virgin females. This effect was evident in an analysis of the slope of decline in the numbers of offspring produced by each female (Figure 3). The slope of decline in female offspring production depended on a female's position in the mating sequence (χ^2_4=174.47, P<0.001). Post hoc Tukey tests showed that this slope of decline was significantly different when comparing across all mate numbers (P<0.01 - P<0.001), with the exception of the fourth versus fifth females to mate with a male (t_{290}=-1.3, P= 0.68). Mating latency and duration were not correlated with offspring production (Appendix, Figure A4).

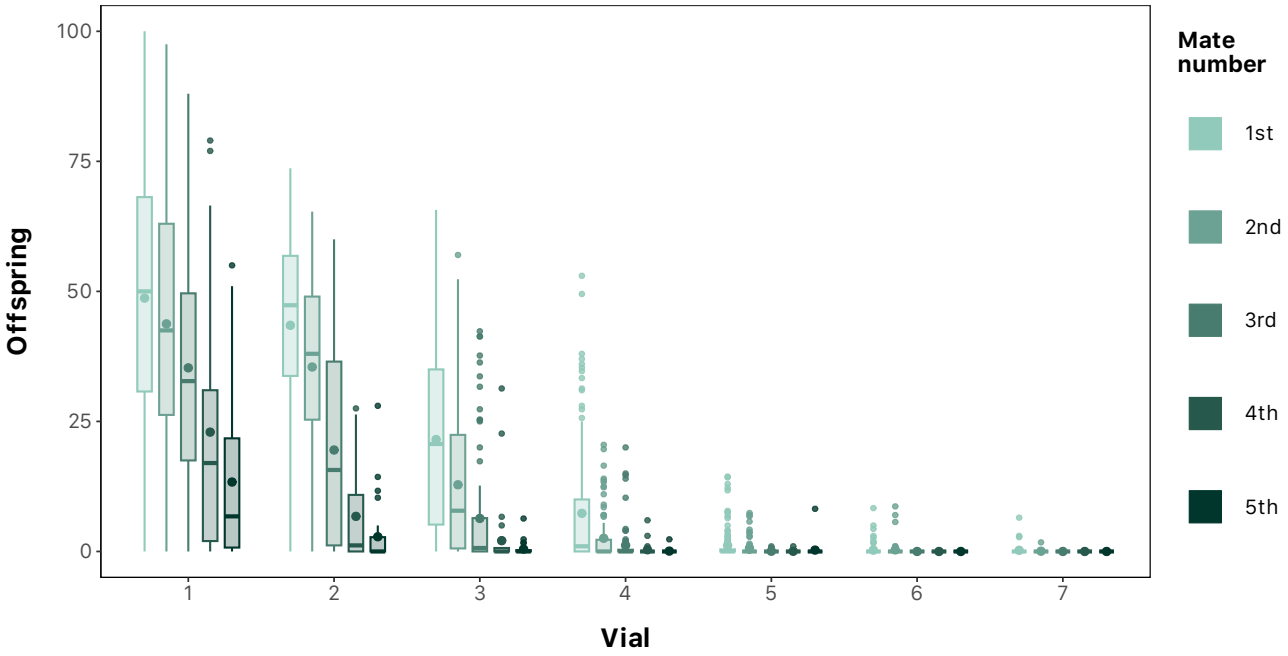


Figure 3

Total numbers of adult offspring produced over 24 h by individual females that were a male's first, second, third, fourth or fifth mate (*N*=387). All females were initially virgins. Offspring were counted from vials in which females had been allowed to lay eggs for 3–5 days for a total of 21 days. These periods were normalized to 24 h by dividing values by the number of days the female laid eggs in that vial. Shown in the figure is the total data set for all females (including those that did and did not have data for all seven vials; data analysis was conducted on the data set that included all seven vials only). Boxes represent the interquartile range (IQR), with medians as thick horizontal lines and means as large, filled circles. Whiskers represent 1.5 x IQR, and small circles are outliers. Data shown are from blocks 1 and 2 where a three times mated treatment was included.

Males took longer to mate if they had previously mated at least twice

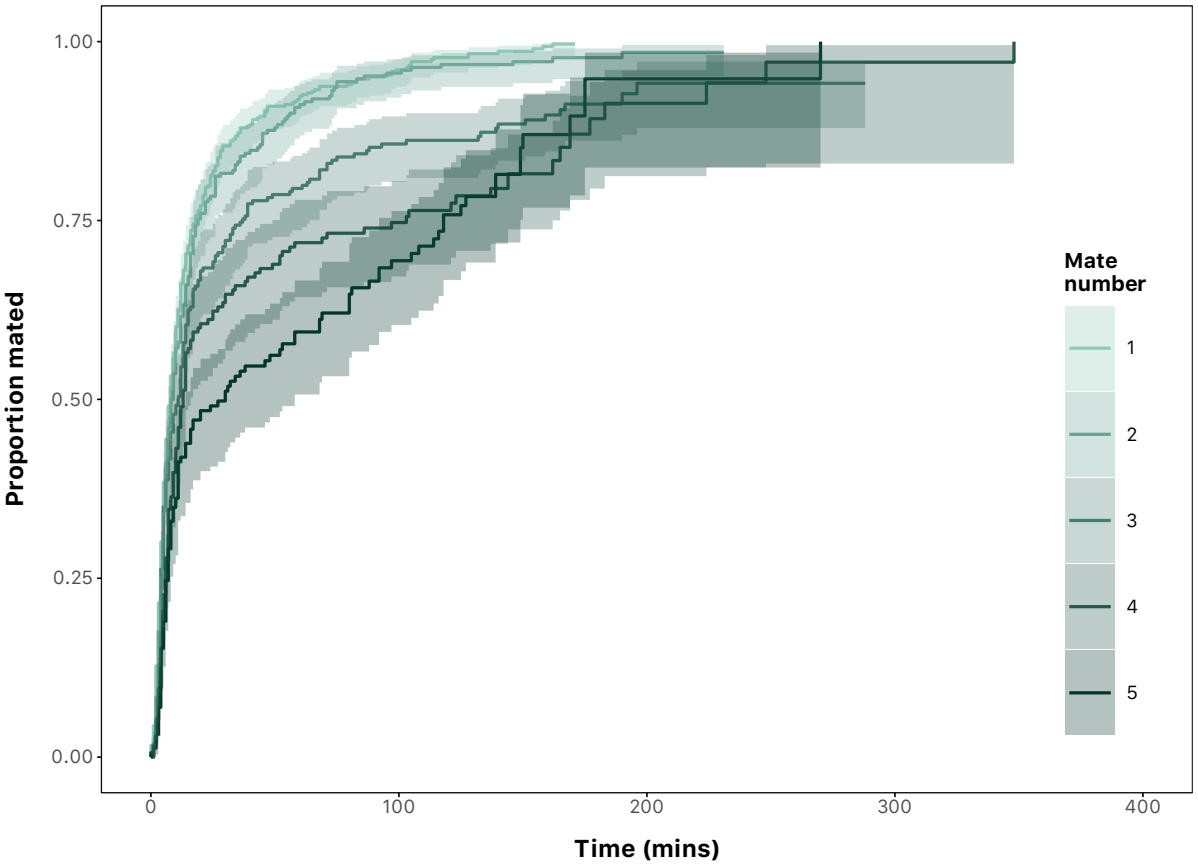


Figure 4

Kaplan–Meier survival curves illustrating the proportion of experimental males initiating their first, second, third, fourth or fifth matings. Solid lines (with surrounding confidence intervals in matching colourways) represent each of the one to five sequential mating treatments, in which males were presented with a new virgin female. Data shown are taken from all four blocks.

Latency to mate became significantly slower as males mated more times in sequence (χ^2_4=108.65, *P*<0.001; Figure 4). The median time to mate was 8.0, 8.5, 11.0, 13.0 and 30.0 min for the first, second, third, fourth and fifth matings, respectively. Latency between consecutive matings was not significantly different, with the exception of the second versus third mating (post hoc Tukey: *Z*=3.5, *P*<0.01), while latency times between non-consecutive matings were significantly different from one another (post hoc Tukey: *P*<0.001 - *P*<0.01). There was significant variation in latency between experimental blocks (χ^2_3=21.31, *P*<0.001). Males also

showed an increased reluctance to mate later in the mating sequence, as evident in the increased requirement to add additional virgin females for experimental matings to occur (Appendix, Figure A5). Latency to mate was not a repeatable individual behaviour (repeatability score: R=0.02, P=0.19).

Mating duration decreased after the first mating

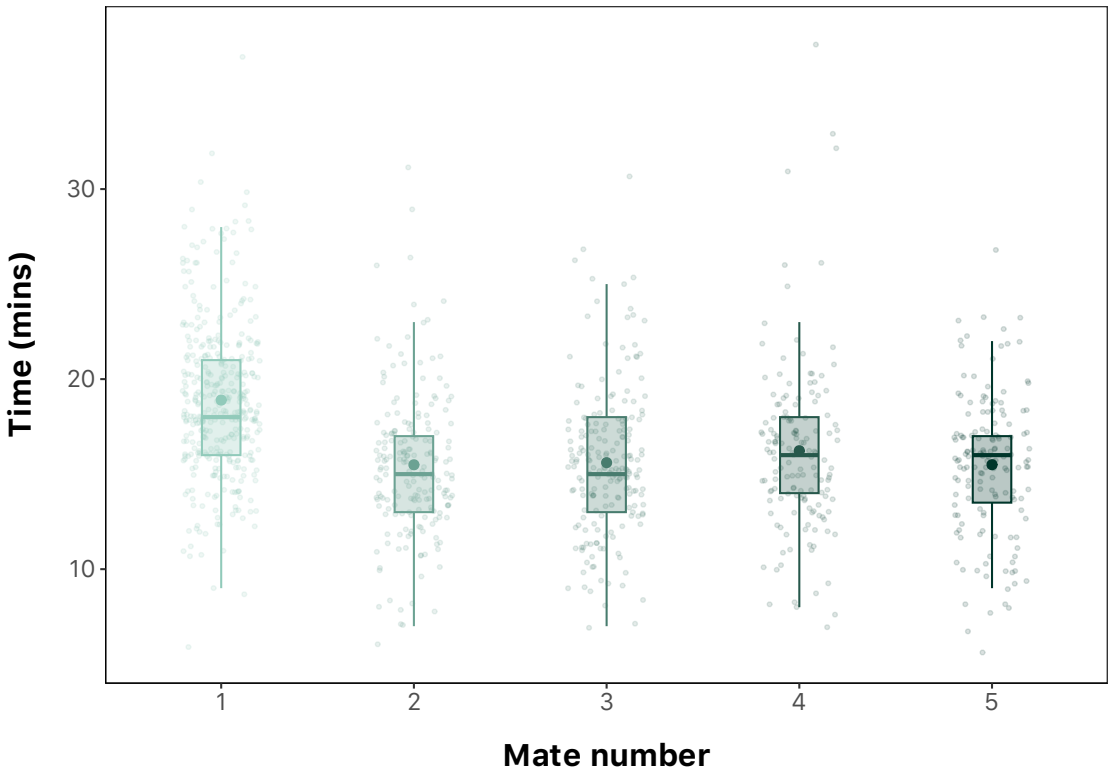


Figure 5

Mating duration of individual males when mating for the first, second, third, fourth or fifth time with a virgin female in a single day. Boxes represent the interquartile range (IQR), with medians as thick horizontal lines and means as large, filled circles. Whiskers represent 1.5 x IQR and jittered raw data are overlaid on each box. Data shown are taken from all blocks.

Mating duration was significantly altered by mate number (χ^2_4=190.39, P<0.001; statistical model run across data from all blocks; Figure 5). However, significant deviation from the

expected distribution of residuals was detected during model fit tests. Therefore, blocks were also analysed separately. These analyses showed that mate number had a significant effect on duration in all blocks (P<0.001 in all cases). Pairwise comparisons showed that first matings were significantly longer than all subsequent matings (post hoc Tukey tests: P<0.001 - <0.05) with the exception of the fourth mating in both block 1 (t_{168}=2.30, P=0.149) and block 2 (t_{178}=2.60, P=0.075). All matings after the first did not differ from each other. There was significant but low repeatability in mating duration across the multiple matings of an individual male, once accounting for the variation caused by mate number (repeatability score: R=0.152, P<0.001).

Discussion

Although it is often assumed that production of sperm incurs little cost to males, ejaculate production costs as a whole can be significant (Dewsbury, 1982). Nutritional demands due to sperm depletion may not be discernible after a single mating (Camus et al., 2018; Perry and Tse, 2013). However, they are predicted to be evident after multiple matings, which males of many species experience in natural settings. To our knowledge, this broad prediction had not previously been tested in species lacking nuptial gifts. We tested whether mating multiply in series would trigger male fruit flies to alter their dietary preference to replenish ejaculate components. We tested three predictions. Consistent with prediction 1, we found that males in the high mating rate treatment were more ejaculate depleted. However, predictions 2 and 3 were not supported: we did not detect evidence for a change in macronutrient preference, nor did males eat more, in the high mating rate treatment compared to the unmated and low mating rate treatments.

Male ejaculate depletion with consecutive matings

We found that males were severely depleted in their ability to transfer ejaculates with fertilization potential by their fifth mating. This was observed in the pattern of offspring production, with females that mated later in a male's mating sequence producing no, or markedly reduced, numbers of adult offspring compared with the first mate. Furthermore, the reduction in offspring produced showed a nonlinear mate number-dependent decline. This is in accord with results on reproductive success in multiply mated males observed previously in several insect species (Abraham et al., 2020; Douglas et al., 2020; Hihara, 1981; Kant et al., 2012; Linklater et al., 2007; Macartney et al., 2021; Savalli and Fox, 1999). This effect is likely to result chiefly from seminal fluid rather than sperm depletion because it has been observed that males retain some sperm in their seminal vesicles, but lack fluid in the accessory gland, after four to five matings (Lefevre and Jonsson, 1962; Gillott, 2003; Macartney et al., 2021; see also Reinhardt, Naylor and Siva-Jothy, 2011). This effect occurs even though *D. melanogaster* males are reported to differentially allocate seminal fluid proteins (Wigby et al., 2009) suggesting that strategic allocation of ejaculates across many matings has some limits. In the common bedbug, Cimex lectularius, reserves of seminal fluid are also reported to decline faster than sperm reserves in the male's reproductive organs, and seminal fluid depletion can even limit male remating (Reinhardt et al., 2011). Similarly, in the South American fruit fly, Anastrepha fraterculus, female fecundity was more strongly linked to male accessory gland size than to sperm transfer (Abraham et al., 2020).

An increased reluctance to mate was evident in males as they mated in sequence, males took longer to mate after the first two matings, later matings showed an increase in male refusal to mate, and fifth matings required more instances of swapping in new virgin females to get matings to occur. Duration of mating was also significantly shorter in all matings after a male's first. This could suggest reduced strategic investment in subsequent matings, or that males are

becoming energetically depleted. To distinguish between these alternatives, it would be interesting to further study the adaptive allocation of behaviour, energy and ejaculate resources across bouts of multiple matings.

Ejaculate depletion does not alter male food intake or preference

We predicted that following the depletion of ejaculate reserves after five matings, males would increase consumption of food, and especially protein, to facilitate efficient recharging of sperm and seminal peptides (Perry et al., 2013). Yeast availability in the adult male diet, as a protein supplement, has been found to influence a male's ability to gain mates and sire offspring (Fricke et al., 2008). However, despite this, males remained on similar P:C trajectories with similar food intake rates regardless of their mating rate. Males were either unable to translate their physiological requirements into dietary preference or gained no benefit from doing so. Higher protein diets limited sperm viability in field crickets, *Teleogryllus oceanicus* (Ng et al., 2018). Dietary preference was consistent between singly mated and unmated male *D. melanogaster*, which is congruent with previous work (Camus et al., 2018) and with the observation that levels of seminal fluid are not significantly depleted after a single mating (Lefevre and Jonsson, 1962).

Males in this study favoured a low P:C ratio of 1:4 in their diet in all mating treatments. This is consistent with previous studies of male *D. melanogaster* (Camus et al., 2018; Jensen et al., 2015; Lee et al., 2013; Ribeiro and Dickson, 2010) and strong carbohydrate preference in other male insects (e.g. field crickets; *T. oceanicus* (Ng et al., 2019, 2018), cockroaches, *B. germanica* (Jensen and Silverman, 2018) and *Nauphoeta cinerea* (South et al., 2011)). Although both sexes of *D. melanogaster* prefer a carbohydrate-biased diet, male fruit flies exhibit an even greater preference for carbohydrate than females. This is thought to result from the demands of performing energetically costly courtship rituals, which are essential for male reproductive

success (Bastock and Manning, 1955; Camus et al., 2018; Lee et al., 2013; Maklakov et al., 2008; von Schilcher, 1976). When placed with nonreceptive females, males continue courting for hours (Bastock and Manning, 1955). Male attractiveness to mates in *N. cinerea*,was also closely aligned with carbohydrate intake (South et al., 2011) and a carbohydrate-rich diet in juvenile male Jamaican field crickets, *Gryllus assimilis*, boosted their courting effort as adults (Reifer et al., 2018). It is possible that males might increase carbohydrate intake after multiple matings, to recoup nutritional resources lost to extra bouts of courtship. However, this prediction was not upheld, as we observed that carbohydrate intake remained unchanged across the treatment groups.

The results from this study suggest that male *D. melanogaster* choose a similar ratio of P:C and dietary intake rate regardless of sexual experience. This could indicate that diet intake in males is a non-plastic trait. Consistent with this, strict self-regulation of protein intake has been previously observed in this species (Rushby et al., 2023). No change in P:C ratio was also observed in male *T. oceanicus* in environments of varying sexual competition (Simmons and Chan, 2023). Some invariance in male diet preference is also suggested by a study of dietary preference following macronutrient deprivation (Ribeiro and Dickson, 2010). After maintenance on a sucrose-only diet, females strongly preferred yeast after only 3 days on sucrose, while males took 10 days to reach an equivalent yeast preference and lost this preference far more rapidly when returned to a yeast medium (Ribeiro and Dickson, 2010). That study provided some evidence that males are able to respond to a severe protein deficit but suggested that males do so more slowly than females. The absence of evidence in our study for increased food or protein ingestion by multiply mated males suggests that multiple matings do not induce such extreme protein limitation in males, even near the physiological limits for mating rate. In another study, five times mated male *D. melanogaster* sired equivalent offspring to their first mating, provided that the fifth mating had taken place after a 24 h respite period

(Loyau et al., 2012). However, males in the Loyau et al. (2012) study remained on a solid cornmeal – agar–yeast diet, so it was unclear whether males had altered their food intake over the 24 h respite period. The reproductive output of multiply mated males over 4 h mating bouts on successive days has also been tested (Douglas et al., 2020). Males were reported to be able to remate multiply on consecutive days but did not necessarily sire more offspring, suggesting incomplete regeneration of ejaculate reserves between days (Douglas et al., 2020). The nutritional intake patterns of males that sustain a high mating rate over multiple days would be interesting to investigate further.

Sexual selection theory suggests that males typically maximize reproductive success by mating as many times as possible (Andersson, 1994; Arnqvist and Rowe, 2005). However, in natural settings, multiple factors may affect a male's ability to gain mating and remating opportunities, including access to females, female mate preferences and male–male competition. It may be that males have not evolved a mechanism to cope with sudden ejaculate depletion because the opportunity for consecutive, multiple rematings is rare in nature. There is also evidence that a mated male is less attractive as a mate to females (Douglas et al., 2020; Loyau et al., 2012; Savalli and Fox, 1999). Hence, in scenarios where access to females is unlimited, males may gain fitness by mating at maximal frequencies regardless of ejaculate depletion. Consistent with this idea, we observed that both multiply mated treatment groups had higher offspring numbers than those only mated once, while five times mated males were still able to sire offspring with their last mate and had a marginally (although not significantly) higher reproductive output than three times mated males. More data on male remating behaviour in natural contexts would be helpful for exploring these ideas.

Our results contrast markedly with findings in females, which respond to nutritional deficit and the initiation of reproduction and dynamically adjust their intake accordingly. Specifically, *D.*

melanogaster females alter their diet preference from a low P:C diet as virgins (from a level that is similar to the observed P:C ratio of males) to a higher P:C ratio after mating (Barnes et al., 2008; Camus et al., 2018; Corrales-Carvajal et al., 2016; Lee et al., 2013; Ribeiro and Dickson, 2010). This sex difference is likely to ultimately result from contrasting reproductive strategies between the sexes, whereby females might gain most reproductive success from limiting their number of mates and have greater nutritional requirements to support offspring production. Proximate explanations for the sex differences in dietary responses to mating include gene expression differences in nutrient sensing pathways between the sexes (Bennett-Keki et al., 2023; Fowler et al., 2019) and the effects of the ejaculate sex peptide transferred to females during mating in the seminal fluid (Chapman et al., 2003; Hopkins and Perry, 2022; Liu and Kubli, 2003). The sex peptide has been identified as the key mediator of an increased preference for protein in females after mating (Carvalho et al., 2006; Hopkins and Perry, 2022; Ribeiro and Dickson, 2010).

In conclusion, we have shown that multiply mated *D. melanogaster* males do not, or cannot, adjust their intake of protein and carbohydrate diets compared with unmated or singly mated males. Surprisingly, this lack of dietary response occurs even despite significant ejaculate depletion. This study highlights a gap in knowledge regarding male nutrient homeostasis in response to sexual experience, in contrast to the data available on females. Given the importance of the relationship between the P:C ratio and mating in many insects, it would be useful to investigate the role of multiple mating in diet choice in additional invertebrate models. Males of many insect species adjust their ejaculate investment in response to the sexual environment (Gage and Barnard, 1996; Gage, 1991; Wigby et al., 2009). For example, males of the West Indian fruit fly, *Anastrepha obliqua*, are able to partition their sperm reserves when transferring ejaculate to multiple females and reserve sperm for possible future matings (Perez-Staples and Aluja, 2006). However, our data suggest that males of *D. melanogaster* do

not support this shift in ejaculate allocation by responding to any nutrient debt it entails. In addition, since five times mated male *D. melanogaster* might still have motile sperm present in their vesicles (Gillott, 2003; Lefevre and Jonsson, 1962), perhaps *D. melanogaster* do not perceive a protein requirement until sperm reserves have been fully depleted. Overall, the results suggest that males remain on a fixed feeding trajectory even when mating close to their daily functional maxima, and do not increase nutrient intake to recoup their reduced ability to transfer ejaculates to females.

References

Abraham, S., Moyano, A., Murillo Dasso, S., Van Nieuwenhove, G., Ovruski, S., Pérez-Staples, D., 2020. Male accessory gland depletion in a tephritid fly affects female fecundity independently of sperm depletion. *Behavioral Ecology & Sociobiology* 74, 60. https://doi.org/10.1007/s00265-020-02835-y

Andersson, M., 1994. *Sexual Selection*. Princeton University Press.

Arnqvist, G., Rowe, L., 2005. *Sexual Conflict*. Princeton University Press.

Barnes, A.I., Wigby, S., Boone, J.M., Partridge, L., Chapman, T., 2008. Feeding, fecundity and lifespan in female *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences* 275, 1675–1683. https://doi.org/10.1098/rspb.2008.0139

Bastock, M., Manning, A., 1955. The Courtship of *Drosophila melanogaster*. *Behaviour* 8, 85–110. https://doi.org/10.1163/156853955X00184

Bateman, A.J., 1948. Intra-sexual selection in *Drosophila*. *Heredity* 2, 349–368. https://doi.org/10.1038/hdy.1948.21

Bennett-Keki, S., Fowler, E.K., Folkes, L., Moxon, S., Chapman, T., 2023. Sex-biased gene expression in nutrient-sensing pathways. *Proceedings of the Royal Society B: Biological Sciences* 290, 20222086. https://doi.org/10.1098/rspb.2022.2086

Bownes, M., Blair, M., 1986. The effects of a sugar diet and hormones on the expression of the *Drosophila* yolk-protein genes. *Journal of Insect Physiology* 32, 493–501. https://doi.org/10.1016/0022-1910(86)90011-9

Brooks, M., E., Kristensen, K., Benthem, K., J. ,van, Magnusson, A., Berg, C., W., Nielsen, A., Skaug, H., J., Mächler, M., Bolker, B., M., 2017. glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. *R Journal* 9, 378. https://doi.org/10.32614/RJ-2017-066

Camus, M.F., Fowler, K., Piper, M.W.D., Reuter, M., 2017. Sex and genotype effects on nutrient-dependent fitness landscapes in *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences* 284, 20172237. https://doi.org/10.1098/rspb.2017.2237

Camus, M.F., Huang, C.-C., Reuter, M., Fowler, K., 2018. Dietary choices are influenced by genotype, mating status, and sex in *Drosophila melanogaster*. *Ecology and Evolution* 8, 5385–5393. https://doi.org/10.1002/ece3.4055

Carey, M.R., Archer, C.R., Rapkin, J., Castledine, M., Jensen, K., House, C.M., Hosken, D.J., Hunt, J., 2022. Mapping sex differences in the effects of protein and carbohydrates on lifespan and reproduction in *Drosophila melanogaster*: is measuring nutrient intake essential? *Biogerontology* 23, 129–144. https://doi.org/10.1007/s10522-022-09953-2

Carvalho, G.B., Kapahi, P., Anderson, D.J., Benzer, S., 2006. Allocrine modulation of feeding behavior by the Sex Peptide of *Drosophila*. *Current Biology* 16, 692–696. https://doi.org/10.1016/j.cub.2006.02.064

Chapman, T., Bangham, J., Vinti, G., Seifried, B., Lung, O., Wolfner, M.F., Smith, H.K., Partridge, L., 2003. The sex peptide of *Drosophila melanogaster*: Female post-mating responses analyzed by using RNA interference. *Proceedings of the National Academy of Sciences of the United States of America* 100, 9923–9928. https://doi.org/10.1073/pnas.1631635100

Chapman, T., Trevitt, S., Partridge, L., 1994. Remating and male-derived nutrients in *Drosophila melanogaster*. *Journal of Evolutionary Biology* 7, 51–69. https://doi.org/10.1046/j.1420-9101.1994.7010051.x

Corrales-Carvajal, V.M., Faisal, A.A., Ribeiro, C., 2016. Internal states drive nutrient homeostasis by modulating exploration-exploitation trade-off. *eLife* 5, e19920. https://doi.org/10.7554/eLife.19920

Dewsbury, D.A., 1982. Ejaculate Cost and Male Choice. *American Naturalist* 119, 601–610.

Douglas, T., Anderson, R., Saltz, J.B., 2020. Limits to male reproductive potential across mating bouts in *Drosophila melanogaster*. *Animal Behaviour* 160, 25–33. https://doi.org/10.1016/j.anbehav.2019.11.009

Drummond-Barbosa, D., Spradling, A.C., 2001. Stem Cells and Their Progeny Respond to Nutritional Changes during *Drosophila* Oogenesis. D*evelopmental Biology* 231, 265–278. https://doi.org/10.1006/dbio.2000.0135

Fanson, B.G., Weldon, C.W., Pérez-Staples, D., Simpson, S.J., Taylor, P.W., 2009. Nutrients, not caloric restriction, extend lifespan in Queensland fruit flies (*Bactrocera tryoni*). *Aging Cell* 8, 514–523. https://doi.org/10.1111/j.1474-9726.2009.00497.x

Fowler, E.K., Bradley, T., Moxon, S., Chapman, T., 2019. Divergence in Transcriptional and Regulatory Responses to Mating in Male and Female Fruitflies. *Scientific Reports* 9, 16100. https://doi.org/10.1038/s41598-019-51141-9

Fricke, C., Bretman, A., Chapman, T., 2008. Adult Male Nutrition and Reproductive Success in *Drosophila melanogaster*. *Evolution* 62, 3170–3177. https://doi.org/10.1111/j.1558-5646.2008.00515.x

Gage, A.R., Barnard, C.J., 1996. Male crickets increase sperm number in relation to competition and female size. *Behavioral Ecology & Sociobiology* 38, 349–353. https://doi.org/10.1007/s002650050251

Gage, M.J.G., 1991. Risk of sperm competition directly affects ejaculate size in the Mediterranean fruit fly. *Animal Behaviour* 42, 1036–1037. https://doi.org/10.1016/S0003-3472(05)80162-9

Gilchrist, A.S., Partridge, L., 2000. Why It Is Difficult to Model Sperm Displacement in *Drosophila melanogaster*: The Relation Between Sperm Transfer and Copulation Duration. *Evolution* 54, 534–542. https://doi.org/10.1111/j.0014-3820.2000.tb00056.x

Gillott, C., 2003. Male Accessory Gland Secretions: Modulators of Female Reproductive Physiology and Behavior. *Annual Review of Entomology* 48, 163–184. https://doi.org/10.1146/annurev.ento.48.091801.112657

Greenspan, L., 1977. Humidity Fixed Points of Binary Saturated Aqueous Solutions. *Journal of Research of the National Bureau of Standards. Section A, Physics and Chemistry*, *81*(1), 89–96. https://doi.org/10.6028/jres.081A.011

Hartig, F., 2022. DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models. R package version 0.4.6. http://florianhartig.github.io/DHARMa/

Hihara, F., 1981. Effects of the Male Accessory Gland Secretion on Oviposition and Remating in Females of *Drosophila melanogaster*. *Zoological Magazine* 90, 307–316. https://doi.org/10.34435/zm004883

Hopkins, B.R., Perry, J.C., 2022. The evolution of sex peptide: sexual conflict, cooperation, and coevolution. *Biology Reviews* 97, 1426–1448. https://doi.org/10.1111/brv.12849

Ja, W.W., Carvalho, G.B., Mak, E.M., de la Rosa, N.N., Fang, A.Y., Liong, J.C., Brummel, T., Benzer, S., 2007. Prandiology of *Drosophila* and the CAFE assay. *Proceedings of the National Academy of Sciences of the United States of America* 104, 8253–8256. https://doi.org/10.1073/pnas.0702726104

Jensen, K., McClure, C., Priest, N.K., Hunt, J., 2015. Sex-specific effects of protein and carbohydrate intake on reproduction but not lifespan in *Drosophila melanogaster*. *Aging Cell* 14, 605–615. https://doi.org/10.1111/acel.12333

Jensen, K., Silverman, J., 2018. Frequently mated males have higher protein preference in German cockroaches. *Behavioral Ecology* 29, 1453–1461. https://doi.org/10.1093/beheco/ary104

Kant, R., Trewick, S. a., Sandanayaka, W. r. m., Godfrey, A. j. r., Minor, M. a., 2012. Effects of multiple matings on reproductive fitness of male and female *Diaeretiella rapae*. *Entomologia Experimentalis et Applicata* 145, 215–221. https://doi.org/10.1111/eea.12007

Lee, K.P., Kim, J.-S., Min, K.-J., 2013. Sexual dimorphism in nutrient intake and life span is mediated by mating in *Drosophila melanogaster*. *Animal Behaviour* 86, 987–992. https://doi.org/10.1016/j.anbehav.2013.08.018

Lee, K.P., Simpson, S.J., Clissold, F.J., Brooks, R., Ballard, J.W.O., Taylor, P.W., Soran, N., Raubenheimer, D., 2008. Lifespan and reproduction in *Drosophila*: New insights from nutritional geometry. *Proceedings of the National Academy of Sciences of the United States of America* 105, 2498–2503. https://doi.org/10.1073/pnas.0710787105

Lefevre, G., Jonsson, U.B., 1962. Sperm Transfer, Storage, Displacement, and Utilization in *Drosophila melanogaster*. *Genetics* 47, 1719–1736.

Lenth, R.V., Buerkner, P., Giné-Vázquez, I., Herve, M., Jung, M., Love, J., Miguez, F., Riebl, H., Singmann, H., 2023. emmeans: Estimated Marginal Means, aka Least-Squares Means. https://CRAN.R-project.org/package=emmeans

Leshner, A.I., Siegel, H.I., Collier, G., 1972. Dietary self-selection by pregnant and lactating rats. *Physiology & Behavior* 8, 151–154. https://doi.org/10.1016/0031-9384(72)90144-8

Lewis, S.M., 2004. Multiple mating and repeated copulations: effects on male reproductive success in red flour beetles. *Animal Behaviour* 67, 799–804. https://doi.org/10.1016/j.anbehav.2003.05.013

Linklater, J.R., Wertheim, B., Wigby, S., Chapman, T., 2007. Ejaculate depletion patterns evolve in response to experimental manipulation of sex ratio in *Drosophila melanogaster*. Evolution 61, 2027–2034. https://doi.org/10.1111/j.1558-5646.2007.00157.x

Liu, H., Kubli, E., 2003. Sex-peptide is the molecular basis of the sperm effect in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America* 100, 9929–9933. https://doi.org/10.1073/pnas.1631700100

Loyau, A., Blanchet, S., Van Laere, P., Clobert, J., Danchin, E., 2012. When not to copy: female fruit flies use sophisticated public information to avoid mated males. *Scientific Reports* 2, 768. https://doi.org/10.1038/srep00768

Macartney, E.L., Crean, A.J., Nakagawa, S., Bonduriansky, R., 2019. Effects of nutrient limitation on sperm and seminal fluid: a systematic review and meta-analysis. *Biological Reviews* 94, 1722–1739. https://doi.org/10.1111/brv.12524

Macartney, E.L., Zeender, V., Meena, A., De Nardo, A.N., Bonduriansky, R., Lüpold, S., 2021. Sperm depletion in relation to developmental nutrition and genotype in *Drosophila melanogaster*. *Evolution* 75, 2830–2841. https://doi.org/10.1111/evo.14373

Maklakov, A.A., Simpson, S.J., Zajitschek, F., Hall, M.D., Dessmann, J., Clissold, F., Raubenheimer, D., Bonduriansky, R., Brooks, R.C., 2008. Sex-Specific Fitness Effects of Nutrient Intake on Reproduction and Lifespan. *Current Biology* 18, 1062–1066. https://doi.org/10.1016/j.cub.2008.06.059

Manier, M.K., Belote, J.M., Berben, K.S., Novikov, D., Stuart, W.T., Pitnick, S., 2010. Resolving Mechanisms of Competitive Fertilization Success in *Drosophila melanogaster*. *Science* 328, 354–357. https://doi.org/10.1126/science.1187096

Mason, J.S., Rostant, W.G., Chapman, T., 2016. Resource limitation and responses to rivals in males of the fruit fly *Drosophila melanogaster*. *Journal of Evolutionary Biology* 29, 2010–2021. https://doi.org/10.1111/jeb.12924

McPherson, S., Wada-Katsumata, A., Hatano, E., Silverman, J., Schal, C., 2021. Comparison of Diet Preferences of Laboratory-Reared and Apartment-Collected German Cockroaches. *Journal of Economic Entomology* 114, 2189–2197. https://doi.org/10.1093/jee/toab139

Newell, N.R., Ray, S., Dalton, J.E., Fortier, J.C., Kao, J.Y., Chang, P.L., Nuzhdin, S.V., Arbeitman, M.N., 2020. The Drosophila Post-mating Response: Gene Expression and Behavioral Changes Reveal Perdurance and Variation in Cross-Tissue Interactions. *G3 GenesGenomesGenetics* 10, 967–983. https://doi.org/10.1534/g3.119.400963

Ng, S.H., Simpson, S.J., Simmons, L.W., 2019. Sex differences in nutrient intake can reduce the potential for sexual conflict over fitness maximization by female and male crickets. *Journal of Evolutionary Biology* 32, 1106–1116. https://doi.org/10.1111/jeb.13513

Ng, S.H., Simpson, S.J., Simmons, L.W., 2018. Macronutrients and micronutrients drive trade-offs between male pre- and postmating sexual traits. *Functional Ecology* 32, 2380–2394. https://doi.org/10.1111/1365-2435.13190

Olsson, M., Madsen, T., Shine, R., 1997. Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. *Proceedings of the Royal Society B: Biological Sciences* 264, 455–459. https://doi.org/10.1098/rspb.1997.0065

Pérez-Staples, D., Abraham, S., 2023. Postcopulatory Behavior of Tephritid Flies. *Annual Review of Entomology* 68, 89–108. https://doi.org/10.1146/annurev-ento-120220-113618

Perez-Staples, D., Aluja, M., 2006. Sperm allocation and cost of mating in a tropical tephritid fruit fly. *Journal of Insect Physiology* 52, 839–845. https://doi.org/10.1016/j.jinsphys.2006.05.007

Perry, J.C., 2011. Mating stimulates female feeding: testing the implications for the evolution of nuptial gifts. *Journal of Evolutionary Biology* 24, 1727–1736. https://doi.org/10.1111/j.1420-9101.2011.02299.x

Perry, J.C., Sirot, L., Wigby, S., 2013. The seminal symphony: how to compose an ejaculate. T*rends in Ecology and Evolution* 28, 414–422. https://doi.org/10.1016/j.tree.2013.03.005

Perry, J.C., Tse, C.T., 2013. Extreme Costs of Mating for Male Two-Spot Ladybird Beetles. *PLoS ONE* 8, e81934. https://doi.org/10.1371/journal.pone.0081934

Piper, M.D.W., Blanc, E., Leitão-Gonçalves, R., Yang, M., He, X., Linford, N.J., Hoddinott, M.P., Hopfen, C., Soultoukis, G.A., Niemeyer, C., Kerr, F., Pletcher, S.D., Ribeiro, C., Partridge, L., 2014. A holidic medium for *Drosophila melanogaster*. *Nature Methods* 11, 100–105. https://doi.org/10.1038/nmeth.2731

Prabhu, V., Perez-Staples, D., Taylor, P.W., 2008. Protein: carbohydrate ratios promoting sexual activity and longevity of male Queensland fruit flies. *Journal of Applied Entomology* 132, 575–582. https://doi.org/10.1111/j.1439-0418.2007.01265.x

Raubenheimer, D., Simpson, S.J., 1993. The geometry of compensatory feeding in the locust. *Animal Behaviour* 45, 953–964. https://doi.org/10.1006/anbe.1993.1114

Reddiex, A.J., Gosden, T.P., Bonduriansky, R., Chenoweth, S.F., 2013. Sex-Specific Fitness Consequences of Nutrient Intake and the Evolvability of Diet Preferences. *American Naturalist* 182, 91–102. https://doi.org/10.1086/670649

Reifer, M.L., Harrison, S.J., Bertram, S.M., 2018. How dietary protein and carbohydrate influence field cricket development, size and mate attraction signalling. *Animal Behaviour* 139, 137–146. https://doi.org/10.1016/j.anbehav.2018.03.010

Reinhardt, K., Naylor, R., Siva-Jothy, M.T., 2011. Male Mating Rate Is Constrained by Seminal Fluid Availability in Bedbugs, *Cimex lectularius*. *PLoS ONE* 6, e22082. https://doi.org/10.1371/journal.pone.0022082

Ribeiro, C., Dickson, B.J., 2010. Sex Peptide Receptor and Neuronal TOR/S6K Signaling Modulate Nutrient Balancing in *Drosophila*. *Current Biology* 20, 1000–1005. https://doi.org/10.1016/j.cub.2010.03.061

Richter, C., P., Barelare, B., JR., 1938. Nutritional requirements of pregnant and lactating rats studied by the self selection method. *Endocrinology* 23, 15–24. https://doi.org/10.1210/endo-23-1-15

Rushby, H.J., Andrews, Z.B., Piper, M.D.W., Mirth, C.K., 2023. Ageing impairs protein leveraging in a sex-specific manner in *Drosophila melanogaster*. *Animal Behaviour* 195, 43–51. https://doi.org/10.1016/j.anbehav.2022.10.013

Savalli, U.M., Fox, C.W., 1999. The effect of male mating history on paternal investment, fecundity and female remating in the seed beetle *Callosobruchus maculatus*. *Functional Ecology* 13, 169–177. https://doi.org/10.1046/j.1365-2435.1999.00287.x

Simmons, L.W., Chan, H.-L., 2023. Male responses to sperm competition risk associated with increased macronutrient intake and reduced lifespan. *Biology Letters* 19, 20230336. https://doi.org/10.1098/rsbl.2023.0336

Simmons, L.W., Ng, S.H., Lovegrove, M., 2022. Condition-dependent seminal fluid gene expression and intergenerational paternal effects on ejaculate quality. *Functional Ecology* 36, 798–811. https://doi.org/10.1111/1365-2435.13987

Simpson, S.J., Le Couteur, D.G., Raubenheimer, D., 2015. Putting the Balance Back in Diet. *Cell* 161, 18–23. https://doi.org/10.1016/j.cell.2015.02.033

Simpson, S.J., Raubenheimer, D., 2012. The Nature of Nutrition: A Unifying Framework from Animal Adaptation to Human Obesity. Princeton University Press.

Simpson, S.J., Raubenheimer, D., 1997. Geometric Analysis of Macronutrient Selection in the Rat. *Appetite* 28, 201–213. https://doi.org/10.1006/appe.1996.0077

Simpson, S.J., Ribeiro, C., González-Tokman, D., 2018. Feeding behavior, in: Córdoba-Aguilar, A., González-Tokman, D., González-Santoyo, I. (Eds.), *Insect Behavior: From Mechanisms to Ecological and Evolutionary Consequences.* Oxford University Press. https://doi.org/10.1093/oso/9780198797500.003.0008

Solon-Biet, S.M., Mitchell, S.J., Coogan, S.C.P., Cogger, V.C., Gokarn, R., McMahon, A.C., Raubenheimer, D., de Cabo, R., Simpson, S.J., Le Couteur, D.G., 2015. Dietary Protein to Carbohydrate Ratio and Caloric Restriction: Comparing Metabolic Outcomes in Mice. *Cell Reports* 11, 1529–1534. https://doi.org/10.1016/j.celrep.2015.05.007

South, S.H., House, C.M., Moore, A.J., Simpson, S.J., Hunt, J., 2011. Male cockroaches prefer a high carbohydrate diet that makes them more attractive to females: implications for the study of condition dependence. *Evolution* 65, 1594–1606. https://doi.org/10.1111/j.1558-5646.2011.01233.x

Stoffel, M.A., Nakagawa, S., Schielzeth, H., 2017. rptR: repeatability estimation and variance decomposition by generalized linear mixed-effects models. Methods in Ecology and Evolution 8, 1639–1644. https://doi.org/10.1111/2041-210X.12797

Suárez, M.D., Sanz, A., Bazoco, J., García-Gallego, M., 2002. Metabolic effects of changes in the dietary protein: carbohydrate ratio in eel (*Angilla anguilla*) and trout (*Oncorhynchus mykiss*). *Aquaculture International* 10, 143–156. https://doi.org/10.1023/A:1021371104839

Therneau, T.M., 2023. A Package for Survival Analysis in R. https://github.com/therneau/survival

Therneau, T.M., Grambsch, P.M., 2000. Modeling Survival Data: Extending the Cox Model. Springer.

Trivers, R.L., 1972. Parental Investment and Sexual Selection, in: Campbell, B. (Ed.), *Sexual Selection and the Descent of Man: The Darwinian Pivot* (p. 137). Taylor & Francis .

Tsukamoto, Y., Kataoka, H., Nagasawa, H., Nagata, S., 2014. Mating changes the female dietary preference in the two-spotted cricket, *Gryllus bimaculatus*. *Frontiers in Physiology* 5.

von Schilcher, F., 1976. The role of auditory stimuli in the courtship of *Drosophila melanogaster*. *Animal Behaviour* 24, 18–26. https://doi.org/10.1016/S0003-3472(76)80095-4

Wigby, S., Sirot, L.K., Linklater, J.R., Buehner, N., Calboli, F.C.F., Bretman, A., Wolfner, M.F., Chapman, T., 2009. Seminal fluid protein allocation and male reproductive success. *Current Biology* 19, 751–757. https://doi.org/10.1016/j.cub.2009.03.036

Appendix for Chapter 2

Table A1

Recipe for essential amino acid and nonessential amino acid stock solutions (Camus et al., 2018)

Amino acid stock	Quantity (g/200 ml)
Essential amino acids	
F (L-phenylalanine)	3.03
H (L-histidine	2.24
K (L-lysine)	5.74
M (L-methionine)	1.12
R (L-arginine)	4.70
T (L-threonine)	4.28
V (L-valine)	4.42
W (L-tryptophan)	1.45
Nonessential amino acids	
A (L-alanine)	5.25
D (L-aspartate)	2.78
G (glycine)	3.58
N (L-asparagine)	2.78
P (L-proline)	1.86
Q (L-glutamine)	6.02
S (L-serine)	2.51

Table A2

Recipe to make 200 ml stocks of protein and carbohydrate diet solutions for *Drosophila* (Piper et al., 20 14; Camus et al., 2017, 2018): protein diet contains L-ile powder (348mg), L-leu powder (492mg) and L-tyr powder (252mg), and carbohydrate diet substitutes the amino acids 1:1 with sucrose (6.5g).

	Method	Quantity
Cholesterol	20 mg/ml in EtOH	3 ml
$CaCl_2$	1000x	200 µl
$MgSO_4$	1000x	200 µl
$CuSO_4$	1000x	200 µl
$FeSO_4$	1000x	200 µl
MnC_{l2}	1000x	200 µl
$ZnSO_4$	1000x	200 µl
H_2O		Up to 50 ml
Autoclave the 50 ml solutions at this stage		
Buffer	10x acetate buffer base	20 ml
Nucleic acid/lipid solution	125x stock	1.6 ml
Essential amino acid solution	Stock (Table A1)	18.154 ml
Nonessential amino acid solution	Stock (Table A1)	18.154 ml
Na glutamate solution	100 mg/ml	5.464 ml
Cys solution	50 mg/ml	1.584 ml
Vitamin mix	47.6x stock	4.2 ml
Folic acid	1000x stock	200 µl
Propionic acid		1.2 ml
Nipagin	100g/litre stock in 95% EtOH	3 ml

Gently warm the protein diet to aid dissolution and make final diets to total volumes of 200 ml with H_2O. Syringe filter into tubes for storage. Add 20% yeast solution to protein diet (at 20% concentration) before use. Ingredients for the vitamin mix can be found in Piper et al., (2014).

Table A3

Statistical models used to analyse data in R version 4.0.4 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org)

Data	Model
Intake data	
Angle	glm(alpha ~ block, data=tidy.alpha)
	glmmTMB(resid ~ treatment*day + (1 | id), data=tidy.alpha2)
Day 1 angle	glm(alpha ~ block, data=alpha1)
	glmmTMB(resid ~ treatment + (1|id), data=alpha1)
Length	glm(distance ~ block, data=tidy.distances)
	lmer(resid ~ treatment*day + (1 | id), tidy.distances2)
Day 1 length	glm(distance ~ block, distances1)
	glmmTMB(resid ~ treatment + (1|id), data=distances1)
Raw protein	glm(P ~ block, data=tidyCP, family = Gamma())
	glmmTMB(resid ~ treatment*day + (1 | id), data=tidyCPP)
Raw carbohydrate	glm(C ~ block, data=tidyCP)
	glmmTMB(resid ~ treatment*day + (1 | id), data=tidyCPC)
MANOVA	manova(cbind(alp, dis) ~ treatment*day, data = tidy.alphdis2)
Offspring data	
Male output	glm(totaloffspring ~ block, data=offspring2)
	glm(resid ~ male, data=offspring2)
Offspring slopes	c1 %>%group_by(id.b) %>% summarize(slope = coef(lm(adultperday ~ vial))[[2]], .groups = 'drop')
	glm(slope ~ block, data=coefs)
	glmmTMB(resid ~ mate + (1 | male), data=coefs)
Mating data	
Latency, survival	coxph(Surv(latency, lat.censor) ~ matenumber + block, data = Lmating.times)
	survfit(Surv(latency, lat.censor) ~ matenumber, data = Lmating.times)
Latency repeatability	rptGaussian(latency ~ matenumber + (1 | id), grname = c('id', 'Fixed'), data = REPmating.times, nboot = 1000, npermut = 0, adjusted = FALSE)
Duration	glm(duration ~ block, data=duration.times.2, family = poisson())
	glmmTMB(resid ~ matenumber + (1 | id), data=duration.times.2)

Duration repeatability	rptGaussian(duration ~ matenumber + (1 | id), grname = c('id', 'Fixed'), data = REPmating.times, nboot = 1000, npermut = 0, adjusted = FALSE)
Offspring x mating	glmmTMB(totaloffspring ~ scaledduration + scaledlatency + matenumber + (1|id.b))

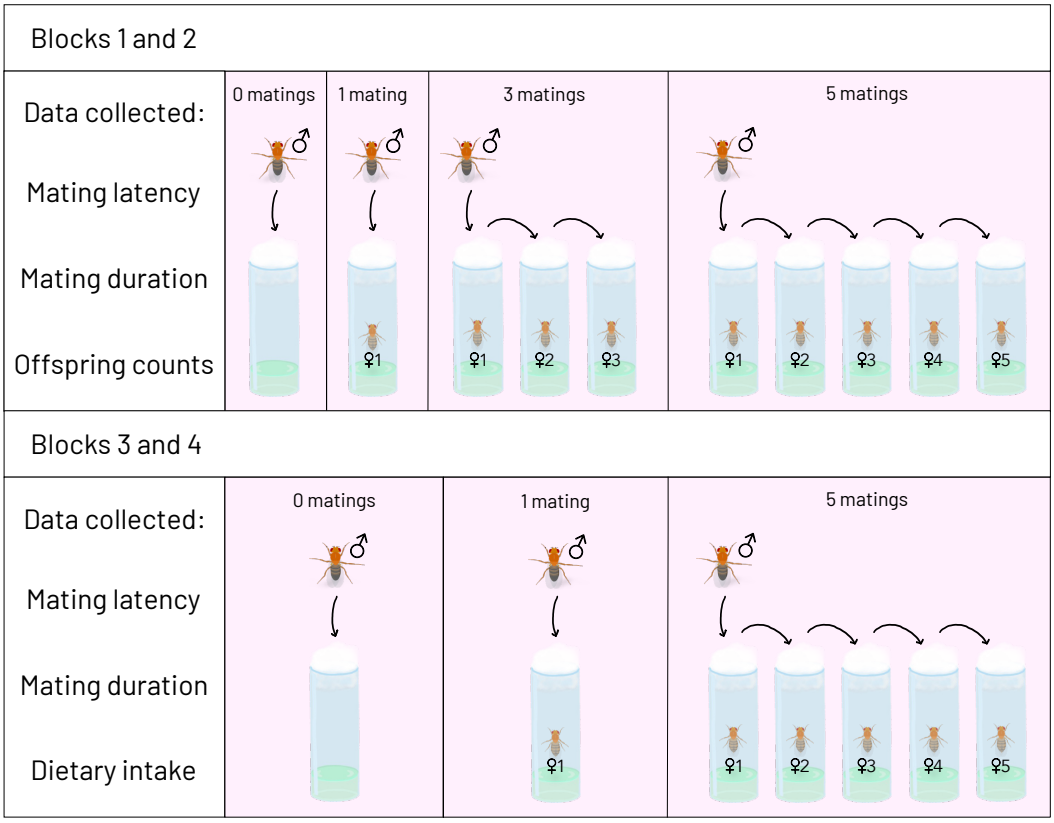


Figure A1

Experimental set-up of male mating treatments and variables collected across the four experimental blocks. Males were assigned at random to treatments of no mating, one, three or five sequential matings, each with a new virgin female, as shown. After the mating treatments, the dietary preferences of males in blocks 3 and 4 were tested using the CAFE assay. Mated females in blocks 1 and 2 were retained and offspring counted.

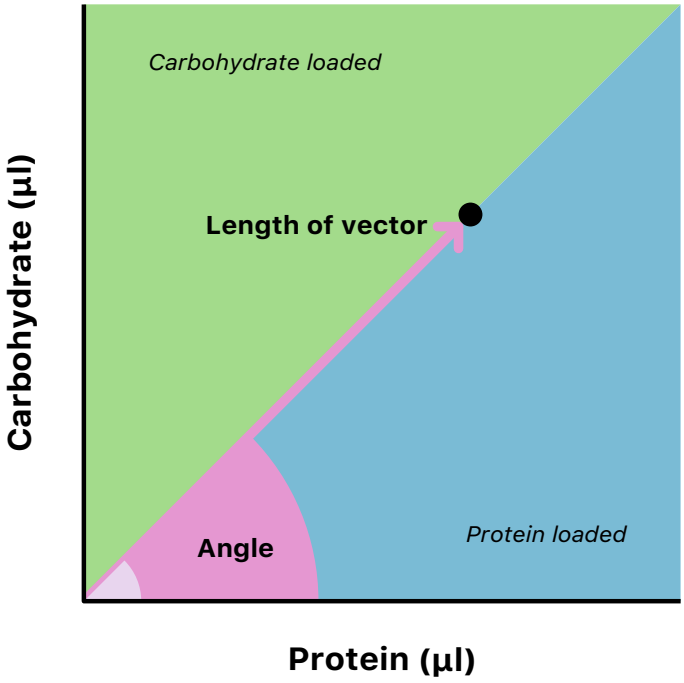


Figure A2

Graphical representation of the trigonometric conversion used to calculate angle and length of the vector from raw feeding data. Protein and carbohydrate intake is represented by the large black circle. The length of the vector for each intake is calculated using distance from the origin (0,0). Points further from the origin signify greater total consumption of macronutrients. The angle between the vector and the x-axis represents composition. Angle values less than 45° denote a greater proportion of protein (the blue space) and values greater than 45° denote a greater proportion of carbohydrate (the green space) eaten.

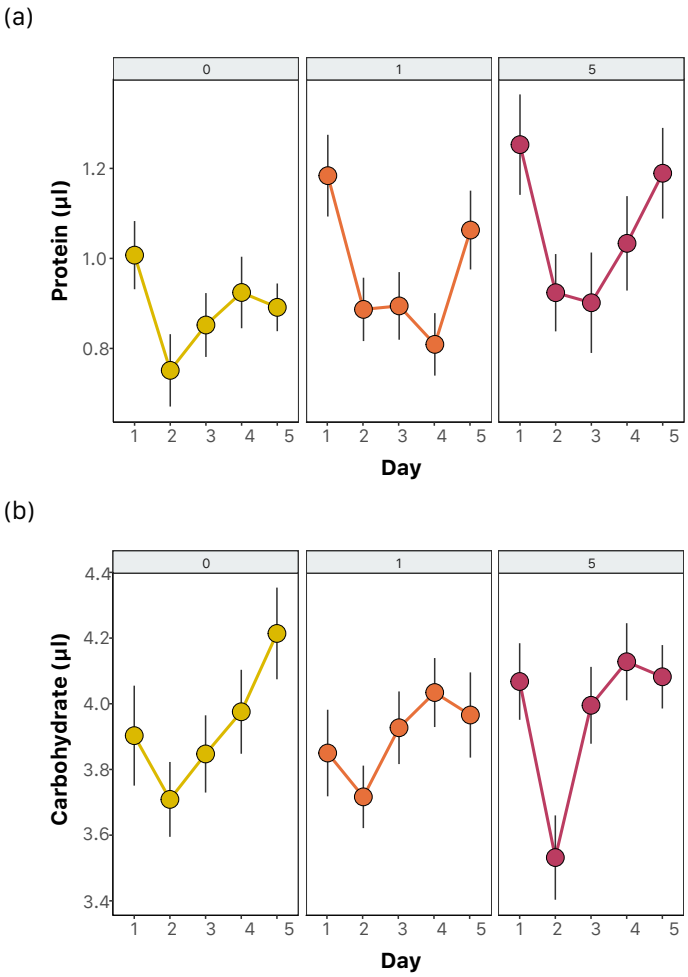
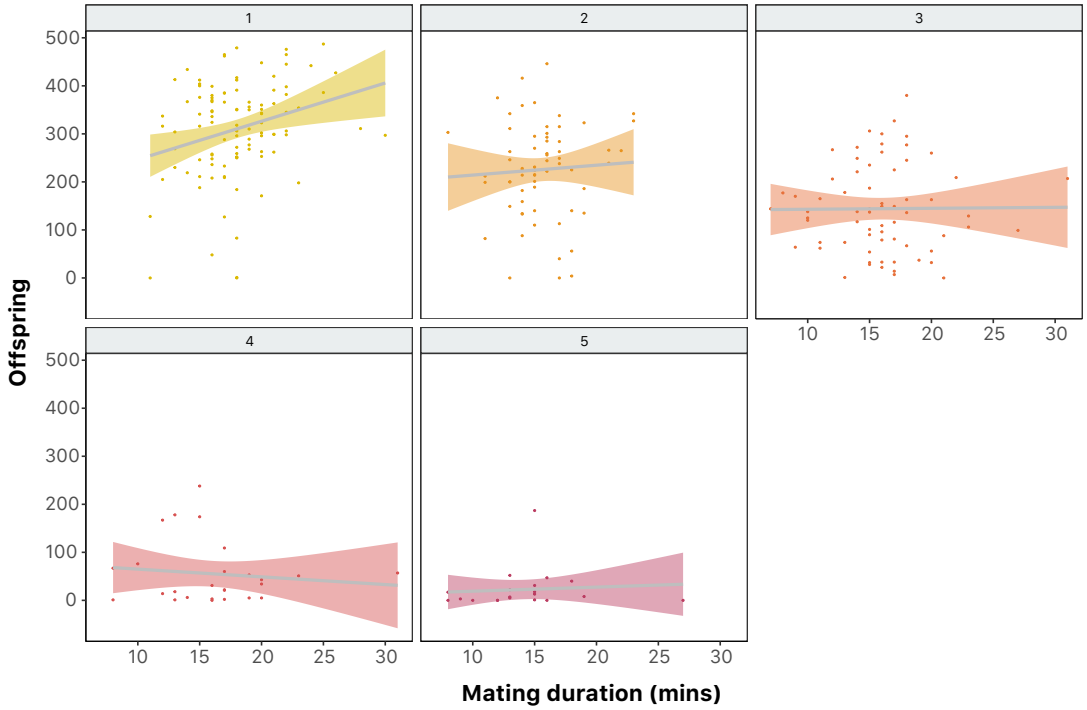


Figure A3

Mean (±SE) intake (µl) of (a) protein and (b) carbohydrate eaten over 24 h periods by experimental males mated zero, one or five times. Experimental males were kept in vials in groups of three and presented with a choice of protein and carbohydrate synthetic liquid diets. Black vertical lines denote the standard error around the mean (large, filled circles). (a) Raw protein intake was not significantly affected by treatment, day or the interaction between treatment and day (treatment: χ^2_2= 4.93, *P*=0.08; day: χ^2_4= 6.25, *P*=0.18; treatment*day: χ^2_8= 8.22, *P*=0.41). Treatment was not significant on day 1 only (χ^2_2= 4.17, *P*=0.12). (b) Raw carbohydrate intake was not significantly affected by treatment or the interaction between treatment and day (treatment: χ^2_2= 1.6, *P*=0.45; treatment*day: χ^2_8=6.42, *P*=0.60). There was a significant effect of day (χ^2_4=11.78, *P*<0.05). Treatment was not significant on day 1 only (χ^2_2= 1.35, *P*=0.51).

(a)


1
2
3
4
5
Offspring
0
100
200
300
400
500
10
15
20
25
30
Mating duration (mins)

(b)

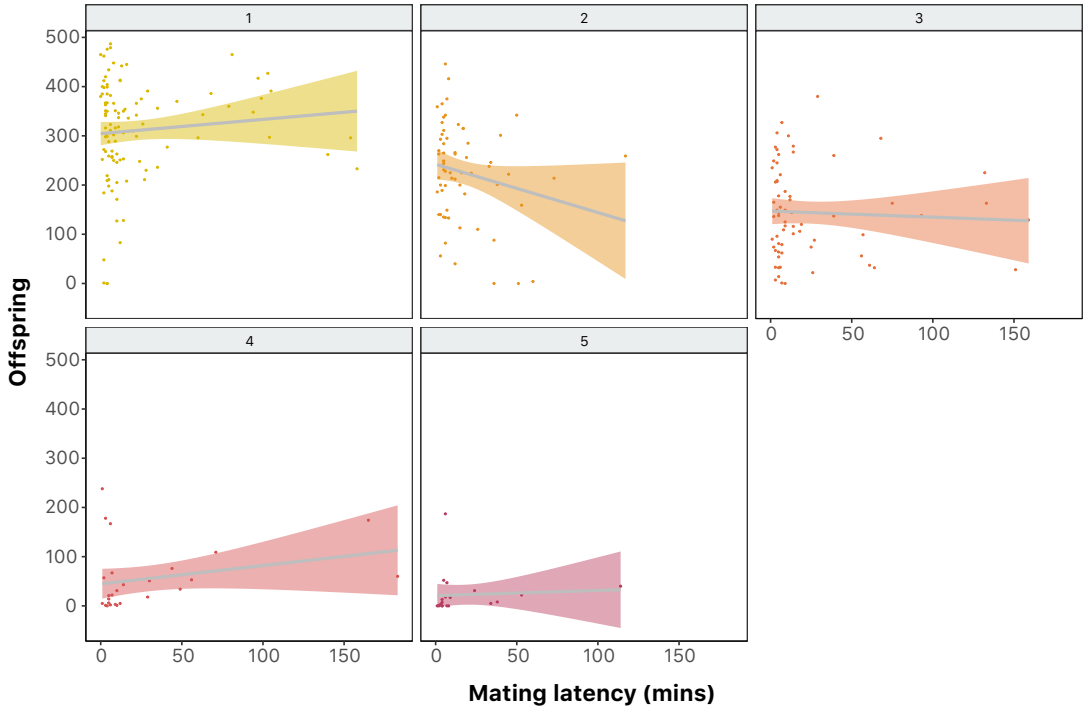

1
2
3
4
5
Offspring
0
100
200
300
400
500
0
50
100
150
Mating latency (mins)

Figure A4

Relationship between mating traits and the offspring produced from a single mating. Data show the offspring produced from a single mating between a male and female fly, where females were mated as virgins and were the first, second, third, fourth or fifth female to mate with a male partner, against the (a) duration and (b) latency to mate of each mating. Raw data points are shown as small circles and linear regression lines are overlaid in grey with coloured confidence intervals. Statistical analysis was carried out with transformed latency and duration data centred around a mean of 0. Preliminary modelling showed a nonsignificant effect of block, which was subsequently removed from the model. There was no significant interaction between duration, latency and mate number when included as a three-way interaction in a generalized linear mixed model (duration*latency*mate number; P=0.41). Model testing showed a reduced model to have the best fit, with mate number having a significant effect on offspring production (χ^2_4=281.77, P<0.001), but not duration (P=0.07) or latency (P=0.96).

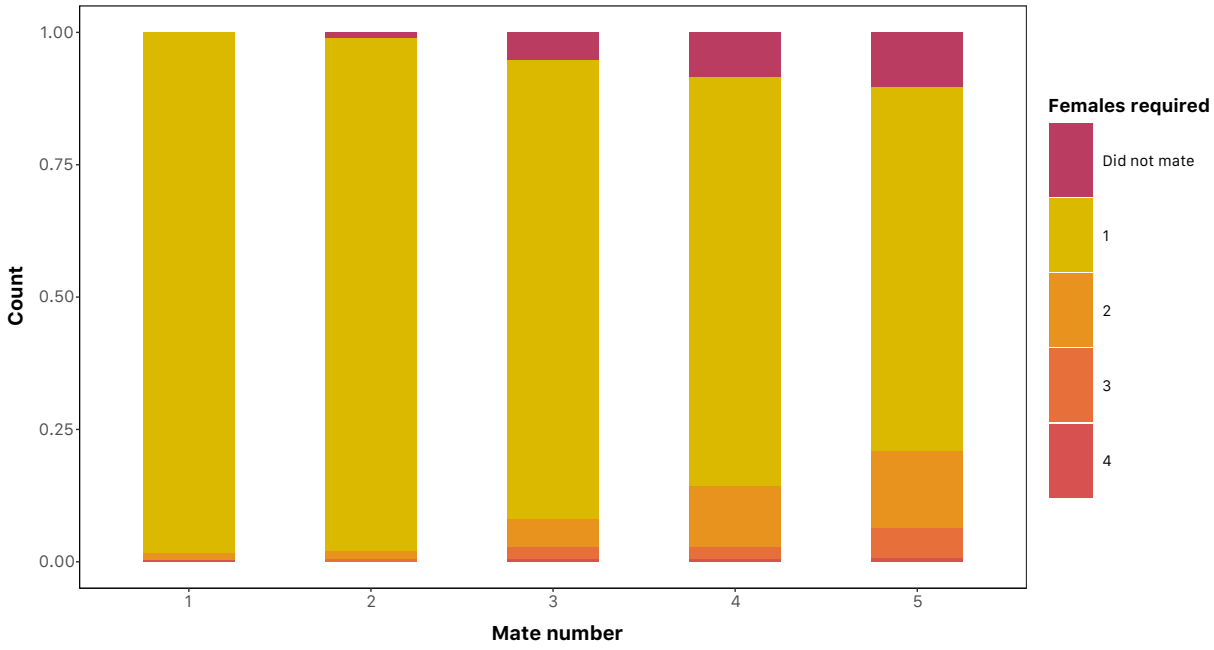


Figure A5

The number of virgin females presented to an experimental male before a successful first, second, third, fourth or fifth mating for that male took place. New, additional virgin females were added to mating arenas containing a single male when latency to mate with the previous female was >60 min. The 'did not mate' category represents males that refused to mate within the period of the mating assay.

Chapter 3

Post-mating switch in diet preference and reproductive behaviour in female *D. melanogaster*

Abstract

Across multiple species, a single, initial mating triggers a shift in female diet preference. In mated female *Drosophila melanogaster* fruit flies, such increases in overall consumption and protein consumption are widely understood to support egg production. However, key aspects of this feeding switch remain unknown. To characterise the effect of mating and remating on female diet choice and its relationship with egg production, we tracked female *D. melanogaster* diet choice and egg laying over one month, using capillary feeding assays with intermittent supply of oviposition substrates. Females were raised on high- and low-yeast diets to alter body condition and were assigned to remain unmated or mate once. A subset of mated females was assigned to mate again halfway through the experiment to test their capacity to express mating dose-dependent feeding. Consistent with predictions, we found increased protein intake and daily egg laying rate upon mating and a second boost to protein consumption after remating. A second boost in egg production upon remating was also observed but was not significant. Contrary to our predictions, female condition had little impact on total macronutrient intake, despite the potentially reduced reproductive capacity of low-quality

females. These results indicate that there is potential for a secondary post-mating feeding switch in female *D. melanogaster*. As such, post-mating switches in females may be dose-dependent and contribute to conflict over optimal diet choice between the sexes.

Introduction

Males and females prefer to feed from diets of different nutrient content. In particular, they consume different amounts of two fundamental macronutrients, protein and carbohydrate (the P:C ratio). Sex differences in diet preference are often only observed after females have mated, whereas the diets of virgin females and virgin or mated males are similar. Differences between virgin and mated female diet selection have been reported predominantly from studies of insects, for example fruit flies, *Drosophila melanogaster* (Carvalho *et al*., 2006; Lee *et al*., 2013), two-spot ladybirds, *Adalia bipunctata* (Perry, 2011), house crickets, *Acheta domesticus* (Woodring *et al*., 1979) and Queensland fruit flies, *Bactrocera tryoni* (Meats & Leighton, 2004). In *D. melanogaster*, virgin female diet preference is comparable to that of males (Camus et al., 2018; Lee et al., 2013), while mated females consume greater amounts of food than do both males and virgin females, specifically increasing their protein consumption (Lee *et al*., 2013; Camus *et al*., 2018). The post-mating feeding switch in female fruit flies is chiefly initiated by the receipt of a single seminal protein within the seminal fluid, the sex peptide, which is passed from the male to the female during mating (Chapman et al., 2003; Liu & Kubli, 2003; Carvalho et al., 2006; Fricke et al., 2010; Hopkins & Perry, 2022). For example, male *Drosophila* lacking sex peptide (while all other accessory gland proteins and sperm were retained) are unable to induce a post-mating feeding response in their female partners equivalent to control males (Carvalho *et al*., 2006; Ribeiro & Dickson, 2010). Meanwhile, female *Drosophila* lacking the sex peptide receptor (Yapici *et al*., 2008) do not increase their yeast preference after mating, unlike control mated females, and instead show a similar level of yeast preference to unmated females (Ribeiro & Dickson, 2010).

Increased female feeding after mating is likely to be important in sustaining reproduction, yet this has not been fully characterised, and many questions remain about the fundamentals of the post-mating feeding response. A key outstanding question is whether the dietary switch

observed upon mating in females is permanent or can be boosted by repeated transfer of the sex peptide in subsequent matings, in a dose-dependent manner. The answer to this question is important because if female feeding responses are mating / sex peptide dose-dependent, this would create an avenue for sexual conflict to arise, whereby males could influence female feeding in a manner that is suboptimal for females' reproductive success (e.g., through dose-dependent female feeding responses to sex peptide (Perry, 2011; Hopkins & Perry, 2022)). This is a research area that has been little studied to date. There is currently limited evidence of the impact of remating on the feeding response. In *A. bipunctata* there was a slight increase in total female food consumption after a second mating, but this was not significant (Perry, 2011). There is some evidence in *D. melanogaster* for a change to protein intake associated with female remating (Bowman & Tatar, 2016), though in that study it was not clear whether altered protein intake was due to re-mating or to variation among treatment groups.

A second key outstanding research question for female post mating feeding responses is that a female's condition is predicted to have significant effects on a female's fecundity and ability to respond to respond to mating signals. The magnitude and persistence of such effects have not previously been tested. Differences in post-mating feeding could occur between females of varying condition due to compensatory feeding behaviours, such that poor-condition females ingest more food or a more protein-rich diet. Alternatively, females in poor or good condition could differ in body size and reproductive potential. For example, female *D. melanogaster* that develop on low-yeast diets have lower reproductive success (Duxbury & Chapman, 2020; Klepsatel *et al.*, 2020) and develop fewer ovarioles (Mirth *et al.*, 2019; Klepsatel *et al.*, 2020; Bath *et al.*, 2023) (but see May et al., 2015). Hence, low condition females may consume less diet overall, and specifically, less protein.

A third key question is whether the observed increase in protein intake triggered in mated females relates to the nutritional demands of increasing the production of large, resource costly gametes (Mirth *et al.*, 2019). It is unclear how closely P:C preference aligns with variation in the rates of egg production in mated females under biologically relevant levels of reproduction. Studies investigating the P:C ratio consumed by mated females in *D. melanogaster* may have underestimated protein consumption linked to egg laying due to limited provision of oviposition substates in the design of assays that measure dietary consumption. The quality and quantity of diet consumed by *D. melanogaster* is commonly measured using the Capillary Feeder assay (CAFE; (Ja *et al.*, 2007), where fruit flies are allowed to feed from synthetic, liquid protein and carbohydrate diets of known nutritional content, allowing precise measurement of protein and carbohydrate intake. However, female *D. melanogaster* reproductive output during CAFE assays declines drastically compared to individuals maintained on standard food media. For example, the median number of eggs laid over 3-day intervals during a 9-day CAFE assay was six eggs for mated female *D. melanogaster* and 0 eggs for virgin females (Lee *et al.*, 2013); in contrast, female *D. melanogaster* can lay upwards of 60 eggs per day on standard food media (Partridge *et al.*, 1987; Trevitt *et al.*, 1988; Duxbury & Chapman, 2020). The precise explanation for this low fecundity is not known but could be due to the low quality oviposition substrate (CAFE assays are carried out using a nutrition free agar-water substrate) (Ja *et al.*, 2007; Becher *et al.*, 2012) or associated with the method of food intake. In addition, mortality is generally observed to be higher in CAFE assays, which may limit tests of the trajectory of female post-mating diet choice over a significant period of time, e.g. to examine whether diet choice returns to the virgin-like state in between matings. These factors may limit CAFE-based investigations into the maintenance or attenuation of female feeding switches.

Here we explored how female diet choice responds to female condition, mating and re-mating in *D. melanogaster* to address the significant gaps identified above in our knowledge of nutritional ecology. To do this, we tracked female consumption of synthetic protein and carbohydrate liquid diets and egg-laying, in an assay in which female mating status and developmental condition were experimentally manipulated. Females were raised as larvae on either low- or high-yeast media, and as adults either remained unmated, mated once (mated), or mated for a second time (remated). Importantly, females were placed onto standard food media in between diet measurements via the CAFE assay, to minimise any deleterious effects of the CAFE assay conditions on survival and to boost egg laying by providing biologically relevant egg laying substrates. We had four main predictions: (1) mated females would consume more overall, and would consume a greater proportion of protein, than unmated females; (2) the post-mating diet switch in females would be stable after a single mating due to consistently high levels of post-mating egg laying, while a second mating would further boost egg production and subsequent feeding in remated females; (3) egg laying rates would align with protein consumption among individuals; (4) female condition would affect the strength of the post-mating diet-switch, as low-quality females would lay fewer eggs than high-quality females, and hence require less protein.

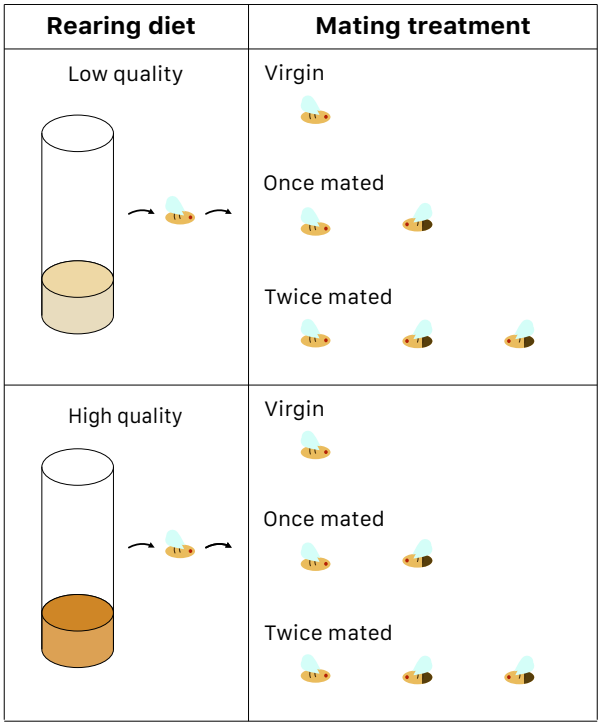


Figure 1

The larval diet and mating treatments for experimental females. Females used in the experiment were raised as larvae on either a low quality (low yeast content) or high quality (high yeast content) diet. Flies from each rearing treatment were then assigned randomly to mating treatments and remained as virgin flies, were mated once at the beginning of the experiment, or were mated once at the beginning and again at day 18 of the experiment.

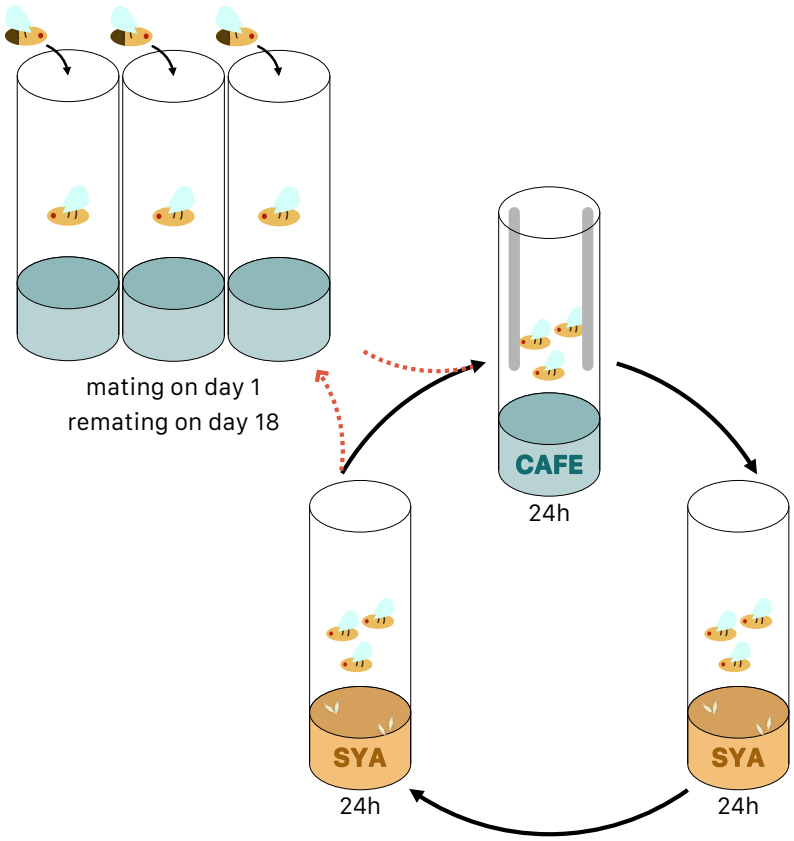


Figure 2

The experimental set-up of CAFE-egg laying rotation of female flies. Females in the mated and remated treatments were mated singly with virgin males on day one of the experiment. Females from all treatments were then added in groups of three females per vial to glass vials containing 7ml 0.75% agar-water. Vials were provided with microcapillary tubes containing carbohydrate and protein liquid diets (CAFE assay). After 24h, females were then moved into fresh sugar-yeast-agar (SYA) vials and allowed to lay eggs for 24h, before moved to new SYA for another 24h. This pattern of CAFE-SYA-SYA was repeated until day 18, when flies in the remated treatment were mated again in individual mating arenas, after which, flies were returned in threes to the CAFE-SYA-SYA cycle. The cycle was repeated until day 33.

Methods

As larval development is slower on low-yeast content diets (May *et al.*, 2015; Duxbury & Chapman, 2020), we staggered the production of low- and high-yeast diet raised experimental flies to standardise age between the two condition treatments. Egg collections for experimental adults were repeated over a 3-day window to buffer any additional variation in development time.

Fly stocks and generation of experimental flies

Experimental individuals were collected from a stock population of the outbred, wildtype, Dahomey line of *D. melanogaster* (Chapman *et al.*, 1994), maintained in large cages on a sugar-yeast-agar diet (SYA: 100 g brewer's yeast, 50 g sucrose, 15 g agar, 30 ml Nipagin (10 % solution), 3 ml propionic acid, 970 ml water). Experimental flies were collected following one generation of standardised rearing to limit parental effects, whereby eggs were first collected from the stock population in bottles containing SYA media. The adults emerging from this collection were used as the parental generation and allowed to lay eggs over three-hour periods on purple grape juice-agar plates with live yeast paste. Eggs were left to develop on grape plates for 24 h, until hatching. To obtain 3-day old virgin male flies for the remating assay, an additional round of parental rearing and egg collections was undertaken in the same manner. Flies collected for body mass measurements were collected in an additional replicate of the experiment, where fly rearing and handling was identical to the main experiment.

Larval rearing diet

First instar larvae in densities of 50 were added to glass vials containing 7 ml of SYA diet of either 20 % or 120 % of the standard yeast content of SYA (20 g/L or 120 g/L of brewer's yeast, respectively, the ratios of all other SYA diet components remained unchanged). Both larval rearing diets are referred to as "low-condition" or "high-condition" due to the impact of yeast

concentration on resource availability. These concentrations of yeast are an established method of condition alteration in *Drosophila* without extreme under or over feeding effects (Duxbury & Chapman, 2020; Bath *et al*., 2023). 120 % yeast was used for the high-condition diet treatment rather than standard SYA (100 % yeast), so that larvae in low-condition and high-condition treatments both experienced developmental conditions that differed from the standard rearing diet. Adults were subsequently collected as virgins from both larval rearing treatments and male mates on the same day, when eclosion was aligned between the treatments. Adults from both larval rearing treatments were allowed to mature for three days in single-sex groups of ten on standard SYA. A sample of females was retained from each treatment when flies were 3 days post eclosion (N = 30 per treatment) for subsequent weighing.

Dry body weight measurements

3-day old virgin females from each larval rearing treatment (N = 60) were anesthetised, frozen at -80 °C, placed into 1.5 ml tubes and heated to 60 °C for approximately 48 h. The dry weight of flies was recorded in micrograms using a 0.001 mg microbalance (BM-20, A&D Instruments).

Mating and remating trials

Approximately 18 h before the mating assay, individual virgin males were aspirated into vials containing 7 ml of 0.75 % agar-water. On the morning of the mating assay, single 3-day old virgin females from either larval diet treatment were added into each vial containing a male. Vials were scan sampled approximately every one minute to record mating latency and duration. Females assigned to the virgin treatment were aspirated into agar-water vials without males to ensure similar levels of mechanical handling between the treatments. After the mating assay was complete, mated females were randomly assigned to the mated once or

remated treatments (per larval rearing treatment) resulting in 6 treatment groups (low-condition virgin females, low-condition single mated females, low-condition remated females, high-condition virgin females, high-condition single mated females and high-condition remated females; Figure 1). All females were transferred in groups of three to new agar-water vials, as previous data suggested that individual flies consume a small quantity of protein over 24 h, which would not be measurable using the CAFE method.

The remating treatment was carried out 17 days after the first mating assay, approximately half-way through the experimental schedule. 3-day old males, obtained from an additional, later round of parental rearing and egg collections, were added to agar-water vials approximately 18 h before remating occurred. Females from the remating treatments were separated from trios and added individually to the mating vials (Figure 2). Scan sampling was used to assay mating latency and duration. Females in the virgin and singly mated treatments were also added to individual agar-water vials that did not contain males to standardize handling disturbance.

CAFE and egg laying assays

To measure consumption of macronutrients by female fruit flies, we used the CAFE method of liquid feeding (Ja *et al.*, 2007; Lee *et al.*, 2008). Two synthetic, chemically defined carbohydrate and protein diets were made using identical components of lipids, vitamins and salts. Amino acids were added to create the protein diet, whereas sucrose was added to create the carbohydrate diet. Recipes for the preparation of both diets are included in the Appendix, Tables A1-A3. Previous work suggested that *Drosophila* would not eat the pure protein diet (Camus *et al.*, 2017, 2018). As a result, the protein diet was supplemented with a 20 % killed yeast suspension and the sugar content of the killed yeast cells resulted in the final protein diet comprising 4 % carbohydrate.

On the day following the first mating assay, two 5 µl microcapillary tubes (Ringcaps™; Hirschmann Instruments™) each holding either the protein diet or the carbohydrate diet, were added to the agar-water vials containing female trios and fixed in place using foam bungs (*N* = 136). Vials containing microcapillaries were subsequently placed into an air-tight box containing a highly concentrated salt solution to increase humidity (Greenspan, 1977) and reduce evaporation of the diets. 10 % of vials used in the CAFE measurements held diet-filled microcapillaries but not flies, to measure evaporative loss independent of feeding. Flies were left undisturbed to feed freely in the CAFE assay for 24 h. The height of the liquid diet was marked on each microcapillary at the start of the assay and again following the 24 h feeding period. The distance between the two points was measured in millimetres using a digital calliper and converted into µl of liquid loss.

After the CAFE measurement, females were moved by inversion into vials containing 7 ml of oviposition substrate (standard SYA) and allowed to lay eggs. After 24 h, females were moved again, into fresh SYA vials, to provide two consecutive measures of 24 h oviposition rates. Once females were removed from oviposition vials, vials were frozen, and numbers of eggs and egg cases counted using a dissecting microscope. This process of a CAFE assay followed by an egg laying was then repeated. Overall, the experimental schedule was based on a 3-day rotation, consisting of a 24 h CAFE assay (*N* = 10) followed by two daily measures of egg laying over 32 days (Figure 2). A subset of oviposition vials was used for the egg counts across the experimental period (*N* = 11 of 24 h egg laying periods).

Statistical analysis

All statistical analyses were carried out using R version 4.3.3 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org). Throughout, generalised linear mixed

models were fit using the package glmmTMB (Brooks *et al.*, 2017) and linear models were fitted using the package lme4 (Bates *et al.*, 2015). In all cases, model fit was checked using the package DHARMa (Hartig, 2022) and post-hoc Tukey testing of estimated marginal means was carried out using the package emmeans (Lenth *et al.*, 2023).

Diet intake

Raw intake values from experimental and control vials over each 24 h period were converted from mm to μl. Protein and carbohydrate values were then adjusted for evaporation, by subtracting the average evaporation of diets in the control vials per day (N = 13 evaporation values per diet, per day). Adjustments for evaporation using this method resulted in negative values of intake (N=10 carbohydrate values, N = 183 protein values). Since useful information can still be gained by consumption of minimal diet, negative values were retained in the dataset, and all intake data points were transformed by adding a constant, using the formula Y+1 – min(Y), where Y is each datapoint and min(Y) is the smallest value. As such, the lowest value was made equal to one.

Daily intake of protein and carbohydrate were analysed separately within generalised linear mixed models. Due to the non-normal distribution of the intake measurements across the 10 CAFE assays, a Box Cox lambda transformation was used separately for both protein and carbohydrate using the R package forecast (Hyndman & Khandakar, 2008; Hyndman *et al.*, 2024). Transformed intake data was then entered as the response variable with mating treatment, female condition and day included as fixed factors, and vial id as a random factor.

Cumulative sums of the intake data were calculated per diet and per vial id, as shown in Figure 3, and used to analyse the total consumption of carbohydrate and total consumption of protein

between the treatment groups in generalised linear mixed models. Treatment was included as a fixed factor and vial id was included as a random factor.

Protein and carbohydrate intake measured in the last CAFE assay before the midpoint and the first CAFE after the midpoint were compared in an additional, separate analysis, to analyse the effect of a second mating in the remating treatments at day 18. Protein and carbohydrate intake values pre- and post-midpoint were analysed separately in generalised linear mixed models, testing for an interaction between day, mating treatment and female condition, with vial id included as a random factor. Pairwise post-hoc Tukey testing was used to compare the estimated marginal means of each day per the treatment levels.

Egg count

Daily egg counts per three females were analysed in a generalised linear mixed model with vial id as a random factor, and mating treatment, female condition and day as fixed factors. The total number of eggs over the first 2-weeks of the experiments (after the initial mating assay) was calculated per vial using 24 h egg counts from experimental days 3, 5, 12 and 15. Vials without egg count data for one or more of the four timepoints were excluded from this analysis (N = 36). The impact of treatment on 14-day egg totals were analysed in a linear model. The number of eggs laid before and after the midpoint of the experiment (when flies in the remating treatment were remated) were compared in an additional generalised linear mixed model, with an interaction between day, mating treatment and female condition, with vial id included as a random factor.

Dry weight

The dried mass of flies was analysed in a linear model with treatment included as a fixed factor.

Results

Manipulation of female condition did not alter body weight

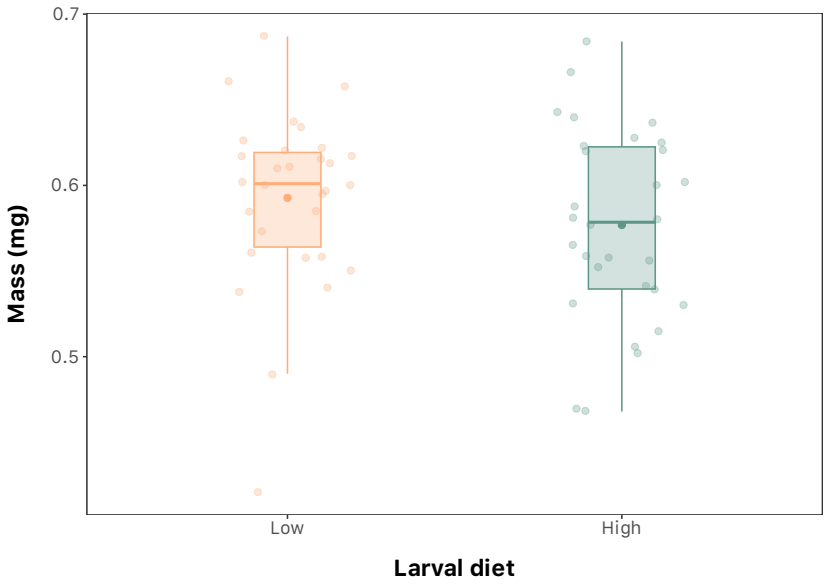


Figure 3

The dry weight in milligrams of individual females collected from each larval diet treatment. Dry weight was collected from 3-day old adult flies raised on either a high or low yeast content diet. Boxes represent interquartile range (IQR) with medians overlaid as thick horizontal lines and means as large, filled circles. Whiskers represent 1.5 x IQR, and small circles represent the raw data.

The dry weight of adult females did not differ significantly depending on the yeast content of larval rearing diet ($F1= 1.3$, $P=0.3$; Figure 3). Mean dry weight for female flies was 0.59 mg (SE±0.01) and 0.58 mg (SE±0.01) for flies raised on the low and high diets, respectively.

Protein but not carbohydrate intake was affected by mating treatment and condition

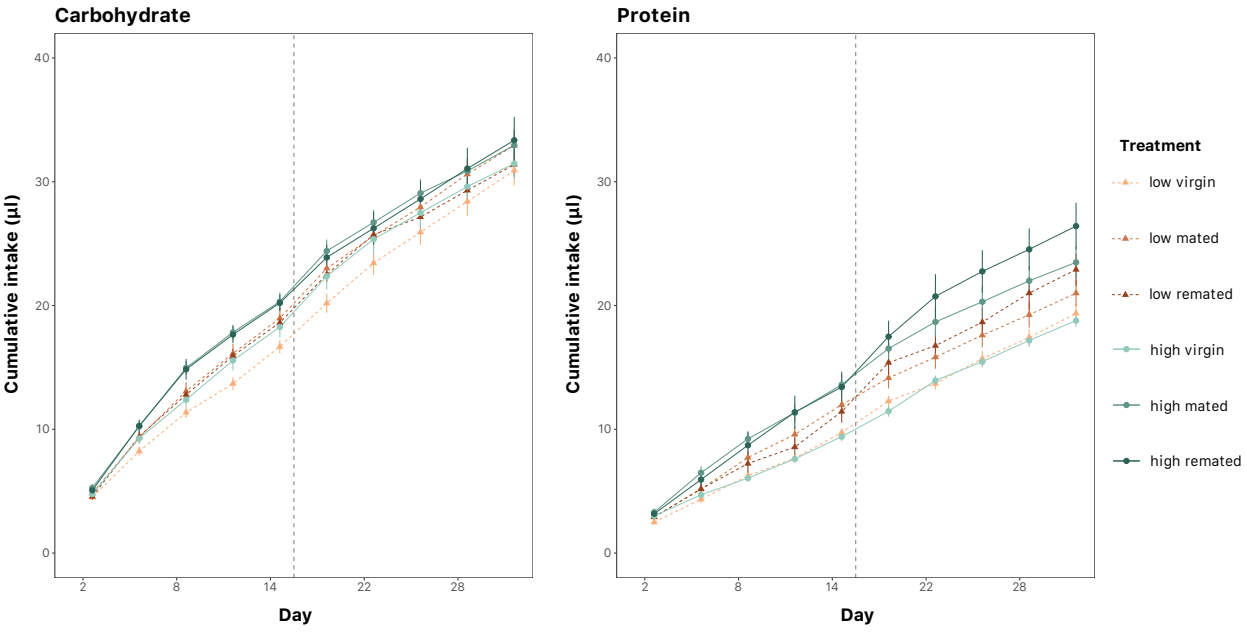


Figure 4

Cumulative intake (µl) of liquid carbohydrate (a) or protein diet (b) by trios of females. Females fed freely within a CAFE assay, choosing between protein or carbohydrate liquid diets for 24 h over a 33-day period. Coloured shapes represent the cumulative mean intake (±SE) per vial, for each day. Triangles connected by dashed lines represent flies reared on low quality larval diet (low condition), while circles connected by solid lines represent flies reared on high quality larval diet (high condition). The grey, vertical, dashed line on each plot denotes the midpoint of the experiment, when flies in the high remated or low remated treatments were mated for a second time, at day 18.

Mating and female condition treatment altered daily protein intake (mating: χ^2_2=28.0, P<0.001, female condition: χ^2_1=6.0, P<0.05; Figure 4) with an interaction between mating and female condition (χ^2_2=7.7, P=0.02). In contrast, daily carbohydrate intake was not altered by mating (χ^2_2=1.5, P=0.5) or condition (χ^2_1=0.5, P=0.5) in female flies. Consumption of both diets varied with day (χ^2_9=215.2, P<0.001 and χ^2_9=820.6, P<0.001, for protein and carbohydrate, respectively; Appendix, Figure A1). Total consumption of both protein and carbohydrate

pooled over the experimental period was also dependent on mating treatment (χ^2_2=11.2, P<0.01; Appendix, Figure A2) but not female condition (χ^2_1=0.8, P=0.4).

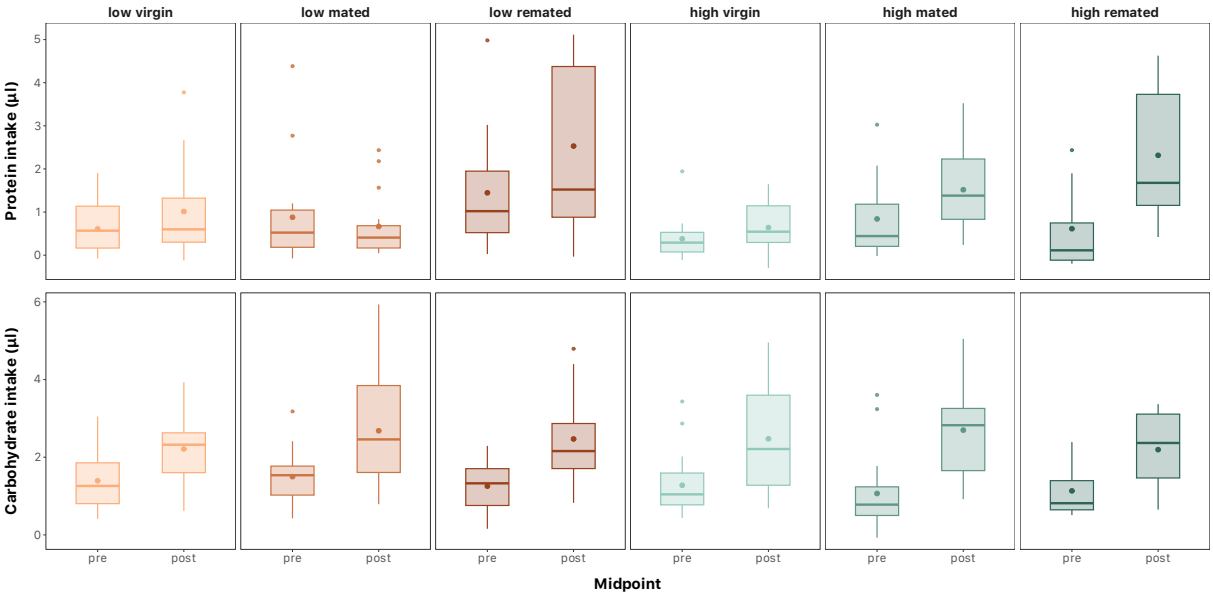


Figure 5

Dietary intake of three females placed in a CAFE assay the day before the high-condition remating or low-condition remating treatments were remated, and the day following the remating. All files were moved into fresh agar vials during the remating assay. Each box represents interquartile range (IQR) with medians as thick horizontal lines and means as large, filled circles. Whiskers represent 1.5 x IQR, and small circles represent outliers.

Pairwise comparisons in post-hoc Tukey tests revealed that daily protein intake of the high-condition virgin treatment was significantly lower than the high-condition mated treatment (t_{866}=-4.8, P=<0.001), high-condition remated treatment (t_{866}=-4.1, P=<0.001) and the low-condition remated treatment (t_{866}=-3.8, P=<0.01). Protein intake in the low-condition virgin treatment was also significantly lower when compared to the high mated treatment (t_{866}=-3.3, P=<0.05) and, marginally, the high remated treatment (t_{866}=-2.8, P=0.05). All other treatment groups were not significantly different from one another in protein consumption (post-hoc Tukey tests: P>0.05).

Flies in the remating treatments were mated for a second time at the midpoint of the experiment, at day 18. To explore the potential for direct, short-term effects of remating, we compared protein and carbohydrate intake from the last CAFE assay before the midpoint, with the first CAFE after the midpoint for all treatments (Figure 5; 'stage'). Analyses of protein intake surrounding the midpoint showed no interaction between the stage, mating treatment and female condition (χ^2_2=3.8, P=0.2) but there was a significant interaction between stage and mating treatment (χ^2_2=7.8, P<0.05) and stage and female condition (χ^2_1=4.9, P<0.05). Post-hoc Tukey tests comparing protein consumption between the stages within each of the 6 treatment combinations (mating treatment x female condition) revealed that protein intake differed significantly between the pre- and post-midpoint in the high condition remated females (t_{156}=-4.4, P=<0.001). Surprisingly, the stage effect was also significant in the high condition mated females, even though individuals did not experience a second mating (t_{156}=-2.8, P<0.01), and was not significant in the low condition remated females (t_{156}=-1.6, P=0.1). Protein intake was not affected by stage in all other treatments (P>0.05).

Carbohydrate intake before and after the midpoint was not significantly dependent on an interaction between stage, mating treatment and female condition (χ^2_2=2.5, P=0.3) or an interaction between stage and mating treatment (χ^2_2=3.6, P=0.2). There was a significant interaction between stage and female condition for carbohydrate consumption (χ^2_1=4.4, P<0.05). Interestingly, post-hoc Tukey test comparing carbohydrate intake before and after the midpoint within each of the 6 treatment combinations (mating treatment*female condition), found a significant difference in all treatments (P<0.05), regardless of mating status.

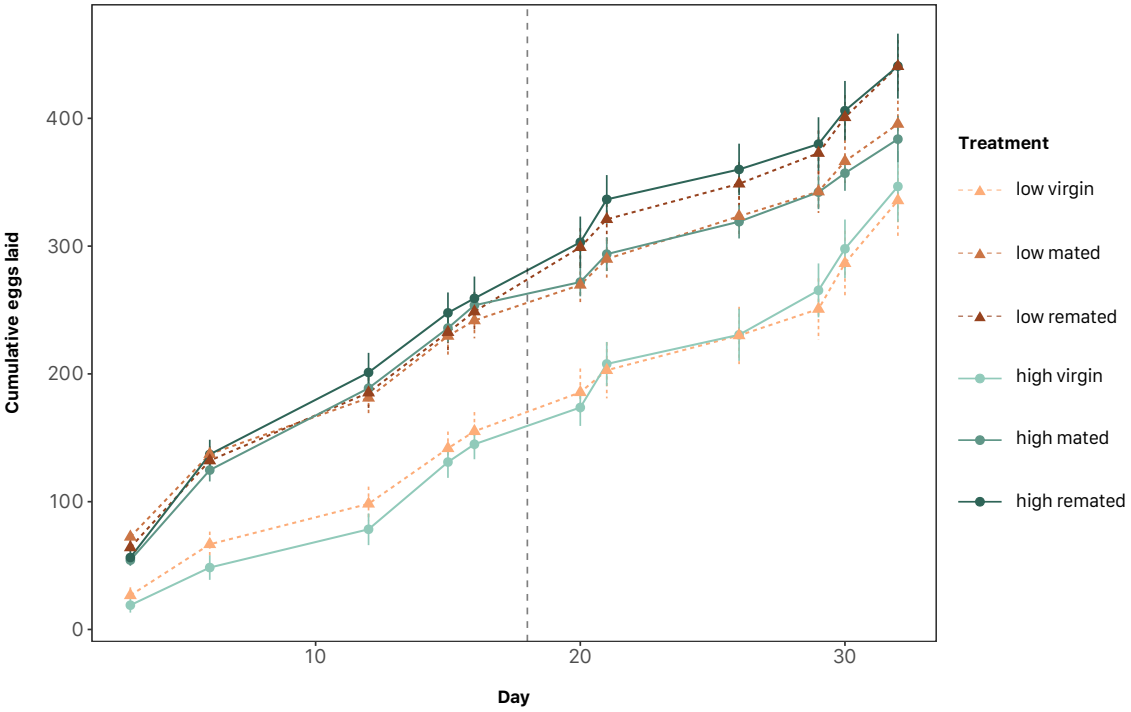


Figure 6

The number of eggs laid per vial over 24 h by three females, plotted cumulatively. Coloured shapes represent the mean (±SE) for each day. Triangles connected by dashed lines represent flies reared on low quality larval diet (low condition), while circles connected by solid lines represent flies reared on high quality larval diet (high condition). The grey, vertical, dashed line denotes when flies in the high remated or low remated treatments were mated for a second time.

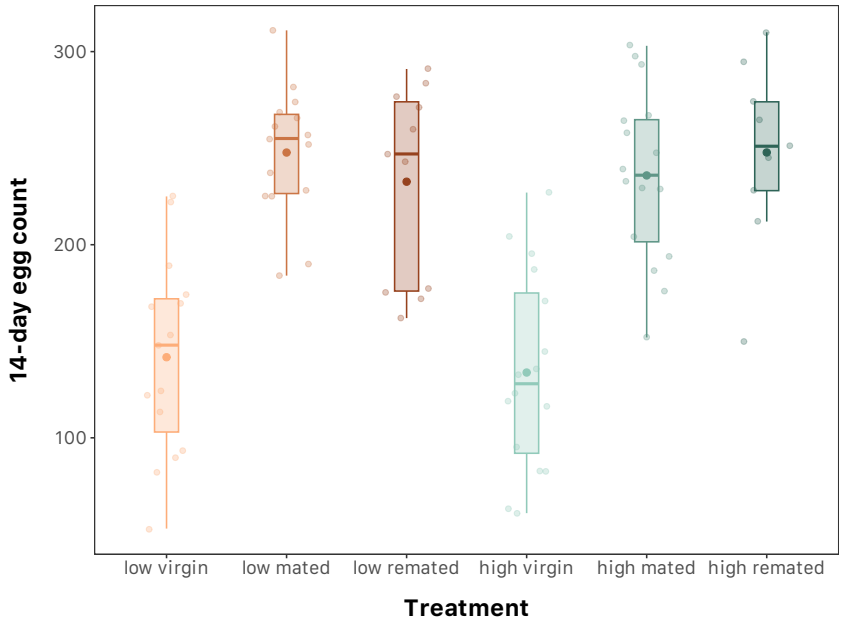


Figure 7

The total number of eggs laid by groups of three females per vial containing SYA media, over the first 14 days of the experiment. Data used are totalled from four, 24 h egg counts on days 3, 6, 12 and 15 of the experiment. 14-day egg counts are grouped and coloured per treatment: female condition (low or high) and mating treatment (virgin, mated or remated). Each box represents interquartile range (IQR) with medians as thick horizontal lines and means as large, filled circles. Whiskers represent 1.5 x IQR, and small circles represent the raw data.

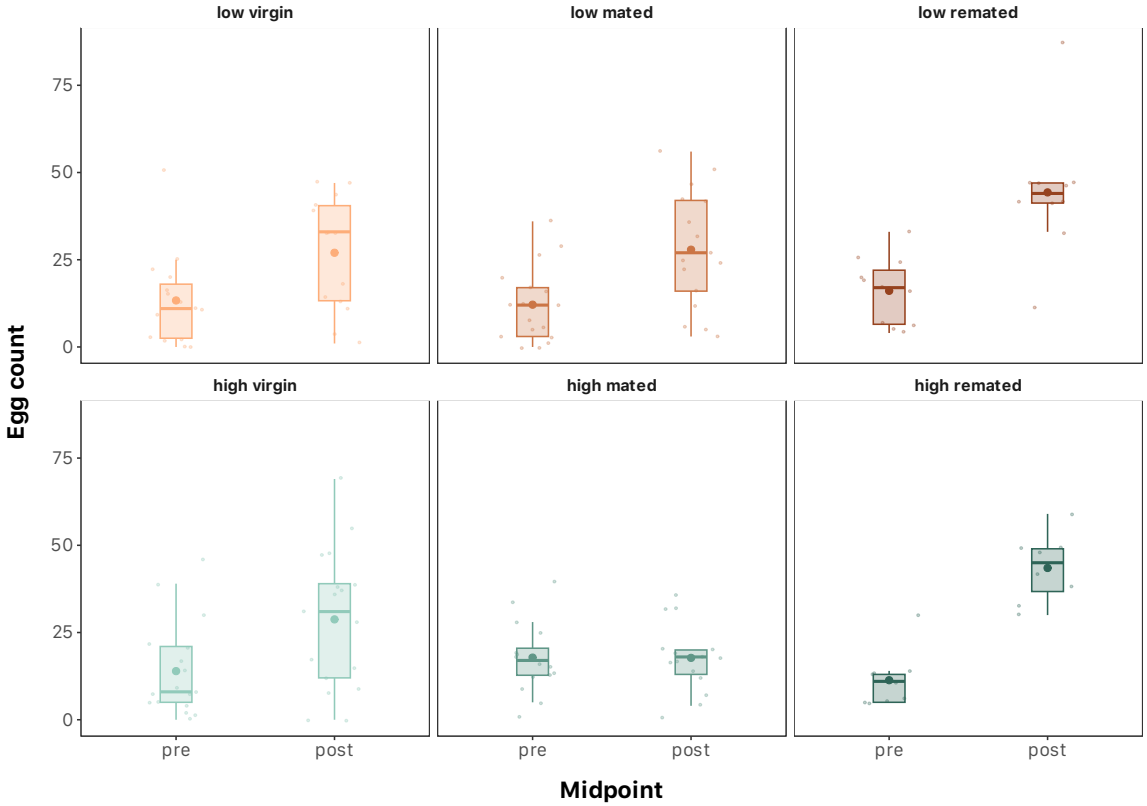


Figure 8

The number of eggs laid by groups of three females in the last measure before the midpoint of the experiment (day 18), and the first measure following the midpoint. The remating stage occurred on day 18 of the experiment, where females from the high remated and low remated treatments were each provided with a virgin male and allowed to mate. Egg counts were taken once females were left in groups of three to lay eggs over 24 h on standard SYA medium. Boxes are separated and coloured per treatment. Each box represents interquartile range (IQR) with medians as thick horizontal lines and means as large, filled circles. Whiskers represent 1.5 x IQR, and small circles represent the raw data.

The daily number of eggs laid per vial was significantly dependent on mating treatment (χ^2_2=23.2, P<0.001; Figure 6) and day (χ^2_{10}=524.8, P<0.001) but not female condition (χ^2_1=1.2, P=0.3). The total number of eggs laid in each treatment in the first 14 days of the experiment (before remating) were tested to compare the impact of female body condition in virgin versus mated flies. The total number of eggs laid over 14-days was dependent on mating treatment

(χ^2_2=99.9, P<0.001; Figure 7) but not female condition (χ^2_1=0.1, P=0.7). Specifically, 14-day egg counts were lower in the virgin treatments when compared to all other mated treatments (post-hoc Tukey: P<0.001) but there was no difference between the two female conditions within the virgin treatments (P>0.5).

The number of eggs laid before and after the remating point (stage) was tested to investigate egg laying before and after flies in the remating treatment were remated (Figure 8). Analyses of egg output surrounding the mid-point found no significant interaction between stage, mating treatment and female condition (χ^2_2=4.9, P=0.09). However, there was a significant interaction between stage and mating treatment (χ^2_2=17.4, P<0.001) and between stage and female condition (χ^2_1=6.1, P<0.05). Post-hoc Tukey testing to compare the number of eggs laid before and after the mid-point, within each of the 6 treatment combinations (mating treatment x female condition) revealed that egg laying before and after the midpoint was significant in all treatments regardless of mating treatment or female condition (P<0.01) other than the high-condition mated females (P>0.5).

Discussion

Nutrition and mating are closely linked in females. In some species, mating triggers a strong feeding response in females, where females increase protein consumption and total dietary intake (Perry, 2011; Lee *et al*., 2013; Camus *et al*., 2018). We investigated the trajectory of post-mating dietary choice and concurrent egg laying rates in trios of unmated, mated and remated females over a one-month period. As predicted, mated flies consumed more protein and carbohydrate and laid more eggs than the unmated treatment groups, but contrary to our second prediction, the post-mating diet switch steadily decreased with time, a second mating boosted both short-term protein intake and egg-laying, indicating a dose-dependent response to mating.

Both mating and remating increase protein intake in females

As expected, female flies experienced a post-mating diet switch, consuming cumulatively more carbohydrate and protein than virgin females. This result is in line with previous findings in *D. melanogaster* (Carvalho *et al*., 2006; Lee *et al*., 2013; Camus *et al*., 2017) and other insect species (e.g. Pérez-Staples and Abraham, 2023). In *D. melanogaster*, the result is consistent with male stimulation of female feeding through the receipt of sex peptide during mating (Chapman *et al*., 2003; Liu & Kubli, 2003; Hopkins & Perry, 2022). In our study, females *D. melanogaster* experienced an additional increase in protein consumption after a second mating, 18 days following their initial mating. The rise in protein consumption at this point suggests that males are able to influence female feeding in a dose-dependent manner, such that post-mating diet preference is not a single, permanent switch but can be further activated by additional matings. This is consistent with a previous study in *D. melanogaster* on P:C ratios consumed after a second mating (Bowman & Tatar, 2016). Protein intake was increased upon mating, and increased further upon remating - virgin flies consumed 1:14 P:C, singly mated flies consumed 1:13 P:C, and twice-mated flies consumed 1:9 P:C (Bowman & Tatar, 2016). However, in that

previous study it was not clear that increased feeding was a response to remating, or a response to group variation, as mated and remated treatment groups were combined into one group before being segregated only after the remating. In addition, yeast rather than a defined protein diet was used, which may have impacted the feeding response. The potential for male-induced dose-dependent diet switches was also investigated in female two-spot ladybird, *A. bipunctata* (Perry, 2011). There was a small increase in the amount of food eaten by remated ladybirds versus singly mated ladybirds, suggesting only a weak effect of dose-dependent male-induced feeding in this species (Perry, 2011).

Surprisingly, we found similar consumption of carbohydrate between mating and diet treatments, which was unexpected, considering mated females typically increase overall consumption of both protein and carbohydrate (Lee *et al.*, 2013). We also found that carbohydrate intake increased across all treatment groups after the remating point, even in groups that did not experience remating. Though males were only provided to females in the remating treatments, females in all treatments were separated into individual agar-water vials to standardise handling. Therefore, the observed increase in carbohydrate intake across all treatment groups, but not protein intake, may be a result of energetic expenditure caused by handling stress.

Egg laying rates and post-mating protein consumption are linked

Females in the remating treatment showed increased protein consumption and egg laying immediately following their second mating, diverging from the singly mated females. Greater protein intake in females post-mating is understood to support the increased production of nutrient rich eggs (see Mirth et al., 2019), and both are manipulated by transferral of the sex peptide from the male's seminal fluid during mating (Chapman *et al.*, 2003; Carvalho *et al.*, 2006; Barnes *et al.*, 2008). The relationship between the post-mating feeding switch and egg

laying in females has been investigated using 'eggless' female *D. melanogaster* with the ovo^{D1} mutation (described in Oliver et al., 1987). Sex peptide transfer did not increase feeding in ovo^{D1} females, which suggested that the strength of the feeding-switch depended on egg-laying capacity (Barnes *et al.*, 2008). However, there is little evidence for a link between egg laying and post-mating diet intake in later studies using the ovo^{D1} mutation. Eggless ovo^{D1} females and wildtype females fed on sucrose for 3 days (yeast deprivation) showed a similar strength in yeast preference when placed in a dietary choice assay (Ribeiro & Dickson, 2010), while mated ovo^{D1} females also preferred a similar P:C ratio to mated wildtype females within a CAFE assay (Bowman & Tatar, 2016).

In this experiment, we stimulated realistic egg-laying rates by providing standard SYA medium to *D. melanogaster* females in between each CAFE measurement. Female propensity to lay on agar-water substrates (as used during CAFE assays) can be increased to some degree by increasing the softness of the oviposition substrate (Zhang et al., 2020), artificially adding yeast volatile cues (Gorter et al., 2016), or using yeast-based liquid protein diets (Bowman and Tatar, 2016). However, egg laying rates on agar are still lower than those observed on standard SYA media. Therefore, we used a design that would induce higher, and potentially more biologically realistic, egg-laying by using a rotation of SYA and CAFE vials. There is the possibility that females were able to fully satisfy their dietary requirements while on SYA and avoid feeding from the liquid diets, which may have impacted the CAFE results. However, since we observed changes in P:C preference between females that were consistent with previous studies, it is likely that such reduction in feeding during the CAFE assay did not strongly influence the results.

The issue of low egg-laying rates for *D. melanogaster* during CAFE assays has been acknowledged in previous studies, and some investigations have used alternative measurements of food intake to gain more realistic egg-laying and survival rates. Methods using solid diets tend to improve survival and oviposition when compared to CAFE, but it

remains difficult to measure precise and dynamic macronutrient consumption with solid diets. The number of times flies extend their proboscis onto solid and liquid food surfaces has been used as a reliable measure of feeding rate (Barnes *et al.*, 2008; Wong *et al.*, 2009). However, there may be undetectable variation in the volume of food consumed per proboscis extension over time, making it difficult to infer precise macronutrient preferences (Wong *et al.*, 2009). In fact, ovo^{D1} eggless females consume more diet per proboscis extension than control females (Wong *et al.*, 2009). In addition, patches of solid diet of differing P:C ratios can be used to measure diet preference (i.e., by counting the number of flies present on one patch versus another, at a given time). However, P:C ratios vary in softness and volatile cues, which would differentially affect oviposition rates.

Female condition and mating status impacted diet choice but not egg laying

Virgin flies from both developmental conditions ate comparable diets. However, female condition influenced post-mating diet preference: low-condition mated and remated females consistently ate less protein than the high-condition flies, in line with our prediction (4). Overall, flies in equivalent mating treatments were more closely aligned than flies in equivalent condition (e.g. high remated = low remated and high mated = low mated etc.). It is possible that reduced protein consumption by low-condition females is due to the development of fewer ovarioles (Bath *et al.*, 2023) and corresponding reductions in protein requirement for egg production (Duxbury & Chapman, 2020). Ovariole number (and fecundity) is also linked to body size in insects (Honěk, 1993), but we did not detect differences in body size between the two developmental conditions, despite previously observed size differences (May *et al.*, 2015; Bath *et al.*, 2023).

Although low-condition females ate less than high-condition females, low-condition females did not lay fewer eggs. Hence, it is possible that their diet response was not a direct result of

reduced capacity for egg-laying. Alternative physiological differences arising from developmental diet such as nutrient processing and foraging (reviewed in Ahmad et al., 2018) may alter female intake. Since larval diet also affects early embryogenesis in the oocytes, condition may have a more pronounced effect on egg-adult viability and adult offspring output, rather than egg laying rates measured in this study (Mirth *et al.*, 2019).

The results from this study suggest that a second mating in females induces a short-term increase in both protein consumption and egg laying. Conversely, remating in male *D. melanogaster* appears to have no effect on male diet choice: male flies do not alter the ratio or amount of P:C consumed over 5 matings (Sydney *et al.*, 2024). Though male gamete production is far less costly than in females, energy expenditure and depletion of proteinaceous seminal fluid components can produce nutritional costs to mating in males. It would be interesting to further explore the potential for dose-dependent male-induced feeding by studying effects of additional female re-mating (i.e., >2 matings), especially because as female fruit flies mate multiply in the wild (Milkman & Zeitler, 1974). In addition, it is unclear whether previously mated males would be differentially able to induce secondary diet-switches in females in ecologically relevant scenarios, considering seminal fluid depletion. However, experiments in this field are limited by female *D. melanogaster* reluctance to remate. Female mating rate can be increased by housing females continuously with males but such studies are limited by eventual trade-offs in reproduction and longevity (Partridge *et al.*, 1986).

References

Ahmad, M., Keebaugh, E.S., Tariq, M. & Ja, W.W. (2018) Evolutionary responses of *Drosophila melanogaster* under chronic malnutrition. *Frontiers in Ecology and Evolution*, **6**.

Barnes, A.I., Wigby, S., Boone, J.M., Partridge, L. & Chapman, T. (2008) Feeding, fecundity and lifespan in female *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences*, **275**, 1675–1683.

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015) Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, **67**, 1–48.

Bath, E., Rostant, W., Ostridge, H.J., Smith, S., Mason, J.S., Rafaluk-Mohr, T., *et al.* (2023) Sexual selection and the evolution of condition-dependence: an experimental test at two resource levels. *Evolution*, **77**, 776–788.

Becher, P.G., Flick, G., Rozpędowska, E., Schmidt, A., Hagman, A., Lebreton, S., *et al.* (2012) Yeast, not fruit volatiles mediate *Drosophila melanogaster* attraction, oviposition and development. *Functional Ecology*, **26**, 822–828.

Bowman, E. & Tatar, M. (2016) Reproduction regulates *Drosophila* nutrient intake through independent effects of egg production and sex peptide: Implications for aging. *Nutrition and Healthy Aging*, **4**, 55–61.

Brooks, M.E., Kristensen, K., Benthem, K.J. van, Magnusson, A., Berg, C.W., Nielsen, A., *et al.* (2017) glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. *The R Journal*, **9**, 378.

Camus, M.F., Fowler, K., Piper, M.W.D. & Reuter, M. (2017) Sex and genotype effects on nutrient-dependent fitness landscapes in *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences*, **284**, 20172237.

Camus, M.F., Huang, C.-C., Reuter, M. & Fowler, K. (2018) Dietary choices are influenced by genotype, mating status, and sex in *Drosophila melanogaster*. *Ecology and Evolution*, **8**, 5385–5393.

Carvalho, G.B., Kapahi, P., Anderson, D.J. & Benzer, S. (2006) Allocrine modulation of feeding behavior by the Sex Peptide of *Drosophila*. *Current Biology*, **16**, 692–696.

Chapman, T., Bangham, J., Vinti, G., Seifried, B., Lung, O., Wolfner, M.F., *et al.* (2003) The sex peptide of *Drosophila melanogaster*: Female post-mating responses analyzed by using RNA interference. *Proceedings of the National Academy of Sciences*, **100**, 9923–9928.

Chapman, T., Trevitt, S. & Partridge, L. (1994) Remating and male-derived nutrients in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **7**, 51–69.

Duxbury, E.M.L. & Chapman, T. (2020) Sex-Specific Responses of Life Span and Fitness to Variation in Developmental Versus Adult Diets in *Drosophila melanogaster*. *The Journals of Gerontology: Series A*, **75**, 1431–1438.

Greenspan, L. (1977) Humidity Fixed Points of Binary Saturated Aqueous Solutions. *Journal of Research of the National Bureau of Standards. Section A, Physics and Chemistry*, **81A**, 89–96.

Hartig, F. (2022) DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models.

Honěk, A. (1993) Intraspecific Variation in Body Size and Fecundity in Insects: A General Relationship. *Oikos*, **66**, 483–492.

Hopkins, B.R. & Perry, J.C. (2022) The evolution of sex peptide: sexual conflict, cooperation, and coevolution. *Biological Reviews*, **97**, 1426–1448.

Hyndman, R., Athanasopoulos, G., Bergmeir, C., Caceres, G., Chhay, L., O'Hara-Wild, M., *et al.* (2024) forecast: Forecasting functions for time series and linear models.

Hyndman, R.J. & Khandakar, Y. (2008) Automatic time series forecasting: the forecast package for R. *Journal of Statistical Software*, **27**, 1–22.

Ja, W.W., Carvalho, G.B., Mak, E.M., Rosa, N.N. de la, Fang, A.Y., Liong, J.C., *et al.* (2007) Prandiology of *Drosophila* and the CAFE assay. *Proceedings of the National Academy of Sciences*, **104**, 8253–8256.

Klepsatel, P., Knoblochová, D., Girish, T.N., Dircksen, H. & Gáliková, M. (2020) The influence of developmental diet on reproduction and metabolism in *Drosophila*. *BMC Evolutionary Biology*, **20**, 93.

Lee, K.P., Kim, J.-S. & Min, K.-J. (2013) Sexual dimorphism in nutrient intake and life span is mediated by mating in *Drosophila melanogaster*. *Animal Behaviour*, **86**, 987–992.

Lee, K.P., Simpson, S.J., Clissold, F.J., Brooks, R., Ballard, J.W.O., Taylor, P.W., *et al.* (2008) Lifespan and reproduction in *Drosophila*: New insights from nutritional geometry. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 2498–2503.

Lenth, R.V., Buerkner, P., Giné-Vázquez, I., Herve, M., Jung, M., Love, J., *et al.* (2023) emmeans: Estimated Marginal Means, aka Least-Squares Means.

Liu, H. & Kubli, E. (2003) Sex-peptide is the molecular basis of the sperm effect in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, **100**, 9929–9933.

May, C.M., Doroszuk, A. & Zwaan, B.J. (2015) The effect of developmental nutrition on life span and fecundity depends on the adult reproductive environment in *Drosophila melanogaster*. *Ecology and Evolution*, **5**, 1156–1168.

Meats, A. & Leighton, S.M. (2004) Protein consumption by mated, unmated, sterile and fertile adults of the Queensland fruit fly, *Bactrocera tryoni* and its relation to egg production. *Physiological Entomology*, **29**, 176–182.

Milkman, R. & Zeitler, R.R. (1974) Concurrent Multiple Paternity in Natural and Laboratory Populations of *Drosophila melanogaster*. *Genetics*, **78**, 1191–1193.

Mirth, C.K., Nogueira Alves, A. & Piper, M.D. (2019) Turning food into eggs: insights from nutritional biology and developmental physiology of *Drosophila melanogaster*. *Current Opinion in Insect Science*, Insect genomics: Development and regulation, **31**, 49–57.

Oliver, B., Perrimon, N. & Mahowald, A.P. (1987) The ovo locus is required for sex-specific germ line maintenance in *Drosophila*. *Genes & Development*, **1**, 913–923.

Partridge, L., Fowler, K., Trevitt, S. & Sharp, W. (1986) An examination of the effects of males on the survival and egg-production rates of female *Drosophila melanogaster*. *Journal of Insect Physiology*, **32**, 925–929.

Partridge, L., Green, A. & Fowler, K. (1987) Effects of egg-production and of exposure to males on female survival in *Drosophila melanogaster*. *Journal of Insect Physiology*, **33**, 745–749.

Pérez-Staples, D. & Abraham, S. (2023) Postcopulatory Behavior of Tephritid Flies. *Annual Review of Entomology*, **68**, 89–108.

Perry, J.C. (2011) Mating stimulates female feeding: testing the implications for the evolution of nuptial gifts. *Journal of Evolutionary Biology*, **24**, 1727–1736.

Ribeiro, C. & Dickson, B.J. (2010) Sex Peptide Receptor and Neuronal TOR/S6K Signaling Modulate Nutrient Balancing in *Drosophila*. *Current Biology*, **20**, 1000–1005.

Sydney, M.C., Chapman, T. & Perry, J.C. (2024) Diet choice is insensitive to mating in male fruit flies. *Animal Behaviour*, **214**, 73–86.

Trevitt, S., Fowler, K. & Partridge, L. (1988) An effect of egg-deposition on the subsequent fertility and remating frequency of female *Drosophila melanogaster*. *Journal of Insect Physiology*, **34**, 821–828.

Wong, R., Piper, M.D.W., Wertheim, B. & Partridge, L. (2009) Quantification of Food Intake in *Drosophila*. *PLOS ONE*, **4**, e6063.

Woodring, J.P., Clifford, C.W. & Beckman, B.R. (1979) Food utilization and metabolic efficiency in larval and adult house crickets. *Journal of Insect Physiology*, **25**, 903–912.

Yapici, N., Kim, Y.-J., Ribeiro, C. & Dickson, B.J. (2008) A receptor that mediates the post-mating switch in Drosophila reproductive behaviour. *Nature*, **451**, 33–37.

Appendix for Chapter 3

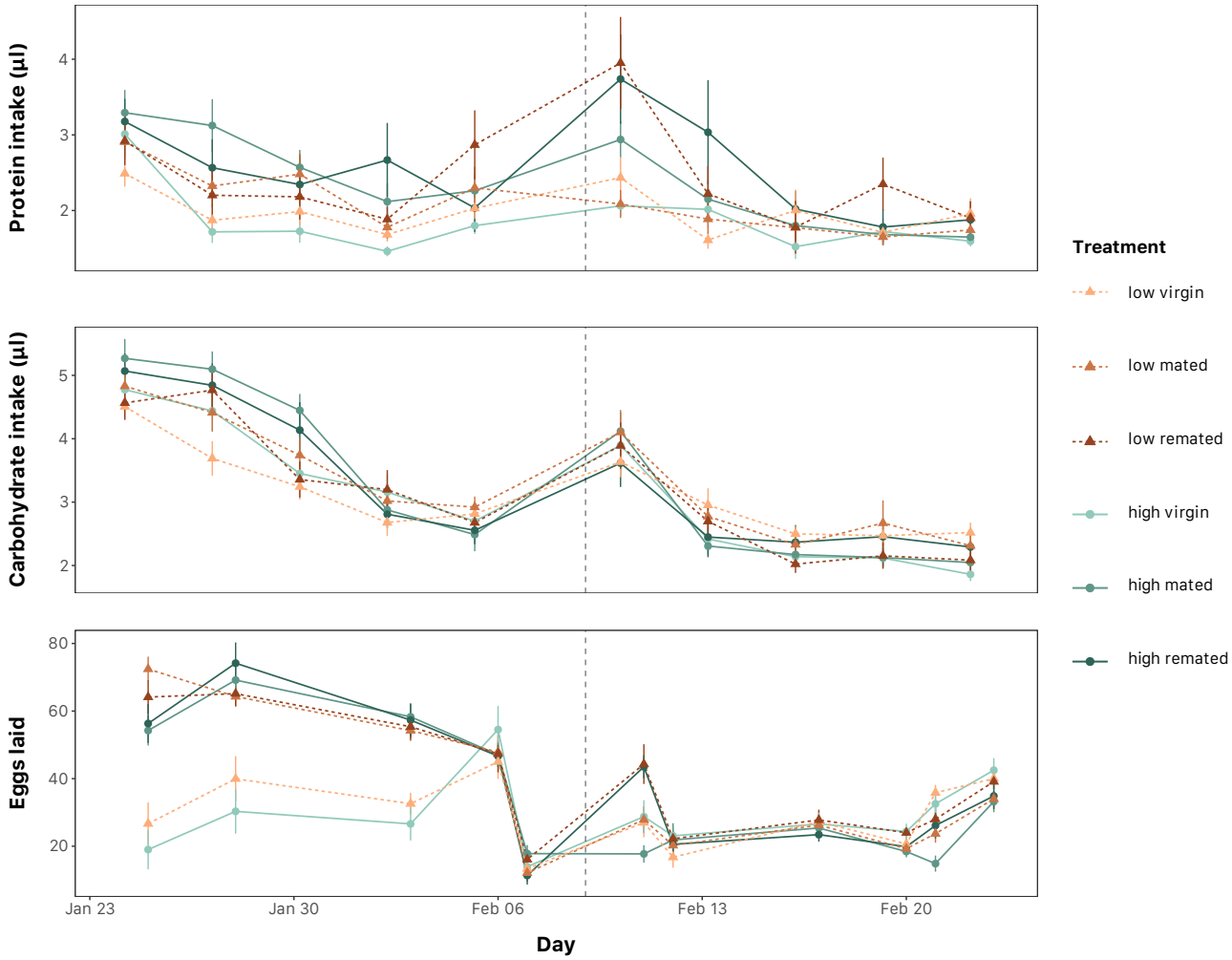


Figure A1

Daily consumption of protein diet, carbohydrate diet and egg laying across the 33-day experimental period (23rd January 2023 to 24th February 2023). Triangle points denote the mean consumption or egg number of females in each group (±SE), with triangles for females raised on a low-yeast diet, and circles for females raised on high-yeast diet. Grey, dashed line indicates the midpoint of the experiment, where females in the remated treatment were mated for a second time with virgin male flies.

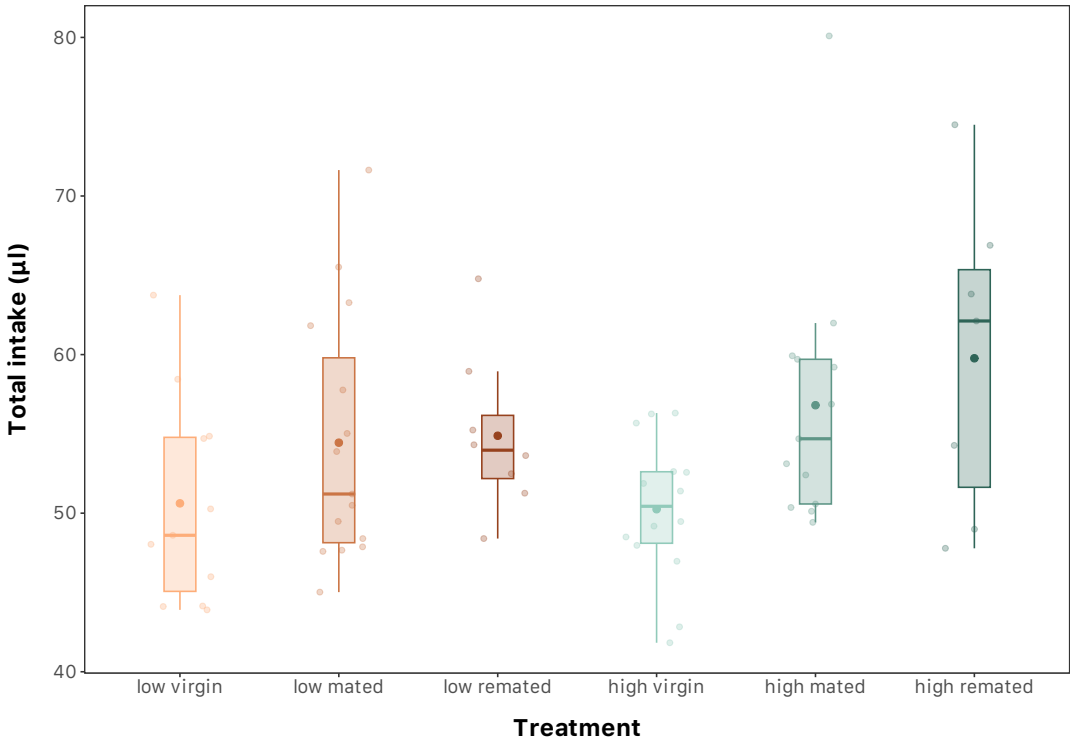


Figure A2

Total protein and carbohydrate intake of female flies across 10 CAFE assays. Intake is measured from three females per vial. Data is limited to IDs with both protein and carbohydrate intake values for all 10 CAFE assays only. Treatment represents female condition (low or high) and mating treatment (virgin, mated or remated). Each box represents interquartile range (IQR) with medians as thick horizontal lines and means as large, filled circles. Whiskers represent 1.5 x IQR, and small circles represent outliers.

Chapter 4

Female feeding and oviposition site preference is influenced by male social cues

Abstract

Sexual conflict over mating receptivity can be an important driver of mating behaviour in animals. For example, if sexual receptivity of females and males is mismatched, it can result in males repeatedly courting resistant females. This form of sexual harassment can disturb optimal feeding behaviour in females; for example, when male harassment drives females away from preferred foraging patches. Females are able to regulate their macronutrient intake by choosing to feed from diets of differing quality. However, it is unknown how females weigh costs of sexual harassment against macronutrient composition in foraging decisions. In this study, we investigated female feeding choice in response to male harassment risk, while controlling for physical displacement from direct interactions between the sexes. Female feeding and oviposition site choice of *Drosophila melanogaster* were measured between two diet patches differing in macronutrient balance, in experimentally controlled socio-sexual groups of single-sex female flies, mixed-sex flies, or restricted male flies. Restricted males were enclosed on diet patches and were unable to physically interact with females. Contrary to our predictions, females did not avoid restricted males and prioritized social information above direct information on nutrient quality and above harassment risk and moved to the most populous patch in response, regardless of macronutrient content. In addition, diet preference

was not altered by the presence of unrestricted males despite observed harassment behaviour. Female movement to patches containing males suggests that females used the presence of males as a social cue of foraging patch quality.

Introduction

Mating is often costly for females. In fact, when matings are frequent, the benefits of mating for females may even be outweighed by the costs. This can contribute to a mismatch in mating rate optima between the sexes and hence the potential for sexual conflict. Females may mate at a low rate to avoid costs arising from energy expenditure (Watson *et al.*, 1998), reduced longevity (Fowler & Partridge, 1989), predation (Wing, 1988; Herberstein *et al.*, 2002), disease transmission (Thrall *et al.*, 1997) or physical damage during mating (Stutt & Siva-Jothy, 2001). In contrast, per mating costs may often be lower for males, and their fitness instead is more likely to be limited by the number of times they are able to mate. In species where the cost to females of resisting mating is less than the cost of mating itself, sexual harassment can occur, whereby males attempt to coerce resistant females into mating. Sexual harassment can persist as an adaptive strategy in males when the costs of finding a new potential mate outweigh energy wasted on unsuccessful courtship attempts. As such, levels of harassment are positively correlated with the number of successful matings (Rowe, 1992).

The consequences of this type of sexual harassment can be far reaching. For example, there is evidence that it can disrupt female nutrient intake. This is expected to have important effects, as nutrient intake and diet composition are important determinants of fitness, with the balance of two major macronutrients (protein and carbohydrate) altering reproductive success and longevity in many species (Simpson & Raubenheimer, 2012). For example, female egg production is maximised on high-protein diets, whereas female lifespan is maximised on high-carbohydrate diets (Lee *et al.*, 2008; Maklakov *et al.*, 2008; Fanson & Taylor, 2012). In *Drosophila melanogaster*, females subjected to harassment from males reduced time spent on their preferred food and laid fewer eggs, potentially as a result of continually dodging males without pausing to feed for long (Teseo *et al.*, 2016). Similar observations have been made in mixed-sex groups of *Gerris buenoi* water striders (Rowe, 1994). In live-bearing poeciliid fish,

sexual harassment reduced foraging time of female guppies, *Poecilia reticulata* (Magurran & Seghers, 1997) regardless of hunger levels (Griffiths, 1996). Foraging efficiency also decreased in female mosquitofish, *Gambusia holbrooki*, when a male was present (Pilastro *et al*., 2003).

Despite the range of reported direct effects of sexual harassment on female feeding, few studies have investigated whether females dynamically offset their nutrient intake if males are nearby. High quality feeding sites might often be occupied by multiple conspecific and heterospecific individuals (Stone, 1995; McLain & Pratt, 1999; Tinette *et al*., 2004), increasing the incidence of harassment. It is unknown whether females make dietary trade-offs by feeding on low-quality patches where fewer males are present to avoid costly harassment, versus bearing the costs of harassment on high-quality patches in order to access high-quality diets. Individuals are known to be able to dynamically adjust their food intake under different social contexts. For example, individuals can adjust their feeding habits depending on social cues, such as the number of individuals present (as studied within the recent field of 'social nutrition') (Lihoreau *et al*., 2015, 2016a, 2018) and harassment risk (Stone, 1995; McLain & Pratt, 1999). Specifically, females of *Anthophora plumipes* flower bees fed on flowers sheltered further within plant bushes when males were present on the outer, more accessible flowers (Stone, 1995), while prey capture decreased in *Argiope keyserlingi* orb-web spiders when a courting male was present on a female's web (Herberstein *et al*., 2002).

In this study, we measured the diet preference of female *D. melanogaster* when they were provided with a choice of diets that differed in protein to carbohydrate ratio (P:C) under different regimes of male harassment risk. Our aim was to test whether the presence of males alters female foraging behaviour in a manner that pushes females off their dietary optima. *D. melanogaster* is a highly suitable system for this study due to established methods of testing dietary preference (Piper, 2017) and easily observable harassment behaviour. Females of this

species are reluctant to remate for around 5 days after their first mating (Singh & Singh, 2004), while males can mate multiply in quick succession (up to 11 times) (Douglas *et al.*, 2020). This can result in continuous sexual harassment of females by males. In this experiment, female *D. melanogaster* were assigned to arenas containing two solid foraging patches of high P:C and low P:C content. Specifically, P:C ratios of 4:1 and 1:4 were chosen to represent contrasting high and low protein solid diets, since previous studies indicate increased preference for, and reproductive fitness on, protein-rich diets (Jang & Lee, 2018). Hence, these two patches each represented a preferred and non-preferred diet. These ratios also fall within a range of solid diets for *D. melanogaster* females tested previously with contrasting impacts on life-history traits – female lifespan was maximised on a diet of 1:4, while early life egg production was maximised on a diet of 8:1 (Jang & Lee, 2018). In addition, expected preference for the 4:1 solid diet over the 1:4 solid diet was observed in the choice assay described below, which used coloured dyes to alter the colour of the patches (Diet preferences – colour assay). We manipulated female exposure to harassment by males in experimentally controlled socio-sexual environments (same sex or exposed directly or indirectly to the opposite sex). We then recorded the dietary and oviposition preferences of groups of wild type, mated females. To verify that female location was a reliable indication of foraging choice, we conducted an additional experiment using dyes to alter the colour of diet patches, as diets coloured with dye are visible externally in fly abdomens once ingested (Ribeiro & Dickson, 2010). This method allowed us to compare whether behavioural data on fly location correlated with the colour of the diet consumed and controlled for the slight colour differences between the high P:C and low P:C diets. Overall, we predicted that: (1) females in the same sex treatment would show a strong preference for the preferred, high protein diet (in agreement with previous results); (2) females in treatments exposed directly or indirectly to males would avoid patches with a strong male presence, or with cues of male presence, and thus show an increase in dietary

preference for the alternative less preferred diet; (3) females would show stronger alterations in preferences when in direct contact with courting males, as opposed to only male cues.

Methods

Fly stocks and rearing

Experimental flies were maintained on a standard sugar-yeast-agar (SYA) diet (50 g sucrose, 100 g brewer's yeast, 15 g agar, 30 ml Nipagin (10 % solution), 3 ml propionic acid, 970 ml water) from a large stock population of outbred wildtype Dahomey flies (Chapman, Trevitt and Partridge, 1994). Eggs were collected from the stock population in glass bottles containing 70 ml SYA medium and allowed to develop over a 10-day period to standardise parental age and rearing conditions. The resulting adults were used as parents of the experimental flies to limit parental carry over effects. Eggs were collected from the parental generation using dishes of purple-grape-juice medium supplemented with live yeast paste. Eggs were allowed to develop for 24 h, and controlled densities of 50 first instar larvae were transferred to glass vials containing 7 ml of SYA medium. Experimental adults were collected as virgins using ice anaesthesia and housed in vials of SYA in groups of 10 virgin males or mixed sex groups containing 20 females and five males. Following a 3-day period, mated females were moved from the mixed sex groups into new vials for 24 h before the start of the experiment. A subset of females was retained in individual vials and allowed to lay eggs to confirm that females were fertile (N = 54, 98 % of vials contained offspring). All flies and experiments were maintained in a controlled environment at 50 % humidity and 25 °C, under a 12 h light-dark schedule.

Diets

Solid meridic diets for the main experiments were made following a recipe adapted from Piper and colleagues (2014). Recipes are included in the Appendix, Tables A1 and A2. Both diets were made to 1 L with equal amounts of cholesterol, lecithin, agar, water, preservatives and essential vitamins and salts, while amounts of sucrose (C) and bovine casein (P) were adjusted to alter the protein: carbohydrate (P:C) ratio (24 g bovine casein and 96 g sucrose in 1:4 diet,

96 g bovine casein and 24 g sucrose in 4:1 diet). For the additional colour assay using dye to confirm diet intake, 4:1 and 1:4 diets were dyed with red and blue pigment, which can subsequently be observed externally within fly guts following ingestion of those diets (Ribeiro & Dickson, 2010). 125 mg of powdered red dye (amaranth, Sigma A1016) and 50 mg of powdered blue dye (indigo carmine, Sigma 131164) were added to separate 250 ml aliquots of each diet, to create four diet types (red 1:4 and 4:1, blue 1:4 and 4:1). All diets were autoclaved at 120 °C for 15 minutes for sterilisation and poured into trays at approximately 5 mm thickness to set. Diets were stored at 2 °C until use.

On the morning of observations, patches were cut from 1:4 and 4:1 diets using a 26 mm circular cutter. One patch from each diet was placed at equal distances into plastic petri dish observation arenas (100 mm x 15 mm) and moved to room temperature. Pin holes in the sides of these petri dish arenas allowed for the insertion of a CO_2 gun for anaesthetising the flies.

Diet preferences – main experiment

Data on the effect of direct or indirect exposure to males on female dietary and oviposition preferences were collected across two experimental blocks. Observations were taken over three days in block one (five observations on day one, three on day two and three on day three) and block two (five observations on day one, five on day two and six on day three). All other methods were identical. Offspring data was collected from the first day of block two only. The aim was to test the diet preferences of females for high P versus high C diets in scenarios where they were exposed to males directly (females + males in the arenas), indirectly (males confined to a portion of either food patch within an acetate arena, preventing direct contact with females) or not exposed to males. The experimental treatments (Figure 1) were: single sex females, freely interacting mixed sex groups, males enclosed on the preferred diet, and males enclosed on the non-preferred diet.

Strips of acetate transparency film were formed into 150 mm diameter rings, which were pressed into each patch of food, so that roughly 1/3 of the patch was also enclosed. In the relevant treatments, males were placed within one of these acetate barriers to prevent direct harassment and physical interaction with females. This allowed visual, pheromone and auditory cues of males to be detected, potentially, by females in the wider arenas (Bretman *et al.*, 2011; Fowler *et al.*, 2022) without any direct contact between the sexes. To control for the presence of the acetates, each observation arena contained two non-focal fly acetate enclosures, regardless of whether any males were held within the enclosures. Rings were cut to 13 mm high to ensure close contact with the lid to prevent files escaping and perforated using a mounted needle.

Four-day old experimental mated females were added to each of the arenas in groups of ten, using CO_2 anaesthesia (block 1 N = 52 arenas, block 2 N = 64 arenas). In all treatments, 10 females were added to the centre of each arena and had access to both diet patches. In the male restricted treatments, 10 males were added to either the 1:4 or 4:1 patch enclosure. In the mixed-sex treatment, 10 males were added to the centre of the arenas alongside females (Figure 1). Flies were then allowed to acclimatise for one hour before observations began.

Behavioural observations of female patch preference and sexual harassment

Arenas were scan sampled every 0.5 - 1 h and the number of female flies resting on the 1:4 patch, the 4:1 patch, or not on the food (i.e., on the dish or next to the acetate barrier) were recorded. At each timepoint, a two-second video was recorded for each arena containing the mixed-sex treatment in order to assess the level of courtships and potential for sexual harassment of females. Sexual harassment was defined as observations of male files engaging

in courtship towards a female (licking, wing-vibrating, orientation, or chasing) which did not result in mating (Bastock & Manning, 1955). Any matings were also recorded.

Flies remained in the arenas overnight after the first day of observations. Flies were anaesthetised using CO_2 in the morning of the second day, to allow replacement of the food patches by fresh diet from the same food batches. Diet patches were swapped out in between the daily behavioural measurements to control for the effect of any larvae present on female patch preferences, as larval digestion could alter diet composition and contribute additional social cues. Following anaesthetisation, flies were allowed to acclimatise for one hour, before behavioural scans began following the same protocol as for day one. Food patch replacement and behavioural observations were then repeated for a third day.

Offspring development

Diet patches were retained following the first day of behavioural observations and placed in individual SYA vials to allow larval development on a standardised food source and thus to record the number of offspring emerging from each of the diet patches. Offspring were left to develop over 12 days to ensure eclosion of adults, and vials were subsequently frozen. Numbers of adult offspring per vial were then counted to profile reproduction on each patch in the 24 h window (N = 64 patches per diet).

Diet preferences – colour assay

Diet preference

I next tested whether the location preference of females was aligned to diet intake, and whether preference was influenced by differences in food colours. Diet patches dyed with either red or blue dye were cut from each P:C diet type using a 26 mm circular cutter. Two food

patches were added to each observation arena (*N = 28*) prior to the introduction of groups of the females and the start of the preference tests. Half of the arenas contained one patch of red 4:1 and one of blue 1:4, while the other half of arenas contained one patch of blue 4:1 and one of red 1:4. Arenas were then randomised using coded identifiers, to anonymise the treatments from the perspective of the observer.

Behavioural observations of female patch preference

Three-day old, mated females were collected from a second generation of controlled rearing (by the method described above) and added to the blue and red food arenas in single sex groups of 10 using CO_2 anaesthesia (*N* = 64). Arenas were then moved to 25 °C and left to acclimatise for 2 hours. Food patch preference scores were obtained for each arena by scan sampling the number of individuals on each location (blue patch/red patch/dish) every 0.5 h, for 2.5 h.

After the last observation, flies were anaesthetised using CO_2 and photos were taken of each individual female's abdomen using a digital microscope camera (GXCAM, GT Vision Ltd; *N* = 280 photos). Camera settings were fixed for all photos, and the origin arena was recorded for later identification of treatment. Photos were edited post-production to adjust contrast and tone blue light levels, using the Photos app (Version 9.0, Apple Inc.) to apply edits identically to all photos (Appendix, Figure A1). To determine abdomen hue, five observers were each provided with the photos in a randomised order in PowerPoint (Microsoft) and were asked to score the colour of each fly's abdomen using a reference colour score bar; 0 – no colour, 1 – blue, 2 – purple or 3 – red (Appendix, Figure A2).

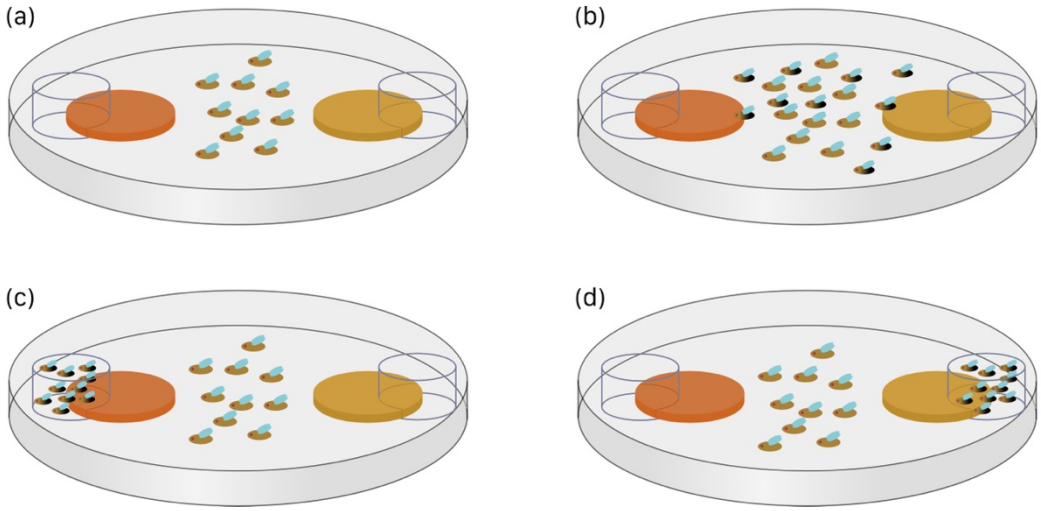


Figure 1

Experimental design of the behavioural assay in the sexual harassment experiment. 10 females were added to Petri dishes containing two discs of 4:1 and 1:4 diet, each supporting an acetate ring. (a) In the single sex treatment, females remained in a single sex group throughout the observation period. (b) In the mixed-sex treatment, 10 males were added to the females in main arena space and the sexes were able to interact. Two treatments included patches containing enclosed males: (c) 10 males were added within the acetate barrier on the 4:1 diet and 10 females to the main arena, (d) similar to c, but 10 males were added within the acetate barrier on the 1:4 diet.

Statistical analysis

All statistical analyses were carried out in R version 4.0.4 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org) and all statistical models can be found in the appendix for Chapter 4. Model fit was checked using the R package DHARMa (Hartig, 2022), and post-hoc Tukey testing using estimated marginal means package emmeans (Lenth *et al.*, 2023), in all cases.

Diet preferences – main experiment

The number of flies present on the 1:4 food, 4:1 food or not on the food was recorded, over 11 and 13 observations for block 1 and 2, respectively. Total counts for arenas that did not contain 10 females were excluded as these represented arenas where flies had escaped or where non-focal flies had entered. To calculate % preference for the expected preferred diet of 4:1, observations were filtered to arenas in which 50 % or more of the females were present on either food patch (i.e., arenas showing evidence for diet preferences) (*N* = 1060 observations). Arenas where 50 % or more of flies were not on a food patch were not included in this analysis (*N* = 377 observations total). The response variable % preference, was calculated for each arena at each observation:

$$\frac{N\text{♀ on 4:1}}{N\text{♀ on 4:1} + N\text{♀ on 1:4}} \times 100$$

To assess the effect of treatment on % preference for the 4:1 P:C diet, we ran generalised linear mixed models (GLMM) using the R package glmmTMB (Brooks et al., 2017). To account for repeated measures of the arenas over each observation, the arena ID and observation number were included as random effects. Block was first included as a fixed effect, and subsequently removed from the final model, as it was not significant.

Offspring counts – main experiment

The effect of treatment group on the number of offspring per patch was analysed separately per diet type in two models: the number of offspring on each 4:1 patch was compared across the treatment groups and the number of offspring on each 1:4 patch was compared across the treatment groups. Data were analysed in GLMMs using the R package glmmTMB (Brooks et al., 2017), where arena ID was included as a random effect in both models.

Diet preferences – colour assay

The effect of diet colour on diet preference was analysed by using a binomial GLMM, in which the number of flies on each patch was combined using the cbind function. Arena ID and observation were included as nested random factors.

Results

Female patch preference was dependent on male location not macronutrients

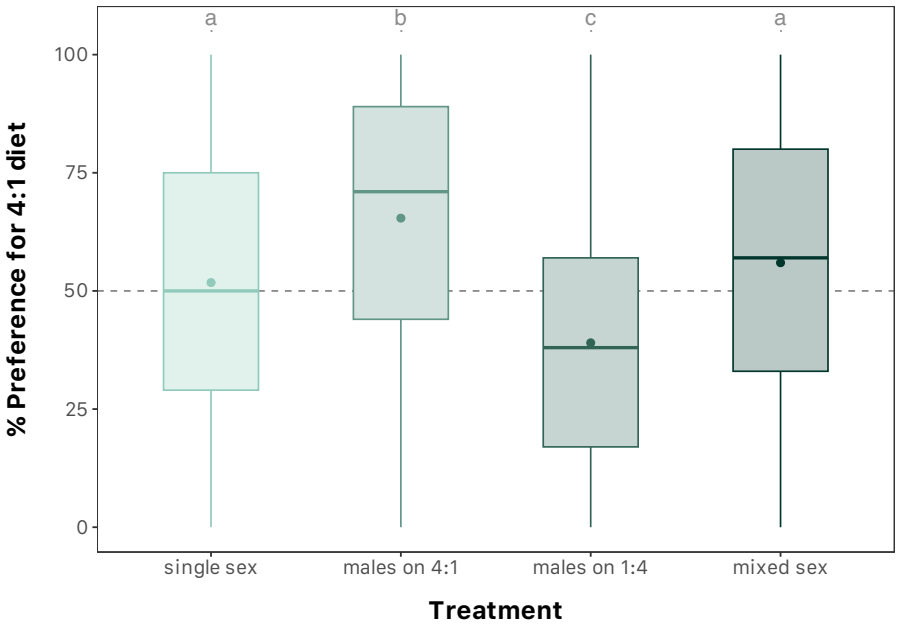


Figure 2

The percentage of females resting on the 4:1 patch versus the 1:4 patch under varying socio-sexual treatments. Shown are preferences from arenas in which 50 % of flies had expressed a dietary choice. Measures of female location were collected using scan sampling over three days. Boxes represent interquartile range (IQR) with whiskers as 1.5 x IQR. Medians are presented as thick horizontal lines and means as large, filled circles. Letters above boxes denote statistically significant differences between treatment groups for 4:1 diet preference.

Overall, preference for the 4:1 P:C diet was altered significantly depending on the presence and location of males in an arena (χ^2_3=48.6, P<0.001) (Figure 2). Specifically, female preference for 4:1 diets was actually increased when males were enclosed on the preferred 4:1 patches, compared to all other treatments (post-hoc Tukey tests: males on 4:1 versus single-sex: t_{1053}=-3.8, P<0.001, males on 4:1 versus mixed-sex: t_{1053}=3.1, P=0.01, males on 4:1 versus males on 1:4: t_{1053}=-6.9, P<0.001). Similarly, females also shifted their diet preference towards the non-preferred 1:4 patch when males were enclosed on those 1:4 patches (post-hoc Tukey tests: males on 1:4 versus single-sex: t_{1053}=3.2, P=0.008, males on 1:4 versus mixed-sex: t_{1053}=-3.9, P<0.001).

Unexpectedly, counter to hypothesis 1, there was no overall female preference for the 4:1 diet in the single sex treatment (mean = 52.6 %), despite previous work establishing a consistent strong preference for similar protein rich diets. There was no difference in preference for 4:1 between the single-sex and mixed-sex treatments (post-hoc Tukey test: t_{1053}=-0.7, P=0.98), with no effect of block on preference (χ^2_1=1.6, P=0.21).

At least one incidence of harassment was observed in 83 % of observations across the “mixed” treatment (N = 348 observations). The maximum number of observed courtship events in a single arena observation was 8, over the 10 females.

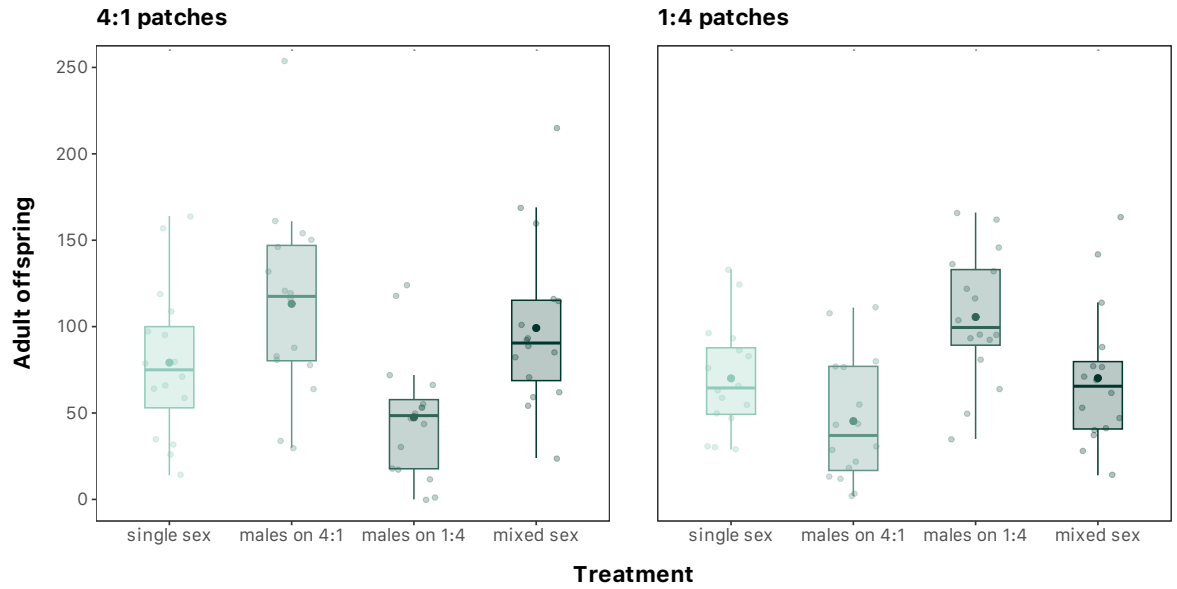


Figure 3

Number of adult offspring produced from diet patches taken from observation arenas containing 10 females, exposed directly or indirectly to 10 males per patch, or to no males. Boxes represent interquartile range (IQR) with whiskers as 1.5 x IQR and small grey circles are the raw data overlaid on their respective group. Medians are presented as thick horizontal lines and means as large, filled circles. Letters above boxes denote statistically significant differences between treatment groups, for 4:1 and 1:4 patches respectively.

Offspring production was highest on diet patches with enclosed males

Overall, the mean total offspring on each diet was 85 ±SE 0.7 and 73 ±SE 0.6, for 4:1 and 1:4 respectively (N=64 patches; χ^2_1=2.1, P=0.14) (Figure 3). The number of adult offspring produced from the food patches varied significantly according to treatment, for both 4:1 (χ^2_3=15, P=0.002) and 1:4 (χ^2_3=24.3, P<0.001) diets. The average number of offspring emerging from 4:1 patches that also held confined males was higher than for 4:1 patches under all other treatments (113 ±SE 13.9 offspring when males were enclosed on 4:1 versus 79±SE 10.9 for single-sex groups, 53±SE 9.3 when males were enclosed on 1:4 and 99 ±SE 12 for mixed-sex groups). However, there was no significant difference in number of offspring emerging from

4:1 when males were also enclosed on 4:1, versus the single-sex and mixed-sex treatments (post-hoc Tukey tests: single-sex t_{56}=-2.1, P=0.15, mixed-sex: t_{56}=0.9, P=0.81). The number of offspring from the 4:1 patch when males were also enclosed on 4:1, was significantly higher than the offspring from 4:1 when males were instead enclosed on the 1:4 patch (males confined on 4:1 versus 1:4 treatment, post-hoc Tukey tests: t_{56}=3.6, P<0.01).

Across the 1:4 patches collected from each treatment, the average number of offspring collected from 1:4 patches that also held confined males was higher than for 1:4 patches under all other treatments (107 ±SE 8.5 offspring when males were enclosed on 1:4 versus 70±SE 7.8 for single-sex groups, 45 ±SE 8.9 when males were enclosed on 4:1 and 70 ±SE 10.2 for mixed-sex groups). The number of offspring collected from 1:4 patches when males were also confined to the 1:4 patch was found to be significantly higher in post-hoc Tukey tests (males held on 4:1 vs males held on 1:4; t_{56}=-4.9, P<.0001, males held on 1:4 vs mixed-sex groups; t_{56}=2.9, P<0.05, single sex groups vs males held on 1:4; t_{56}=-2.9, P<0.05).

Overall, the results did not support the hypotheses that females show a strong preference for high protein diets (4:1) consistent with previous study. In addition, females did not avoid patches with a strong male presence for foraging or egg laying. Instead, the results suggest that females preferred to visit patches with a strong male presence.

Location of females aligned with the colour of dye in their abdomen

Unlike in the main experiment, here, females in all treatments showed the strong, expected preference for the high protein 4:1 food (Figure 4). This preference was observed regardless of the colour of the diet patches. Treatment (colour of the 4:1 food) did not affect the number of females on each diet patch (χ^2_1=0.2, P=0.69). The proportion of abdominal images scored as

red/blue within each treatment also closely aligned to the observed preferences for 4:1 observed in the behavioural data (Figure 5).

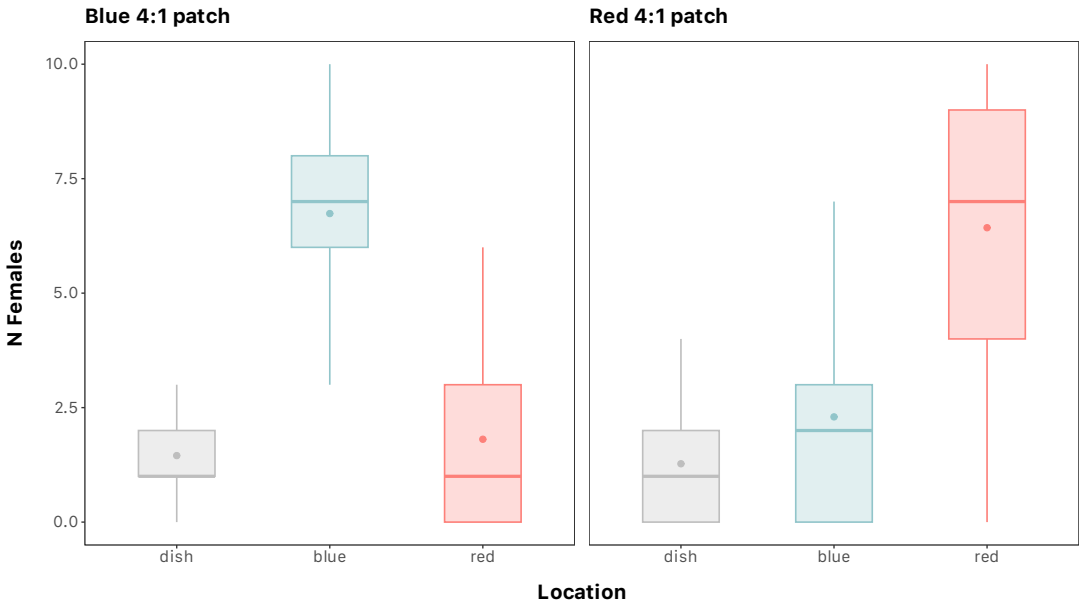


Figure 4

The number of female flies resting on the 1:4 and 4:1 blue and red diets or on the dish across six observations of 28 arenas. Boxes represent interquartile range (IQR) with whiskers as 1.5 x IQR and small grey circles are the raw data overlaid on their respective group. Medians are presented as thick horizontal lines and means as large, filled circles.

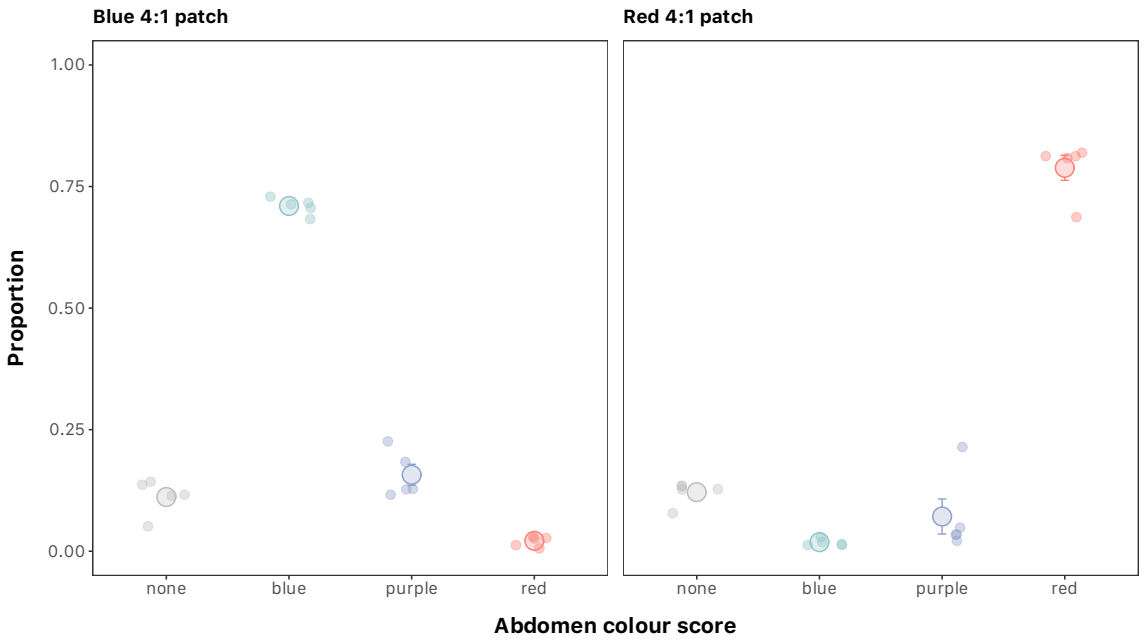


Figure 5

The proportion of scores assigned to photos of female flies after feeding in arenas with two patches of dyed diets (either blue 4:1 and red 1:4 patch, or red 4:1 and blue 1:4 patch) (*N* = 280 females). Scores were obtained from observers marking the colour of each fly's abdomen in photos as none, blue, purple or red. Large, coloured circles represent the average proportion of photos scored as either none, blue, purple, or red, with black, vertical lines as standard error. Small circles represent the proportion per observer.

Discussion

Time spent in superfluous matings, or in countering the effects of high levels of male courtship, can disrupt female feeding rates. In insects, copulation may involve physical constraints of carrying mounted males, which can reduce female foraging ability (Rowe, 1992). Pre-copulatory sexual harassment can also reduce female feeding across taxa (Stone, 1995; Magurran & Seghers, 1997; Herberstein *et al.*, 2002; Teseo *et al.*, 2016). However, little is known about whether females dynamically change their nutrient intake in response to sexual harassment, including whether females might feed on sub-optimal food patches to avoid males or because they have been physically displaced onto those diets by males. Here, I tested whether female diet preference was altered in socio-sexual settings in direct response to the risk of sexual harassment. We predicted that (1) females in single-sex groups would show a strong preferred for the high protein (4:1) diet; (2) females in mixed-sex groups would alter their preference away from diet patches with strong male presence or strong cues of male presence, towards an alternative diet patch; (3) direct, physical contact with males in mixed-sex groups would strongly disrupt female feeding and diet preference, compared to treatments with cues of males only. Contrary to the predictions (1 and 2), I did not see strong preference for the high-protein 4:1 diet in the main experiment in the absence of males in the single-sex treatment. Diet preference was also not reduced when males were present enclosed on a patch behind an acetate arena. In fact, the data show the opposite. Females were significantly attracted to diet patches that contained males within the acetate arenas, and this was the case for both the 4:1 and the 1:4 diets. Prediction 3 was also not met as preference for the high-protein diet was the same between the single-sex and mixed-sex treatments in this main experiment.

Females showed preference for high protein diets with dye, but not the main experiment

There was no strong preference for either the 4:1 or 1:4 diet when females were held in single-sex groups. Therefore, we would not have been able to see disruption to a high protein preference in the main experiment when males were freely able to interact with females in the mixed-sex treatment – because that preference was unexpectedly not observed. However, females in the coloured diet experiment did show the strong, expected preference for the 4:1 diet. The reason for the discrepancy is not clear. Mated females tend to prefer higher protein diets, which increase their reproductive output (Maklakov *et al.*, 2008; Lee *et al.*, 2013), and the P:C ratios chosen for the solid diets in this experiment reflect the nutritional range previously found to support *D. melanogaster* egg production (high protein) versus lifespan (low protein) (Jang & Lee, 2018). However, a contrasting preference for high carbohydrate solid foods has been identified in females when given a choice of eight patches from 1:16 to 8:1 P:C for both feeding and oviposition (Lihoreau *et al.*, 2016b). In addition, it is possible that the overall effect of acetate strips in each of the arenas has an unanticipated effect on preferences, and further work would be needed to test this.

Diet preference depended on male location

Female diet preference altered significantly according to whether females were directly or indirectly exposed to males. However, rather than avoiding patches containing males, we detected a significant increase in female preference for feeding patches where males were also present within the acetate arenas. This attraction of females could result from social aggregation, consistent with experiments testing the collective decisions made by groups of female *D. melanogaster* when provided with a choice between identical food patches (Lihoreau *et al.*, 2016a). This earlier study shows that, after the first flies had made a choice for a patch, new individuals were more likely to join a patch that contained over four individuals, rather than randomly distribute themselves (Lihoreau *et al.*, 2016a). By this mechanism, female

preference for space or nutrient quality could be overridden by a drive to join the most populous patch (Lihoreau *et al.*, 2016a). This could be especially important considering possible competition for food and oviposition sites between foraging female flies. In the study by Lihoreau and colleagues (2016a), females showed a mild preference for joining social groups when held in groups of ten (as in this study), but the preference for joining groups was stronger in larger groups (up to 100 females; (Lihoreau *et al.*, 2016a). Hence, it is possible that a social aggregation effect contributed to our finding that females preferred food patches containing males. It is also possible that this effect could be stronger with larger numbers of same-sex conspecifics, but this remains to be tested.

Female preference for locations containing males could occur as a result of the detection of odorant cues emitted by males to attract females (Landolt, 1997). Insect odorant cues are commonly emitted by female insects to attracting males and avoid costly mate searching. However, male emitted odorant cues occur widely in *D. melanogaster* (Bartelt *et al.*, 1985), medfly, *Ceratits capitata* (Light *et al.*, 1999) and multiple species of moth (Greenfield, 1981). Cuticular hydrocarbons from male *D. melanogaster* act as both close- and long-range pheromones for females (Bartelt *et al.*, 1985; Dweck *et al.*, 2015). Female responses to these pheromones are also connected to nutritional status and dietary odours (Lebreton *et al.*, 2015). Fed females show a much lower preference for a food odorants than do starved females, but addition of the *D. melanogaster* sex pheromone, cis-vaccenyl acetate, increased attractiveness of food odorants to fed females (Lebreton *et al.*, 2015). Preference for the diets in our study could have been shaped by *D. melanogaster* pheromones, with the potential to over-ride nutrient content in the patches.

We expected to see disrupted preferences for 4:1 P:C in the mixed-sex treatment, as males were able to move freely and disturb female feeding or resting. Observationally, activity levels

were much higher in the mixed-sex group than the other treatments, but further study is needed to fully capture the spatial and temporal movement of females (e.g., filming to track individual movement). Reduced feeding and foraging time as a result of sexual harassment has been observed extensively in poecilids (Magurran & Seghers, 1997). In the mosquitofish, *G. holbrooki*, female fish took longer to take the first bite of food and ate less per minute when a male was added to a tank containing a single focal female, although not when another female was added (Pilastro *et al.*, 2003). There is also evidence for sexual harassment disturbing female feeding in a study of *D. melanogaster* (Teseo *et al.*, 2016). Groups of females were given a choice of two fruit-based diets, one containing an aversive stimulus (quinine) under low or high levels of harassment, created via different sex-ratios (6 females:24 males versus 24 females:6 males) (Teseo *et al.*, 2016). When females were separated and allowed to choose either the aversive or attractive diet, following a stimulus learning period, proportionally fewer females from the high harassment groups correctly chose the diet without quinine (Teseo *et al.*, 2016). Females from the high harassment treatment also laid fewer eggs (Teseo *et al.*, 2016). The authors suggested that this resulted from harassed females having less time to associate the aversive stimulus with the diet or oviposit (Teseo *et al.*, 2016). It would be interesting to expose females to these differing levels of harassment and allow them to choose between diets of differing nutritional quality, as an alternative to aversive stimuli. Perhaps females experiencing high levels of harassment during a learning phase would also be slower to select a beneficial diet and oviposition site.

Offspring numbers were highest on patches containing males, regardless of P:C content

The number of offspring from 4:1 patches was highest when males were also confined on the 4:1 patches, and similarly, more offspring emerged from 1:4 patches containing the confined males. This result is consistent with the behavioural data on female patch preference collected from experiment one and suggests that females lay eggs on the same patch on which they feed.

However, there is evidence that female *D. melanogaster* are able to make oviposition choices depending on nutrient quality for larvae (Lihoreau *et al.*, 2016b). In that study, females laid proportionally more eggs on high-carbohydrate patches (1:16 P:C) but were also observed feeding on patches of a higher concentration of protein, suggesting their choices for oviposition and feedings weren't inextricably linked (in choice assays, females ate a ratio of 1:1.6) (Lihoreau *et al.*, 2016b). Despite this, the authors found that higher protein diets were better for larvae fitness: 1:2 was best for egg to adult survival, while 8:1 resulted in the fastest development times (Lihoreau *et al.*, 2016b), in agreement with other reports of larval nutrition (Rodrigues *et al.*, 2015; Jang & Lee, 2018). Female choice for carbohydrate rich oviposition sites is perhaps in line with protein availability in rotting fruit: while ripe fruit is initially rich in sugars, the quantity and diversity of yeast increases as decomposition progresses (Morais *et al.*, 1995; Matavelli *et al.*, 2015). In our study, females were attracted to locations containing males, regardless of nutrient quality for larvae. This could suggest that females are not assessing diets in terms of larval diet quality, or alternatively, that the presence of males (or conspecifics more generally) on a patch is beneficial to both mother and offspring.

Total offspring in the mixed-sex treatment was not significantly different to the single sex treatment, which suggests that sexual harassment did not affect oviposition rates in our study. This is in contrast to results from Teseo (2016), where females under high-harassment pressure laid fewer eggs. However, the sex ratios tested previously were more extreme (24:6 males to females) compared to our study (10:10 males to females). In addition, any effect on oviposition in the mixed-sex treatment could have been masked by the potential for female re-mating.

The data presented here suggest that female diet patch preferences are altered by the presence of males or male cues, but that more work is required to investigate drivers of this

pattern. We did not see evidence that females dynamically adjusted their foraging behaviour to avoid harassment. However, harassment avoidance behaviour in the context of feeding has been observed in a solitary bee *A. plumipes* (Stone, 1995). Female bees compromised feeding from easily accessible flowers with a greater nectar content and high harassment rates, instead preferring to forage from flowers sheltered from males, further within the plant (Stone, 1995). Similar compromises to oviposition site choice have been observed in female seed bugs, *Neacoryphus bicrucis* (McLain & Pratt, 1999). Behavioural changes as a result of sexual harassment risk have also been observed in the Eastern Mosquito fish *G. holbrooki*. Females altered their behavioural strategies in the presence of males, by approaching groups of males more often than individual males, when presented with males through a transparent plastic barrier (Dadda *et al*., 2008). Male-male competition dilutes sexual harassment in this species. It would be interesting to know whether females of this species would prioritise these harassment-reducing behaviours ahead of foraging.

Due to the drive for social aggregation previously observed in *D. melanogaster* females, it is plausible that the movement of females in this study was an effect of preference for the most populous food patch. Food patches containing multiple individuals could provide social information on quality for new individuals (Lihoreau *et al*., 2018), as seen in the foraging patch decisions of cockroaches, *Blattella germanica* (Lihoreau *et al*., 2010), desert locusts, *Schistocerca gregaria* (Günzel *et al*., 2023), and fruit flies (Lihoreau *et al*., 2016a) but it is unclear whether the dynamics of social aggregation are altered in mixed-sex groups.

Further work is required to establish the extent to which social make-up of a group on a feeding site influences subsequent preference, e.g. whether individuals are drawn to patches that appear popular, but only depending on the sex and species of the individuals present. The presence of harassing heterospecific males can affect female oviposition site choice (McLain &

Pratt, 1999), while a female's movement toward groups of female conspecifics can help to ease individual sexual harassment pressure. In a behavioural study of *G. holbrooki*, females moved closer to conspecific females when a male, but not a female, was presented (Dadda *et al*., 2005, 2008). Overall, instances of sexual harassment in *G. holbrooki* increased when more females were present in the tank, but in turn, this reduced the number of sexual harassment interactions experienced by each individual female, as harassment was divided among them (Pilastro *et al*., 2003). However, the non-focal female fish used in these studies were fed to satiation and therefore presented no competition for feeding resources, which would be unlikely in wild scenarios. It is unclear whether female *D. melanogaster* experience a similar level of sexual harassment dilution when in a group, or whether females in this experiment would aggregate to a similar extent if females were included in the acetate barriers, rather than males. If female *D. melanogaster* conspecifics do offer a level of harassment protection, it would be interesting to test whether a lone female would prefer a patch of food on which males were present to the same extent we observed.

In conclusion, the presence of males influenced the observed preference of feeding and oviposition site selection of females, despite differences in site nutrient quality. This is surprising considering that sexual harassment is detrimental to female feeding in multiple animal systems (Rowe, 1992; Stone, 1995; Pilastro *et al*., 2003; Dadda *et al*., 2008; Teseo *et al*., 2016) and the impact of macronutrient consumption on both female and larvae traits (Jang & Lee, 2018). It is possible that females perceived that there was no risk of harassment when males were restricted and instead gained social information on feeding site value from the presence of conspecifics. When females did face harassment in mixed-sex groups, this pattern was absent. However, it is difficult to disentangle macronutrient preference from the physical disturbance caused by sexual harassment, when males are present. In this study, we confined males behind barriers to induce sexual harassment 'risk' to females, with the goal of

disentangling active female choice from physical displacement. It would be interesting to investigate female choice under differing rates of sexual harassment by altering sex ratio and limit the influence of social aggregation by testing fewer females.

References

Bartelt, R.J., Schaner, A.M. & Jackson, L.L. (1985) cis-Vaccenyl acetate as an aggregation pheromone in *Drosophila melanogaster*. *Journal of Chemical Ecology*, **11**, 1747–1756.

Bastock, M. & Manning, A. (1955) The Courtship of *Drosophila melanogaster*. *Behaviour*, **8**, 85–110.

Bretman, A., Westmancoat, J.D., Gage, M.J.G. & Chapman, T. (2011) Males use multiple, redundant cues to detect mating rivals. *Current biology: CB*, **21**, 617–622.

Dadda, M., Pilastro, A. & Bisazza, A. (2005) Male sexual harassment and female schooling behaviour in the eastern mosquitofish. *Animal Behaviour*, **70**, 463–471.

Dadda, M., Pilastro, A. & Bisazza, A. (2008) Innate responses to male sexual harassment in female mosquitofish. *Behavioral Ecology and Sociobiology*, **63**, 53–62.

Douglas, T., Anderson, R. & Saltz, J.B. (2020) Limits to male reproductive potential across mating bouts in *Drosophila melanogaster*. *Animal Behaviour*, **160**, 25–33.

Dweck, H.K.M., Ebrahim, S.A.M., Thoma, M., Mohamed, A.A.M., Keesey, I.W., Trona, F., *et al.* (2015) Pheromones mediating copulation and attraction in *Drosophila*. *Proceedings of the National Academy of Sciences*, **112**, E2829–E2835.

Fanson, B.G. & Taylor, P.W. (2012) Protein:carbohydrate ratios explain life span patterns found in Queensland fruit fly on diets varying in yeast:sugar ratios. *AGE*, **34**, 1361–1368.

Fowler, E.K., Leigh, S., Rostant, W.G., Thomas, A., Bretman, A. & Chapman, T. (2022) Memory of social experience affects female fecundity via perception of fly deposits. *BMC Biology*, **20**, 244.

Fowler, K. & Partridge, L. (1989) A cost of mating in female fruitflies. *Nature*, **338**, 760–761.

Greenfield, M.D. (1981) Moth Sex Pheromones: An Evolutionary Perspective. *The Florida Entomologist*, **64**, 4–17.

Griffiths, S.W. (1996) Sex differences in the trade-off between feeding and mating in the guppy. *Journal of Fish Biology*, **48**, 891–898.

Günzel, Y., Oberhauser, F.B. & Couzin-Fuchs, E. (2023) Information integration for decision-making in desert locusts. *iScience*, **26**.

Hartig, F. (2022) DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models.

Herberstein, M., Schneider, J. & Elgar, M. (2002) Costs of courtship and mating in a sexually cannibalistic orb-web spider: female mating strategies and their consequences for males. *Behavioral Ecology and Sociobiology*, **51**, 440–446.

Jang, T. & Lee, K.P. (2018) Comparing the impacts of macronutrients on life-history traits in larval and adult *Drosophila melanogaster*: the use of nutritional geometry and chemically defined diets. *Journal of Experimental Biology*, **221**, jeb181115.

Landolt, P.J. (1997) Sex Attractant and Aggregation Pheromones of Male Phytophagous Insects. *American Entomologist*, **43**, 12–22.

Lebreton, S., Trona, F., Borrero-Echeverry, F., Bilz, F., Grabe, V., Becher, P.G., *et al.* (2015) Feeding regulates sex pheromone attraction and courtship in *Drosophila* females. *Scientific Reports*, **5**, 13132.

Lee, K.P., Kim, J.-S. & Min, K.-J. (2013) Sexual dimorphism in nutrient intake and life span is mediated by mating in *Drosophila melanogaster*. *Animal Behaviour*, **86**, 987–992.

Lee, K.P., Simpson, S.J., Clissold, F.J., Brooks, R., Ballard, J.W.O., Taylor, P.W., *et al.* (2008) Lifespan and reproduction in *Drosophila*: New insights from nutritional geometry. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 2498–2503.

Lenth, R.V., Buerkner, P., Giné-Vázquez, I., Herve, M., Jung, M., Love, J., *et al.* (2023) emmeans: Estimated Marginal Means, aka Least-Squares Means.

Light, D.M., Jang, E.B., Binder, R.G., Flath, R.A. & Kint, S. (1999) Minor and Intermediate Components Enhance Attraction of Female Mediterranean Fruit Flies to Natural Male Odor Pheromone and Its Synthetic Major Components. *Journal of Chemical Ecology*, **25**, 2757–2777.

Lihoreau, M., Buhl, J., Charleston, M.A., Sword, G.A., Raubenheimer, D. & Simpson, S.J. (2015) Nutritional ecology beyond the individual: a conceptual framework for integrating nutrition and social interactions. *Ecology Letters*, **18**, 273–286.

Lihoreau, M., Clarke, I.M., Buhl, J., Sumpter, D.J.T. & Simpson, S.J. (2016a) Collective selection of food patches in *Drosophila*. *Journal of Experimental Biology*, **219**, 668–675.

Lihoreau, M., Deneubourg, J.-L. & Rivault, C. (2010) Collective foraging decision in a gregarious insect. *Behavioral Ecology and Sociobiology*, **64**, 1577–1587.

Lihoreau, M., Gómez-Moracho, T., Pasquaretta, C., Costa, J.T. & Buhl, C. (2018) Social nutrition: an emerging field in insect science. *Current Opinion in Insect Science*, Vectors and medical and veterinary entomology * Social insects, **28**, 73–80.

Lihoreau, M., Poissonnier, L.-A., Isabel, G. & Dussutour, A. (2016b) *Drosophila* females trade off good nutrition with high-quality oviposition sites when choosing foods. *Journal of Experimental Biology*, **219**, 2514–2524.

Magurran, A.E. & Seghers, B.H. (1997) A cost of sexual harassment in the guppy, *Poecilia reticulata*. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **258**, 89–92.

Maklakov, A.A., Simpson, S.J., Zajitschek, F., Hall, M.D., Dessmann, J., Clissold, F., *et al.* (2008) Sex-Specific Fitness Effects of Nutrient Intake on Reproduction and Lifespan. *Current Biology*, **18**, 1062–1066.

Matavelli, C., Carvalho, M.J.A., Martins, N.E. & Mirth, C.K. (2015) Differences in larval nutritional requirements and female oviposition preference reflect the order of fruit colonization of *Zaprionus indianus* and *Drosophila simulans*. *Journal of Insect Physiology*, **82**, 66–74.

McLain, D.K. & Pratt, A.E. (1999) The cost of sexual coercion and heterospecific sexual harassment on the fecundity of a host-specific, seed-eating insect (*Neacoryphus bicrucis*). *Behavioral Ecology and Sociobiology*, **46**, 164–170.

Morais, P.B., Martins, M.B., Klaczko, L.B., Mendonça-Hagler, L.C. & Hagler, A.N. (1995) Yeast succession in the Amazon fruit *Parahancornia amapa* as resource partitioning among *Drosophila* spp. *Applied and Environmental Microbiology*, **61**, 4251–4257.

Pilastro, A., Benetton, S. & Bisazza, A. (2003) Female aggregation and male competition reduce costs of sexual harassment in the mosquitofish *Gambusia holbrooki*. *Animal Behaviour*, **65**, 1161–1167.

Piper, M.D. (2017) Using artificial diets to understand the nutritional physiology of *Drosophila melanogaster*. *Current Opinion in Insect Science*, Global change biology * Molecular physiology, **23**, 104–111.

Piper, M.D.W., Blanc, E., Leitão-Gonçalves, R., Yang, M., He, X., Linford, N.J., *et al.* (2014) A holidic medium for *Drosophila melanogaster*. *Nature Methods*, **11**, 100–105.

Ribeiro, C. & Dickson, B.J. (2010) Sex Peptide Receptor and Neuronal TOR/S6K Signaling Modulate Nutrient Balancing in *Drosophila*. *Current Biology*, **20**, 1000–1005.

Rodrigues, M.A., Martins, N.E., Balancé, L.F., Broom, L.N., Dias, A.J.S., Fernandes, A.S.D., *et al.* (2015) *Drosophila melanogaster* larvae make nutritional choices that minimize developmental time. *Journal of Insect Physiology*, **81**, 69–80.

Rowe, L. (1992) Convenience polyandry in a water strider: foraging conflicts and female control of copulation frequency and guarding duration. *Animal Behaviour*, **44**, 189–202.

Rowe, L. (1994) The costs of mating and mate choice in water striders. *Animal Behaviour*, **48**, 1049–1056.

Simpson, S.J. & Raubenheimer, D. (2012) *The Nature of Nutrition: A Unifying Framework from Animal Adaptation to Human Obesity*. Princeton University Press.

Singh, S.R. & Singh, B.N. (2004) Female remating in *Drosophila*: Comparison of duration of copulation between first and second matings in six species. *Current Science*, **86**, 465–470.

Stone, G.N. (1995) Female foraging responses to sexual harassment in the solitary bee *Anthophora plumipes*. *Animal Behaviour*, **50**, 405–412.

Stutt, A.D. & Siva-Jothy, M.T. (2001) Traumatic insemination and sexual conflict in the bed bug *Cimex lectularius*. *Proceedings of the National Academy of Sciences*, **98**, 5683–5687.

Teseo, S., Veerus, L., Moreno, C. & Mery, F. (2016) Sexual harassment induces a temporary fitness cost but does not constrain the acquisition of environmental information in fruit flies. *Biology Letters*, **12**, 20150917.

Thrall, P.H., Antonovics, J. & Bever, J.D. (1997) Sexual Transmission of Disease and Host Mating Systems: Within-Season Reproductive Success. *The American Naturalist*, **149**, 485–506.

Tinette, S., Zhang, L. & Robichon, A. (2004) Cooperation between *Drosophila* flies in searching behavior. *Genes, Brain and Behavior*, **3**, 39–50.

Watson, P.J., Stallmann, R.R. & Arnqvist, G. (1998) Sexual Conflict and the Energetic Costs of Mating and Mate Choice in Water Striders. *The American Naturalist*, **151**, 46–58.

Wing, S.R. (1988) Cost of Mating for Female Insects: Risk of Predation in *Photinus collustrans* (Coleoptera: Lampyridae). *The American Naturalist*, **131**, 139–142.

Appendix for Chapter 4

Table A1. Measurements needed for 1.5 L of solid meridic diet of varying P:C ratios, based on a combined caloric concentration of 120 g/L.

P:C ratio	1:2	1:4	1:5	1:8	4:1
Casein (g)	60	36	30	20	120
Sucrose (g)	120	144	150	160	60
Cholesterol (g)	0.45	0.45	0.45	0.45	0.45
Lecithin (g)	6	6	6	6	6
Agar (g)	30	30	30	30	30
KH_2PO_4 (ml)	150	150	150	150	150
K_2HPO_4 (ml)	150	150	150	150	150
$MgSO_4$ (ml)	150	150	150	150	150
$NaHCO_3$ (ml)	150	150	150	150	150
Nucleic acid sol (ml)	150	150	150	150	150
De-ionised H_2O (ml)	300	300	300	300	300
Autoclave the mixture at this stage					
10% Nipagin-ethanol solution (ml)	15	15	15	15	15
Propionic acid (ml)	4.5	4.5	4.5	4.5	4.5
Vitamin mix (ml)	225	225	225	225	225
De-ionised H_2O (ml)	Top up to 1.5 L	Top up to 1.5 L	Top up to 1.5 L	Top up to 1.5 L	Top up to 1.5 L

Salt solutions contain 2 L de-ionised H_2O and each salt in solid form (14.22 g KH_2PO_4, 74.6 g K_2HPO_4, 12.4 g $MgSO_4$, 20 g $NaHCO_3$). Nucleic acid solution contains 11.4 g Uridine, 12.8 g Inosine, 2 litres de-ionised H_2O. Diet adapted from Piper and colleagues (Piper *et al.*, 2014).

Table A2. Recipe to make 2 L vitamin mix

Ingredient	Quantity
Thiamine (g)	0.0267
Riboflavin (g)	0.133
Nicotinic acid (g)	0.16
Ca Pantothenate (g)	0.222
Pyridoxine (g)	0.033
Biotin solution (ml)	133
Folic acid solution (ml)	133
De-ionised H2O (ml)	1733

Biotin solution made from 0.01g Biotin and 500ml de-ionised H_2O. Folic Acid solution made from 0.119g Folic Acid, 80ml ethanol and 320ml de-ionised H_2O. Both made in 500ml conical flasks and stored in fridge.

Figure A1

Example subset of 9 images scored during the diet preference for coloured patches assay. Images of female flies were presented to scorers as slides using Microsoft PowerPoint (version 16.86) and each image contained the reference colour scoring bar, and a randomised ID.

Figure A2

Extract of scoring instructions provided to scorers during colour preference assay. Scorers were instructed to give each fly a score of 0 (no food), 1 (blue abdomen), 2 (purple abdomen) or 3 (red abdomen).

Chapter 5

Differences in nutrient sensing gene expression as a result of diet and sex in two experimentally evolved populations

Abstract

Diet composition helps determine lifespan and reproduction in animals. The ratios of macronutrients (chiefly protein and carbohydrate) that maximise life history components often differ between the sexes. The sex differences in optimal diet compositions for maximising lifespan and reproduction are supported by sex-specific signalling in the nutrient sensing pathways, insulin/insulin like growth factor (IIS) and target of rapamycin (TOR) in *Drosophila melanogaster*. However, the effect of the local nutritional environment on the evolution of sex differences in nutrient-sensing pathways is less well understood. Therefore, we measured the relative expression of key genes involved in the IIS/TOR pathways (*dilp2*, *dTOR* and *dfoxo*) in male and female *D. melanogaster* from populations experimentally evolved on one of two diets of varying protein: carbohydrate ratio (P:C). Each diet represented either a high protein diet, typically preferred by females (1:2 P:C), or a low protein diet, typically preferred by males (1:8 P:C). We then compared the lifespan, reproduction and activity of individuals from experimentally evolved populations to investigate the relationship between nutrient signalling and traits associated with fitness. The relative expression of nutrient-

sensing genes *dilp2*, *dTOR* and *dfoxo* in head and thorax samples evolved in response to dietary macronutrients and expression patterns were consistent between the sexes. However, in abdomen samples the relative expression of *dTOR* was sex-specific and the relative expression of *dfoxo* was both sex- and body part- specific. Lifespan also depended on the long-term history of diet composition and sex: lifespan was greatest in flies that had evolved on a 1:8 P:C diet compared to flies that had evolved on a 1:2 diet, but the lifespan of males that had evolved on a 1:2 diet was considerably lower than females. These results further the view that sex differences in diet and lifespan are underpinned by nutrient signalling and contributes novel data on the evolution of these pathways in environments that differ in nutritional resources.

Introduction

The success and survival of an individual is strongly influenced by their diet, particularly by the macronutrients they choose to eat. Animals are able to vary their macronutrient consumption to reach an optimal ratio of key components protein and carbohydrate (P:C ratio) that maximizes lifetime reproduction. Studies investigating the role of P:C ratios on proxies for fitness have found dietary effects on lifespan and reproduction. Longevity increased on relatively low P:C diets in mice (Solon-Biet *et al.*, 2014), *Drosophila melanogaster* fruit flies (Jensen *et al.*, 2015; Jang & Lee, 2018), *Bactrocera tryoni* Queensland fruit flies (Fanson & Taylor, 2012) and *Teleogryllus commodus* field crickets (Maklakov *et al.*, 2008). The P:C ratio is also important for maximising reproduction, though optimal diets for lifetime reproduction vary between the sexes. For example, in *T. commodus*, male calling rate is maximised on low P:C diets, while female egg production is maximised on high P:C diets (Maklakov *et al.*, 2008). Similarly, in *D. melanogaster*, male reproduction is maximised on a low P:C diet, while female reproduction is maximised on a higher P:C diet (Carey *et al.*, 2022). Different macronutrient ratios also maximise female fecundity versus male sperm viability in *Teleogryllus oceanicus* field crickets (Ng *et al.*, 2019).

Differences in macronutrient optima between the sexes are thought to be underpinned by patterns of nutrient sensing in the insulin and/or insulin-like growth factor (IIS) and target of rapamycin (TOR) pathways (Bennett-Keki *et al.*, 2023). Distinct patterns of sex bias in gene expression were found in *D. melanogaster* across 10 different genes involved in the IIS/TOR pathways (Bennett-Keki *et al.*, 2023). IIS/TOR are highly evolutionarily conserved pathways that respond to macronutrients via a complex network of cellular signalling cascades (Tatar *et al.*, 2014) to regulate growth, metabolism and reproduction (by growth and maintenance of the germline) (Templeman & Murphy, 2017). IIS/TOR pathways also form an important link between nutrition and longevity (Templeman & Murphy, 2017). As such, multiple studies have

focussed on the role IIS/TOR plays in lifespan extension and reproduction following dietary restriction (reviewed in Solon-Biet et al., 2015).

Despite the critical influence of diet on animal health, little is known about the evolution of sex-specific nutrient processing in response to variation in the local nutritional environment. It is also largely unknown how evolved differences in nutrient processing translate to sex-specific fitness outcomes. In a relatively high P:C nutritional environment, which typically maximises lifetime reproduction rates in females, males might initially experience depressed reproductive performance. Over time, the evolution of nutrient sensing and processing pathways might allow males to achieve better reproductive performance, narrowing an initial sex difference in dietary optima. Similarly, initial sex differences in the expression of nutrient pathways might diminish over time if males shift towards a female nutrient-processing profile. Analogous evolutionary change might arise when both sexes evolve in a relatively low P:C environment.

In this study, we investigated gene expression within the IIS/TOR pathways and phenotypes of males and females from replicated populations of *D. melanogaster* that had experimentally evolved under distinct dietary regimes. Populations had evolved for approximately 78 generations on nutritionally defined (meridic) diets at macronutrient ratios of either 1:2 P:C, representing a high protein diet (typically beneficial for female fitness) or 1:8 P:C, representing a high carbohydrate diet (typically beneficial for male fitness). *D. melanogaster* is an ideal model for this research, due to its short generation times and the extensive previous study of sex-specific dietary optima and choice in this species (Lee *et al.*, 2008, 2013; Jensen *et al.*, 2015; Camus *et al.*, 2018). We used quantitative RT-PCR to measure the relative expression of genes involved in the IIS/TOR pathway in adult male and female flies. We focused on *dilp2*, *dTOR* and *dfoxo* to investigate possible interactions between diet and sex on gene expression, because

dilp2, *dTOR* and *dfoxo* show strong sex-biased gene expression in *D. melanogaster* in a previous experiment (Bennett-Keki *et al.*, 2023). These three genes were additionally chosen to represent the different cellular locations of the IIS/TOR network: extracellular (*dilp2*), intracellular (*dTOR*, *dFOXO*) and nuclear (*dFOXO)* sections of the pathway. In separate, earlier experiments, we also investigated the potential for sex-specific adaptation to diet macronutrient content (after 48 generations) by measuring three proxies for fitness under common garden dietary conditions: lifespan, offspring production and negative geotaxis. Negative geotaxis assays offer a robust and established indicator of senescence in fruit flies (Gargano *et al.*, 2005), as wildtype *D. melanogaster* have an innate response to mechanical stimulation (e.g. when knocked to the base of a glass vial), by climbing against the direction of gravity (Gargano *et al.*, 2005). Hence, the rate at which individuals climb up to a fixed distance (negative geotaxis), is recorded to investigate physical symptoms of ageing.

We predicted that the experimental evolution of flies on distinct macronutrient ratios would alter the expression of genes in the IIS/TOR pathway. In addition, due to the effects of macronutrient balance on individual reproductive success and longevity in short-term dietary regimes, we predicted that offspring production and survival would differ between 1:2 and 1:8 populations. This is because diets that change life history traits within one generation will initially have the same effect in longer term regimes. It is also possible that the impacts of P:C ratios will then be replicated across and persist within each generation in populations fed the same P:C diet over many generations. Therefore, we predicted that females that evolved on a low protein 1:8 diet would show reduced offspring production, but increased longevity compared to females that evolved on a high protein 1:2 diet. We also predicted that males that evolved on the low protein 1:8 diet would show increased reproduction and lifespan compared to males that evolved on the high protein 1:2 diet.

Methods

Fly stocks

Experimental lines were derived from wild-type stocks of *D. melanogaster* originally collected in Dahomey in 1970 (Chapman *et al.*, 1994). These stocks were maintained in large population cages on a sugar-yeast-agar (SYA) diet (50 g sucrose, 100 g brewer's yeast, 15 g agar, 30 ml Nipagin (10 % solution), 3 ml propionic acid, 970 ml water).

Replicate populations for the experimental evolution lines were established in large cages using WT as the ancestral stock, in March 2020. Four replicate populations were established for each chemically-defined diet treatment. Both diets were composed of chemically-defined protein: carbohydrate ratios (Piper *et al.*, 2014; recipe and protocol in Appendix, Table A1 – A2) at either 1:2 P:C or 1:8 P:C by varying casein and sucrose quantities at a concentration of 120 g/L. Fresh diet media (2 bottles per week) was added to each replicate population cage weekly in glass bottles containing ~70 ml diet. Populations evolved under conditions of overlapping generations and under uncontrolled densities. All populations were maintained under a 12 h: 12 h light: dark schedule and 50 % (±10 %) humidity at 25 °C.

Quantitative RT-PCR

Experimental flies

Individuals for experiments were reared on a "common garden" diet of an intermediate 1:5 P:C to ensure that all populations experienced a novel, intermediate diet but which nevertheless contained the same ingredients as their ancestral diets. Individuals were generated in a staggered format (due to differences in development times of the populations from the two treatments) following a generation of standard rearing. Bottles of 1:5 P:C food were placed

into each population cage (8 cages total) and removed once sufficient numbers of eggs had been laid on the surface. The adults collected from these bottles were used as the parental generation. Parental flies were anaesthetised using CO_2 and added to small egg collection cages over petri dishes containing solid purple grape juice-agar-water. Adults were left to acclimatise for 24 h and eggs were collected on a fresh grape-agar-water dish on day 2. Adults were removed and eggs were allowed to develop for 24 h. First-instar larvae were transferred into glass vials containing 7 ml of 1:5 P:C diet at a density of 50 larvae. Adult experimental flies from both diet treatments were collected on the same day after 10-12 days and placed onto new food in mixed-sex groups to mate for 24 h. Mated flies were then separated into single-sex groups of 5. 5-day old adults were transferred in single sex groups of 5 into 1.5 ml plastic tubes, by aspiration using an electronic pooter, immediately snap frozen in liquid nitrogen, and stored in a -80 °C freezer to prevent degradation of RNA. Flies were placed on dry ice and their bodies divided at the mid-point between the thorax and abdomen within their groups of 5. This resulted in samples of 5 head-thoraxes and 5 abdomens, separated by diet treatment and replicate population, which were placed into new 1.5 ml tubes and stored at -80 °C until RNA extractions.

RNA extractions

RNA was extracted from groups of flies, from each combination of sex and replicate population (2 x diet treatments, 4 x population replicates, 2 x body parts, 2 x sexes = 32 extractions) using the miRvana kit and associated protocol (Ambion, AM1561). Fly samples were broken down using an electric micropestle in an initial 50 µl of lysis material and then 150 µl added after grinding (Bennett-Keki *et al.*, 2023). RNA was eluted into 50 µl RNA storage solution (1 mM sodium citrate, pH 6.4 ± 0.2, Ambion) and DNase treated using TURBO DNA-free kit (Invitrogen, AM1907). A NanoDrop 8000 spectrophotometer (Thermo Scientific) was used to

assess RNA concentrations. The Revertaid RT kit (Thermo Scientific, K1632) and associated protocol were used for reverse transcription synthesis of cDNA, which was stored at -20 °C.

RT-PCR

5 µl template cDNA samples were pipetted into a 96 well plate, with 10 µl iTaq universal SYBR green supermix (Bio-rad #1725121), 1 µl forward primer, 1 µl reverse primer and 3 µl H_2O. Three technical replicates per sample were used to check pipetting consistency. The total number of samples required per primer exceeded a single 96-well plate (2 diets x 4 biological replicates x 2 sexes x 2 body parts x 3 technical replicates = 96 + 3 control H_2O wells). Therefore, we separated samples among 10 plates as follows: *dilp2* abdomen, *dilp2* head-thorax, *dTOR* abdomen, *dTOR* head-thorax, *dfoxo* abdomen, *dfoxo* head-thorax, and reference genes; *αTub84B* abdomen, *αTub84B* head-thorax, *eIF1A* abdomen, and *eIF1A* head-thorax. *αTub84B* and *eIF1A* were chosen as reference genes as expression is stable between males and females (Bennett-Keki *et al.*, 2023). 96-well plates were covered with a film sealant (Bio-Rad MSB-1001) for quantitative RT-PCR using a Bio-Rad CFX Connect Thermal Cycler (software CFX maestro).

Life history assays

Experimental fly collections

Experimental adults for the fitness tests were collected from the Dahomey wild-type, 1:2 and 1:8 populations following 2 generations of standardised rearing on a common garden diet. We chose 1:5 P:C as an intermediate, common garden diet (note that the casein: sucrose base of this diet was novel to wild-type Dahomey files). Glass bottles containing 70 ml of 1:5 P:C diet were placed into each population cage of the experimentally evolved populations, along with four stock Dahomey population cages, resulting in 12 populations in total. Eggs laid on the 1:5

bottles developed into the parental generation. Parental adults were placed into small egg collection cages containing grape juice-agar plates by CO_2 anaesthesia. Parental flies were left to acclimatise for 24 h and eggs were collected on the second day. First instar larvae were transferred at a density of 50 larvae into glass vials containing 7 ml of 1:5 diet. Adult flies were collected after ~12 days, at peak eclosion, and placed into fresh bottles of 1:5 diet in mixed sex groups to mate for 24 h. Individuals were then transferred into individual vials containing 1:5 diet. Fresh vials containing 7 ml 1:5 diet were provided every 3-4 days for two weeks, after which 4 ml of 1:5 was provided every 7 days until the end of the experiment.

Negative geotaxis

A subset of flies was randomly assigned to activity trials during the experimental period. The negative geotaxis response was measured in 5 individuals of each sex from each population. *Drosophila* adults have an innate behaviour to continually climb upwards, against gravity (negative geotaxis) when startled or physically displaced within a chamber. The speed of the negative geotaxis response is an established measure of activity and shows a consistent decline with ageing (Gargano *et al.*, 2005). Negative geotaxis was measured sequentially for each individual by timing the time taken for a fly to move from the surface of the food to a 3 cm line drawn on each vial, and was repeated every seven days, for 5 weeks using the same individuals (Figure 1).

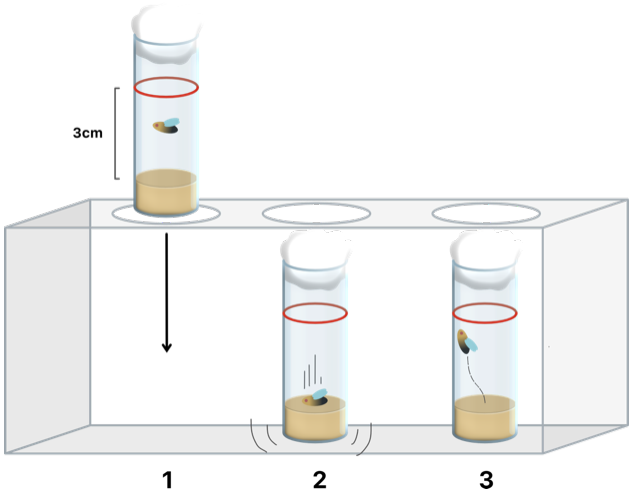


Figure 1

Experimental set up of negative geotaxis assay. Glass vials containing individuals selected for the negative geotaxis assay were marked with a solid line, 3cm above the food surface and placed into a plastic observation rack of 11cm in height. Each vial was held at the top of the rack and released (1), to rapidly, physically displace the fly down onto the surface of the food (2). The time taken for the fly to climb to the 3cm line was recorded in seconds using a stopwatch (3). The assay was repeated for each individual fly.

Survival

Vials of experimental individuals (held separately) were monitored for survival every 1-2 days. Date of death or escape was recorded.

Offspring production

Adult flies were moved to fresh food every 3-4 days for the first two weeks of the experiment. Vials were retained and after 12 days, frozen at -2 °C. Offspring were counted to give four measures of offspring production across the two weeks.

Statistical analysis

All analyses were carried out using R version 4.3.3 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org). Throughout, model fit was checked using the R package DHARMa (Hartig, 2022) and post-hoc Tukey testing was carried out using estimated marginal means to run pairwise comparisons using the R package emmeans (Lenth et al., 2023).

Quantitative RT-PCR

Melting point temperature curves for each sample were checked manually using the Bio-Rad CFX maestro software. Samples with a second peak, indicating the formation of a primer-dimer during the PCR, were removed from the analysis (N = 15, all *dilp2* abdomen). C_t (cycle threshold) expression values for remaining samples were checked for any outlying values between technical replicates by calculating standard deviation and excluding standard deviations above 0.5 cycles, and any significant outliers were removed (Nolan *et al.*, 2006). Mean expression values for the target genes *dilp2*, *dTOR* and *dfoxo* were calculated per sample from the technical replicates, and mean expression values for both the reference genes *eIF1A* and *αTub84B* were calculated per sample across the two genes. Relative C_t change was calculated using the ΔC_t method (Pfaffl, 2001). ΔC_t per sample was calculated by subtracting mean reference gene expression from mean target gene expression (both *eIF1A* and *αTub84B*). Relative expression was then calculated within $2^{-\Delta Ct}$ formula (Pfaffl, 2001).

Relative expression was analysed by fitting generalised linear mixed models (GLMM) using the R package glmmTMB (Brooks *et al.*, 2017) for each PCR. $2^{-\Delta Ct}$ was included as the response variable and tested against diet line and sex as fixed factors with an interaction, and population replicate as a random factor. Fixed and random factors were identical across the analyses for each PCR.

Survival

Survival was analysed as a function of diet treatment and sex using the R package survival (Therneau & Grambsch, 2000; Therneau, 2023) and visualised using a Kaplan-Meier curve in the R package Survminer (Kassambara *et al.*, 2021). Instances where flies were lost or escaped during the measurements were censored.

Offspring

Offspring counts per female over the four time points were analysed using glmmTMB (Brooks *et al.*, 2017) to conduct zero-inflated Poisson model testing the interacting effect of time and diet treatment. Individual female ID nested within population replicate was included as random factors.

Negative geotaxis

Negative geotaxis timings where flies were unable to reach the 3 cm line within the 20 second window were classified together as >20-seconds. We calculated the regression slope of their negative geotaxis response over each of the five weeks, to give one slope value per individual. Individuals without all 5 time points were excluded, as these represented flies that were lost mid-way through the assay. We fitted a zero-inflated model using the glmmTMB package (Brooks *et al.*, 2017) to test the interacting effect of treatment and sex on the slope coefficients as the response variable. Individual ID nested within population replicate was included as a random factor.

Results

Gene expression patterns evolved in response to macronutrient content

Significant differences in the relative expression of nutrient-sensing genes as a result of evolving on different diets for all three genes in the head-thorax samples (*dilp2* χ^2_1=9.22, *P*<0.01, *dTOR* χ^2_1=7.25, *P*<0.01, *dfoxo* χ^2_1=18.56, *P*<0.01) and for *dfoxo* expression in the abdomen samples (χ^2_1=4.09, *P*<0.05) (Figure 2). *dTOR* expression in the abdomen did not differ between diet treatments (χ^2_1=2.32, *P*=0.13).

There was a significant sex bias in *dTOR* expression in the abdomen (χ^2_1=144.28, *P*<0.001) with higher relative expression in females, but there was no effect of sex on expression levels of any of the other genes and body parts tested (*dilp2* head-thorax: χ^2_1=2.39, *P*=0.12, *dTOR* head-thorax: χ^2_1=0.05, *P*=0.82, *dfoxo* abdomen: χ^2_1=1.13, *P*=0.29, *dfoxo* head-thorax: χ^2_1=0.02, *P*=0.90). Mean *dilp2* expression was higher in male abdomens than in female abdomens, but we were unable to model this result because we lost several samples that were below the detection limit of our assay.

The relative expression of *dfoxo* in both the head-thorax and abdomen samples appeared to show interacting effects of diet treatment and sex (Figure 2). However, this interaction between treatment and sex was below the statistical significance threshold (Table 2).

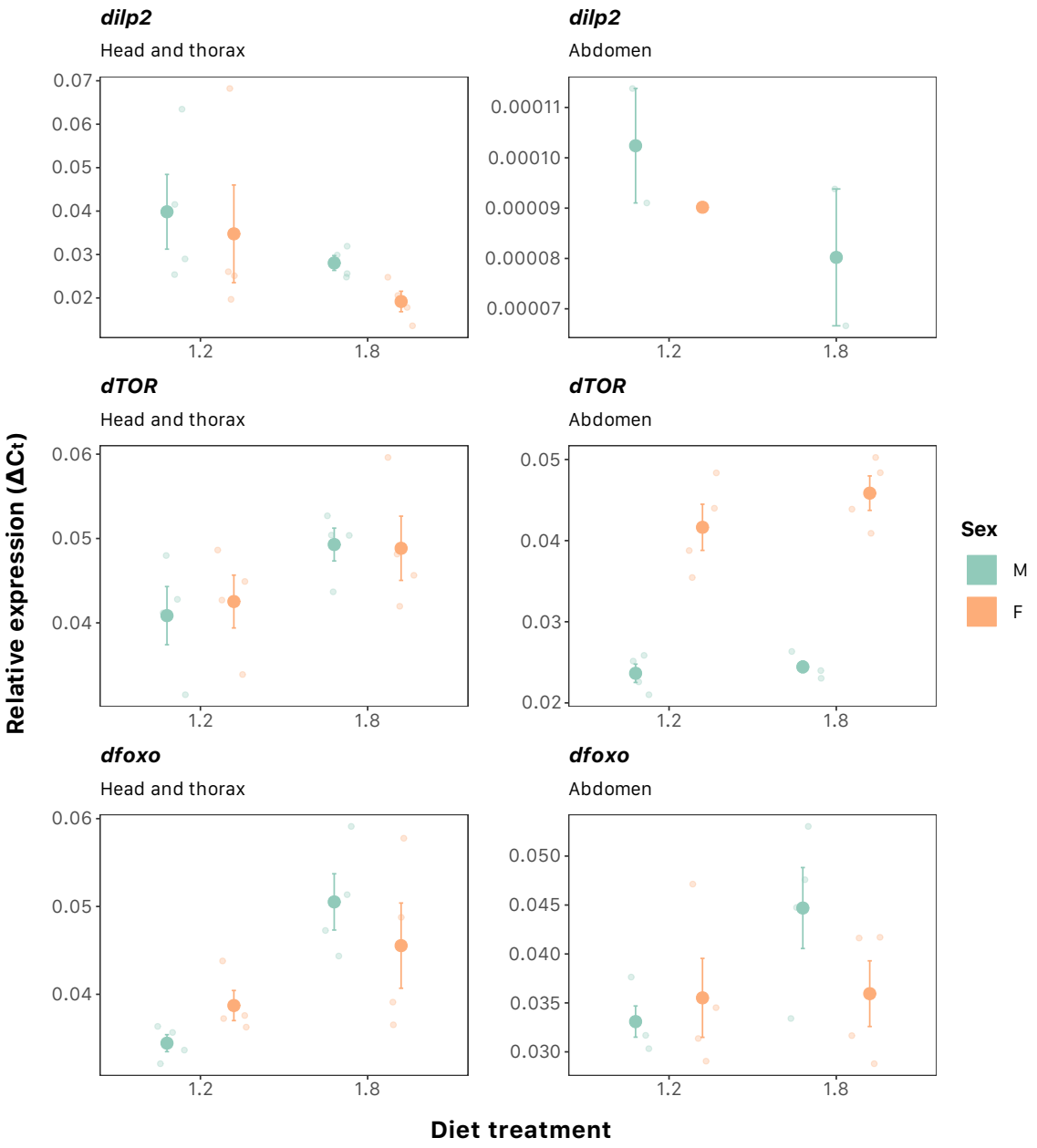


Figure 2

Relative expression levels of genes involved in the IIS/TOR signalling pathways (*dilp2*, *dTOR* and *dfoxo)* in male and female flies, in head and thorax (combined) or abdomen tissue. Flies were sampled from 8 replicate populations experimentally evolved on a diet of either 1:2 or 1:8 P:C (4 replicate populations each). Expression levels were normalised against two reference genes (*eIF1A* and *αTub84B*) using the $2^{-\Delta Ct}$ method. Large, filled circles indicate the mean per sex and diet treatment, with solid lines denoting standard error. Small, transparent circles represent the raw $2^{-\Delta Ct}$ value per replicate population.

Table 1

Model output of the relative expression ($2^{-\Delta Ct}$) of *dilp2*, *dTOR* and *dfoxo*, compared between diet treatment group and sex.

Gene	Body part	Model	Diet	Sex	Diet*sex
dilp2	Head-thorax	glmmTMB(deltaCT ~ diet*sex + (1|population)	χ^2_1=9.22, *P*<0.01	χ^2_1=2.39, *P*=0.12	χ^2_1=0.18, *P*=0.67
dTOR	Head-thorax	glmmTMB(deltaCT ~ diet*sex + (1|population)	χ^2_1=7.25, *P*<0.01	χ^2_1=0.05, *P*=0.82	χ^2_1=1.49, *P*=0.70
dTOR	Abdomen	glmmTMB(deltaCT ~ diet*sex + (1|population)	χ^2_1=2.32, *P*=0.13	χ^2_1=144.28, *P*<0.001	χ^2_1=1.09, *P*=0.30
dfoxo	Head-thorax	glmmTMB(deltaCT ~ diet*sex + (1|population)	χ^2_1=18.56, *P*<0.01	χ^2_1=0.02, *P*=0.90	χ^2_1=3.03, *P*=0.08
dfoxo	Abdomen	glmmTMB(deltaCT ~ diet*sex + (1|population)	χ^2_1=4.09, *P*<0.05	χ^2_1=1.13, *P*=0.29	χ^2_1=3.53, *P*=0.06

Lifespan depended on both evolutionary diet and sex

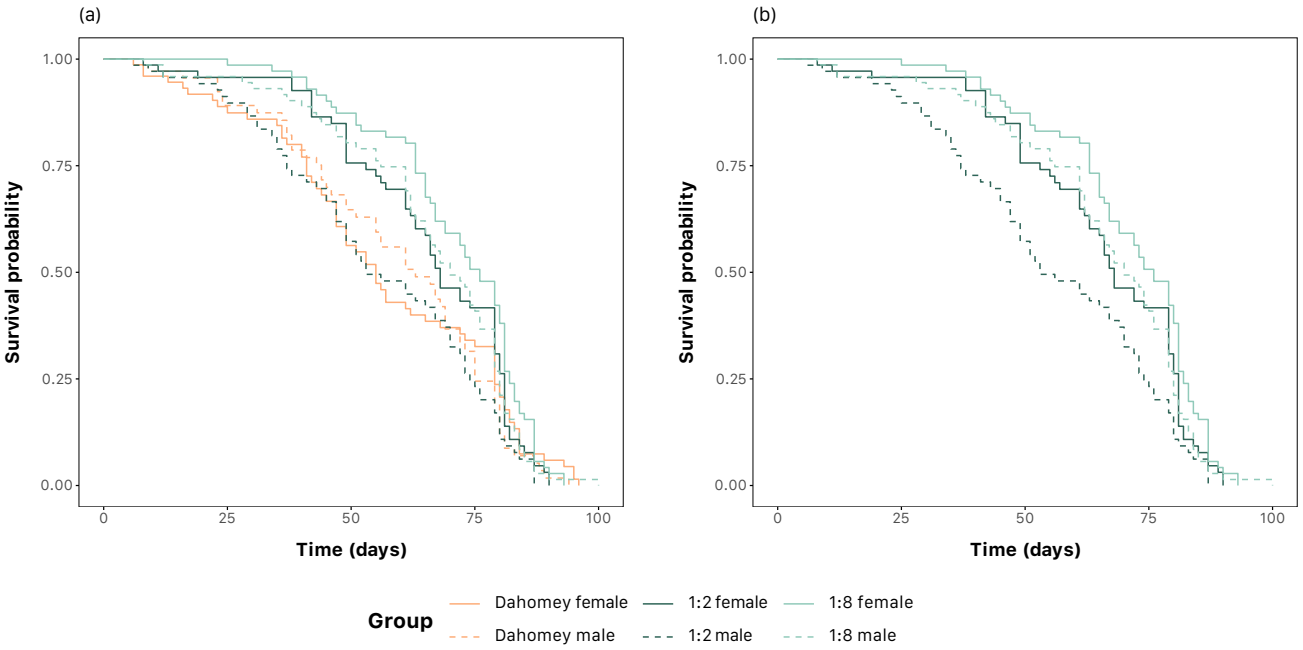


Figure 3

The survival of male and female adult flies from Dahomey, 1:2 and 1:8 populations in a cox model visualised as Kaplan-Meier curves. Each step on the curves denotes the death of an individual, while lost flies were censored. Female flies are presented as the solid, coloured lines, and males are presented as the dashed lines in the corresponding treatment colour. (a) The survival of flies across all three groups tested. (b) The survival of 1:2 evolved versus 1:8 evolved flies only.

Adult survival depended on both diet (χ^2_2=9.35, P<0.01) and sex (χ^2_1=6.30, P<0.05), but there was no significant interaction between diet and sex (χ^2_2=1, P=0.61) when comparing flies that evolved on 1:2 or 1:8 diets and Dahomey flies (Figure 3a). Similarly, in analyses comparing experimentally evolved populations only (Figure 3b), survival also depended on diet (χ^2_1=8.33, P<0.01) and sex (χ^2_1=7.79, P<0.01) but not an interaction between diet and sex (χ^2_1=0.36, P=0.55).

Flies from the 1:8 diet treatment were the longest lived, for both males and females, while survival was lowest in males from the 1:2 treatment. Despite the poor survival of males that evolved on a 1:2 diet, females that evolved on a 1:2 diet showed a higher survival that was similar to that of the males that evolved on a 1:8 diet. This pattern is reflected in the median survival: 1:8 females; 74 days, 1:8 males; 68 days, 1:2 females; 68 days, 1:2 males; 61 days. Dahomey flies had a median survival of 63 and 66 days, for male and female flies, respectively.

Offspring production was similar between the experimentally evolved populations

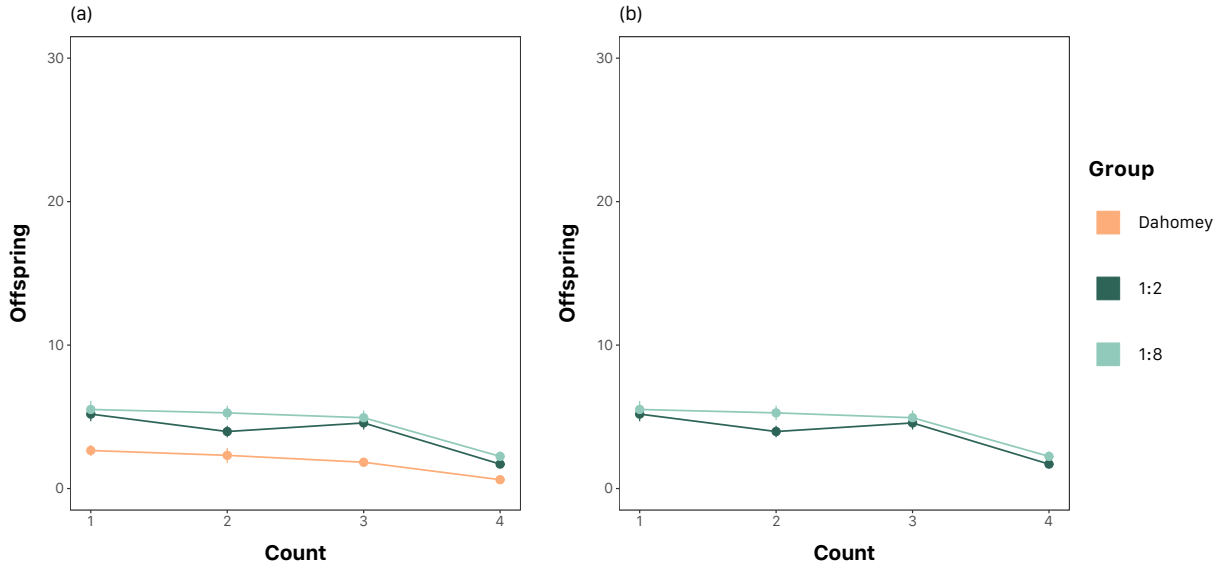


Figure 4

The number of adult progeny collected from vials containing a single mated female for 3-4 days. Data were collected in 4 counts across the first 14 days of experimental observations. All females were held in glass vials containing 7 ml 1:5 P:C diet. Large, filled circles denote mean offspring, linked by solid lines across each day. Pale, small circles are the raw data per female. (a) Adult progeny collected from females of all three lines tested. (b) Adult progeny collected from females of 1:2 evolved and 1:8 evolved lines only.

There was a significant difference in offspring production depending on whether flies had evolved on 1:2 or 1:8 diets or were from a stock population (χ^2_2=44.60, *P*<0.01). The number of offspring produced by Dahomey flies was lower than that of flies from the 1:2 and 1:8 diet treatments (post-hoc Tukey: Dahomey / 1:2 Z = -5.444, *P*<0.001, Dahomey / 1:8 Z= -6.858, *P*<0.001) (Figure 4a). The number of offspring produced was not significantly different between the two experimentally evolved treatments (post-hoc Tukey: 1:2 / 1:8 Z= -1.550, *P*=0.27) (Figure 4b). As expected, the number of offspring produced decreased over the two-week sample period (χ^2_3=177.09, *P*<0.01).

Negative geotaxis is unaffected by evolutionary diet

The rate of increase in time taken for flies to climb to the 3cm line across 5 weeks in the negative geotaxis assay was not affected by evolutionary diet (χ^2_2=0.7, *P*=0.71), sex (χ^2_1=3.34, *P*=0.068) or the interaction between sex and evolutionary diet (χ^2_2=1.68, *P*=0.43) (Figure 5a). This was also evident within analyses comparing 1:2 versus 1:8 populations only (diet: χ^2_1=0.48, *P*=0.71, sex: χ^2_2=0.65, *P*=0.42, diet*sex: χ^2_2=0.63, *P*=0.43) (Figure 5b).

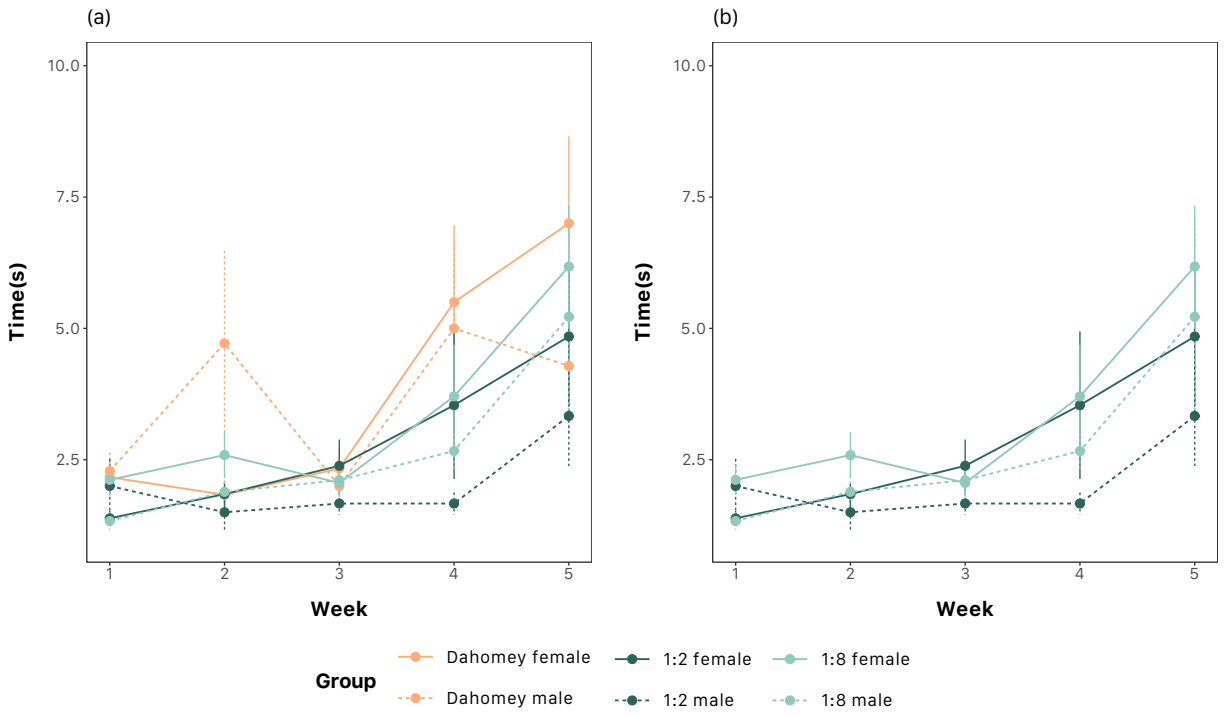


Figure 5

The time taken in seconds for adult flies to climb to a 3cm line during a negative geotaxis assay. Flies were from Dahomey, 1:2 and 1:8 populations. Vials housing individual flies were tapped downwards to displace the fly onto the food surface and we recorded the time taken for the fly to climb 3cm (Figure 1). The assay was repeated weekly for 5 weeks using the same individuals. Data collected from all three groups tested are included in panel (a). Panel (b) shows the data for the 1:2 and 1:8 diet treatments only. Each colour refers to the group tested, with female flies presented as solid-coloured lines, and males presented as dashed lines in corresponding colours. Filled circles represented the mean and vertical lines indicate standard error around the mean. Means are joined point-point by group over each week for each sex x diet sample group.

Discussion

The macronutrients consumed by animals are fundamental to their health, reproduction and lifespan (Simpson & Raubenheimer, 2012). The optimal ratio of protein and carbohydrate required to maximise reproduction often differs between males and females: male reproduction is typically maximised on lower protein diets, while female reproduction is often maximised on a higher protein diet (Lee *et al*., 2008; Maklakov *et al*., 2008). These sex-differences are underpinned by the action of signalling pathways in response to nutrients, such as IIS/TOR in *Drosophila* (Bennett-Keki *et al*., 2023). Despite the significance of diet composition for sex-specific fitness, there is limited information on how sex-specific nutrient processing evolves in response to the local food environment. We found evidence for evolved differences in nutrient sensing gene expression in response to diet macronutrient content of *D. melanogaster*. We also found evidence for sex- and body part-specific expression between diet regimes. In addition, populations that evolve on relatively high or low protein diets differed in their longevity but not offspring production, when measured in a common garden setting. The findings on longevity and offspring production are surprising considering the different dietary requirements for reproduction between the sexes.

Nutrient pathway gene expression evolved in response to diet

We found that the relative gene expression of *dilp2*, *dTOR* and *dfoxo* in the head and thorax, and *dfoxo* in the abdomen, was altered in *D. melanogaster* samples in response to the long-term manipulation of diet. This effect was observed despite two generations of controlled rearing on a common garden diet (1:5 P:C), suggesting that the expression differences represent adaptation to evolutionary diet. However, there was no difference between the diet treatments in the *dTOR* abdomen samples, suggesting that the evolution of *dTOR* gene expression was body part specific. Though the levels of expression in the *dilp2* abdomen samples were below the detection limit of our quantitative RT-PCR methods, there appeared

to be a decrease in relative expression in the 1:8 males versus the 1:2 males. Additional tests of such samples using a higher qPCR template concentration could be useful, to further investigate *dilp2* expression levels.

Nutrient pathway gene expression was sex-specific

Relative gene expression of *dTOR* was strongly female biased in the abdomen samples in both the 1:2 and 1:8 treatments. This result is consistent with previous findings of female-biased expression of *dTOR* in whole body samples of *D. melanogaster* (Bennett-Keki *et al.*, 2023). Interestingly, we did not observe sex-biased *dTOR* expression in head-thorax samples, which suggests that the strong female bias found in the whole fly samples by Bennett-Keki and colleagues (2023) could reflect expression in only the head-thorax body part. Despite strong female biased expression in *dTOR* abdomen samples, we did not see significant sex bias in the other samples tested. It has previously been reported that *dilp2* and *dfoxo* expression is male biased in adult fruit flies (Bennett-Keki *et al.*, 2023). The relative expression between the sexes in the dilp2 abdomen samples was below the detection limit in the female samples, which does give some evidence for sex -specific expression in this gene, with higher expression in males than females.

There was no significant interaction between sex and evolutionary diet in the relative expression of the genes tested here. However, there appeared to be a reversal in the direction of sex-bias in both *dfoxo* samples depending on evolutionary diet: *dfoxo* expression is somewhat female-biased in flies from 1:2 populations versus male-biased in flies from 1:8 populations. This pattern is especially interesting considering that the 1:2 diet is typically favoured by females, who maximise reproduction on higher protein diets, compared to the 1:8 diet, typically favoured by males, who maximise reproduction on lower protein, high carbohydrate diets. The result suggests that sex-biases in IIS/TOR gene expression may be

enhanced or altered over evolutionary time as individuals adapt to local variation in macronutrient availability.

Diet and nutrient pathway gene expression is linked to lifespan but not reproduction

We found that maximum longevity was seen in flies that evolved on the low P:C diet, 1:8, regardless of sex. This finding is consistent with previous findings that lifespan increases under low protein versus high protein diets in single generations of *D. melanogaster* (Jensen *et al.*, 2015; Carey *et al.*, 2022), *T. commodus* (Maklakov *et al.*, 2008), *B. tryoni* (Fanson & Taylor, 2012) and black garden ants *Lasius niger* (Dussutour & Simpson, 2012), for example. The three IIS/TOR pathway genes tested in our study are implicated in longevity responses to nutrients (Solon-Biet *et al.*, 2015). Lifespan extension is widely observed following dietary restriction across multiple taxa (Solon-Biet *et al.*, 2015), and is coupled with reduction in *dTOR* following inhibition by *dTsc1* and *dTsc2* (tuberous sclerosis complex genes 1 and 2) in low nutrient environments (Kapahi *et al.*, 2004; Johnson *et al.*, 2013). However, we did not observe reductions in *dTOR* expression in the 1:8 treatment in this study. Perhaps surprisingly, *dTOR* relative expression increased in the 1:8 populations in the head and thorax sample, while there was no difference between the diet treatments in the abdomen samples. *dfoxo* expression was relatively higher in the 1:8 line in the head-thorax samples. *dfoxo* expression in *D. melanogaster* under protein/yeast restricted diets has previously been found to increase lifespan by repressing *dilp5* (Min *et al.*, 2008) and adipose Dicer1 (*dcr-1*) (Sánchez *et al.*, 2023). This highlights the potential for distinct responses to diet macronutrient content over evolutionary time compared with plastic responses within generation.

Male and female lifespan is typically maximised on diets with a lower P:C ratio (Jensen *et al.*, 2015). Our results are consistent with this pattern: we found that the survival of males from the 1:2 populations was lower than males from the 1:8 populations, and survival of females

from the 1:2 populations was lower than females from the 1:8 populations. The difference in lifespan between diet treatments was less pronounced in females than males, which might indicate sex-differences in adaptation to the high P:C diet. Since 1:2 P:C would typically benefit females in egg laying and reproduction, perhaps females were under stronger selection to better adapt to the 1:2 diet than males. However, *dTOR* expression was significantly female-biased in the 1:2 populations despite evidence that *dTOR* inhibition contributes to lifespan extension (Kapahi *et al.*, 2004; Johnson *et al.*, 2013). Additional genes in the IIS/TOR pathway should be tested to confirm the role of the IIS/TOR pathway in the lifespan results here.

Female *D. melanogaster* reproduction is generally reduced on low protein diets (Lee *et al.*, 2008), due to the protein requirement for egg production (Cummings & King, 1969). Surprisingly, we did not see any changes to offspring production between the 1:2 and 1:8 diet treatments. However, in this study, flies were mated at the beginning of the assay within their populations (1:2♀+1:2♂, 1:8♀+1:8♂). Therefore, any possible costs or benefits of diet composition for females might have been masked by opposing effects of diet composition on male reproductive traits. For example, the increased egg production of females from the 1:2 population could be entangled with reduced courtship and male-male competition males from the 1:2 population due to reduced energy. Male reproductive effort and offspring production rates are maximised on lower protein: higher carbohydrate diets compared to females in *D. melanogaster* (Jensen *et al.*, 2015; Carey *et al.*, 2022), alongside increased male courtship calling effort in *T. commodus* (Hunt *et al.*, 2004; Maklakov *et al.*, 2008). *D. melanogaster* transfer fewer sperm and sire fewer offspring when reared on a low yeast diet (Bath *et al.*, 2023), but diet does not impact mating latency or duration (Macartney *et al.*, 2021). Hence, it would be interesting to measure female and male reproductive traits of the 1:2 and 1:8 treatments separately, by mating evolved flies with stock Dahomey of the opposite sex. Considering the impacts of evolutionary diet observed in the lifespan data, it would be relevant to measure reproductive

output over whole lifetimes to build a more complete picture of the effect of long-term diet on reproduction.

Overall, Dahomey flies from our stock population performed poorly in the fitness assays used here, with low survival and offspring production compared to the diet-evolved populations. This suggests adaptation of 1:2 flies and 1:8 flies to feeding and reproducing on casein:sucrose based diets. It is likely that the Dahomey flies, normally maintained on a sugar-yeast-agar based diet, performed poorly in our assays due to novelty of the casein:sucrose environment for both larvae and adult feeding and as an oviposition substrate. Therefore, it would be interesting to test naïve Dahomey fly performance on 1:2 and 1:8 foods to compare baseline levels of reproduction and lifespan on these diets.

The role of the IIS/TOR pathway in extending lifespan in organisms in starvation conditions has been extensively studied in multiple experimental models, including mice, nematode worms and *Drosophila* (Solon-Biet *et al*., 2015). However, little is known about how the expression of IIS/TOR pathways evolves in response to different ratios of P:C. Our results support the hypothesis that IIS/TOR signalling pathways evolve in response to local variation in diet composition. Our results for *dilp2*, *dTOR* and *dfoxo* gene expression did not precisely match previously observed relationships between relative expression and lifespan within generations. We therefore suggest more extensive study of this field by measuring additional genes of the IIS/TOR pathways following evolution under distinct diet regimes, and consequences for fitness of each life stage and sex.

References

Bath, E., Rostant, W., Ostridge, H.J., Smith, S., Mason, J.S., Rafaluk-Mohr, T., *et al.* (2023) Sexual selection and the evolution of condition-dependence: an experimental test at two resource levels. *Evolution*, **77**, 776–788.

Bennett-Keki, S., Fowler, E.K., Folkes, L., Moxon, S. & Chapman, T. (2023) Sex-biased gene expression in nutrient-sensing pathways. *Proceedings of the Royal Society B: Biological Sciences*, **290**, 20222086.

Brooks, M.E., Kristensen, K., Benthem, K.J. van, Magnusson, A., Berg, C.W., Nielsen, A., *et al.* (2017) glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. *The R Journal*, **9**, 378.

Camus, M.F., Huang, C.-C., Reuter, M. & Fowler, K. (2018) Dietary choices are influenced by genotype, mating status, and sex in *Drosophila melanogaster*. *Ecology and Evolution*, **8**, 5385–5393.

Carey, M.R., Archer, C.R., Rapkin, J., Castledine, M., Jensen, K., House, C.M., *et al.* (2022) Mapping sex differences in the effects of protein and carbohydrates on lifespan and reproduction in *Drosophila melanogaster*: is measuring nutrient intake essential? *Biogerontology*, **23**, 129–144.

Chapman, T., Trevitt, S. & Partridge, L. (1994) Remating and male-derived nutrients in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **7**, 51–69.

Cummings, M.R. & King, R.C. (1969) The cytology of the vitellogenic stages of oogenesis in *Drosophila melanogaster*. I. General staging characteristics. *Journal of Morphology*, **128**, 427–441.

Dussutour, A. & Simpson, S.J. (2012) Ant workers die young and colonies collapse when fed a high-protein diet. *Proceedings of the Royal Society B: Biological Sciences*, **279**, 2402–2408.

Fanson, B.G. & Taylor, P.W. (2012) Protein:carbohydrate ratios explain life span patterns found in Queensland fruit fly on diets varying in yeast:sugar ratios. *AGE*, **34**, 1361–1368.

Gargano, J.W., Martin, I., Bhandari, P. & Grotewiel, M.S. (2005) Rapid iterative negative geotaxis (RING): a new method for assessing age-related locomotor decline in *Drosophila*. *Experimental Gerontology*, **40**, 386–395.

Hunt, J., Brooks, R., Jennions, M.D., Smith, M.J., Bentsen, C.L. & Bussière, L.F. (2004) High-quality male field crickets invest heavily in sexual display but die young. *Nature*, **432**, 1024–1027.

Jang, T. & Lee, K.P. (2018) Comparing the impacts of macronutrients on life-history traits in larval and adult *Drosophila melanogaster*: the use of nutritional geometry and chemically defined diets. *Journal of Experimental Biology*, **221**, jeb181115.

Jensen, K., McClure, C., Priest, N.K. & Hunt, J. (2015) Sex-specific effects of protein and carbohydrate intake on reproduction but not lifespan in *Drosophila melanogaster*. *Aging Cell*, **14**, 605–615.

Johnson, S.C., Rabinovitch, P.S. & Kaeberlein, M. (2013) mTOR is a key modulator of ageing and age-related disease. *Nature*, **493**, 338–345.

Kapahi, P., Zid, B.M., Harper, T., Koslover, D., Sapin, V. & Benzer, S. (2004) Regulation of Lifespan in *Drosophila* by Modulation of Genes in the TOR Signaling Pathway. *Current Biology*, **14**, 885–890.

Kassambara, A., Kosinski, M. & Biecek, P. (2021) survminer: Drawing Survival Curves using "ggplot2."

Lee, K.P., Kim, J.-S. & Min, K.-J. (2013) Sexual dimorphism in nutrient intake and life span is mediated by mating in *Drosophila melanogaster*. *Animal Behaviour*, **86**, 987–992.

Lee, K.P., Simpson, S.J., Clissold, F.J., Brooks, R., Ballard, J.W.O., Taylor, P.W., *et al.* (2008) Lifespan and reproduction in *Drosophila*: New insights from nutritional geometry. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 2498–2503.

Macartney, E.L., Zeender, V., Meena, A., De Nardo, A.N., Bonduriansky, R. & Lüpold, S. (2021) Sperm depletion in relation to developmental nutrition and genotype in *Drosophila melanogaster*. *Evolution*, **75**, 2830–2841.

Maklakov, A.A., Simpson, S.J., Zajitschek, F., Hall, M.D., Dessmann, J., Clissold, F., *et al.* (2008) Sex-Specific Fitness Effects of Nutrient Intake on Reproduction and Lifespan. *Current Biology*, **18**, 1062–1066.

Min, K.-J., Yamamoto, R., Buch, S., Pankratz, M. & Tatar, M. (2008) *Drosophila* lifespan control by dietary restriction independent of insulin-like signaling. *Aging Cell*, **7**, 199–206.

Ng, S.H., Simpson, S.J. & Simmons, L.W. (2019) Sex differences in nutrient intake can reduce the potential for sexual conflict over fitness maximization by female and male crickets. *Journal of Evolutionary Biology*, **32**, 1106–1116.

Nolan, T., Hands, R.E. & Bustin, S.A. (2006) Quantification of mRNA using real-time RT-PCR. *Nature Protocols*, **1**, 1559–1582.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research*, **29**, e45.

Piper, M.D.W., Blanc, E., Leitão-Gonçalves, R., Yang, M., He, X., Linford, N.J., *et al.* (2014) A holidic medium for *Drosophila melanogaster*. *Nature Methods*, **11**, 100–105.

Sánchez, J.A., Ingaramo, M.C., Gervé, M.P., Thomas, M.G., Boccaccio, G.L. & Dekanty, A. (2023) FOXO-mediated repression of Dicer1 regulates metabolism, stress resistance, and longevity in *Drosophila*. *Proceedings of the National Academy of Sciences*, **120**, e2216539120.

Simpson, S.J. & Raubenheimer, D. (2012) *The Nature of Nutrition: A Unifying Framework from Animal Adaptation to Human Obesity*. Princeton University Press.

Solon-Biet, S.M., McMahon, A.C., Ballard, J.W.O., Ruohonen, K., Wu, L.E., Cogger, V.C., *et al.* (2014) The ratio of macronutrients, not caloric intake, dictates cardiometabolic health, aging, and longevity in ad libitum-fed mice. *Cell Metabolism*, **19**, 418–430.

Solon-Biet, S.M., Mitchell, S.J., Cabo, R. de, Raubenheimer, D., Le Couteur, D.G. & Simpson, S.J. (2015) Macronutrients and caloric intake in health and longevity. *The Journal of endocrinology*, **226**, R17–R28.

Tatar, M., Post, S. & Yu, K. (2014) Nutrient control of *Drosophila* longevity. *Trends in Endocrinology & Metabolism*, **25**, 509–517.

Templeman, N.M. & Murphy, C.T. (2017) Regulation of reproduction and longevity by nutrient-sensing pathways. *Journal of Cell Biology*, **217**, 93–106.

Therneau, T.M. (2023) A Package for Survival Analysis in R.

Therneau, T.M. & Grambsch, P.M. (2000) *Modeling Survival Data: Extending the Cox Model*. Springer, New York.

Appendix for Chapter 5

Table A1. Measurements needed for 1.5 L of solid meridic diet of varying P:C ratios, based on a combined caloric concentration of 120 g/L.

P:C ratio	1:2	1:4	1:5	1:8	4:1
Casein (g)	60	36	30	20	120
Sucrose (g)	120	144	150	160	60
Cholesterol (g)	0.45	0.45	0.45	0.45	0.45
Lecithin (g)	6	6	6	6	6
Agar (g)	30	30	30	30	30
KH_2PO_4 (ml)	150	150	150	150	150
K_2HPO_4 (ml)	150	150	150	150	150
$MgSO_4$ (ml)	150	150	150	150	150
$NaHCO_3$ (ml)	150	150	150	150	150
Nucleic acid sol (ml)	150	150	150	150	150
De-ionised H_2O (ml)	300	300	300	300	300
Autoclave the mixture at this stage					
10% Nipagin-ethanol solution (ml)	15	15	15	15	15
Propionic acid (ml)	4.5	4.5	4.5	4.5	4.5
Vitamin mix (ml)	225	225	225	225	225
De-ionised H_2O (ml)	Top up to 1.5 L	Top up to 1.5 L	Top up to 1.5 L	Top up to 1.5 L	Top up to 1.5 L

Salt solutions contain 2 L de-ionised H_2O and each salt in solid form (14.22 g KH_2PO_4, 74.6 g K_2HPO_4, 12.4 g $MgSO_4$, 20 g $NaHCO_3$). Nucleic acid solution contains 11.4 g Uridine, 12.8 g Inosine, 2 litres de-ionised H_2O. Diet adapted from Piper and colleagues (2014).

Table A2. Recipe to make 2 L vitamin mix.

Ingredient	Quantity
Thiamine (g)	0.0267
Riboflavin (g)	0.133
Nicotinic acid (g)	0.16
Ca Pantothenate (g)	0.222
Pyridoxine (g)	0.033
Biotin solution (ml)	133
Folic acid solution (ml)	133
De-ionised H2O (ml)	1733

Biotin solution made from 0.01g Biotin and 500ml de-ionised H_2O. Folic Acid solution made from 0.119g Folic Acid, 80ml ethanol and 320ml de-ionised H_2O. Both made in 500ml conical flasks and stored in fridge.

Chapter 6

General discussion

This thesis aimed to investigate the diet choice of males and females in ecologically relevant socio-sexual environments. In Chapter 1, I described the impacts of dietary macronutrient ratios on fundamental life history traits and the complex factors that impact animal diet choice. For example, the balance of two macronutrients, protein and carbohydrate, can alter reproduction and lifespan in individuals. Often, the ratio of protein and carbohydrate that most benefits reproductive traits can differ between males and females, and when animals are able to choose their diet, they typically prefer to consume a P:C content that will maximise their reproductive success. Therefore, it is important to understand the full picture of why animals choose the diets that they eat, and how this might be altered under different social or sexual scenarios. In this thesis, I studied diet choice (P:C content and quantity) in *D. melanogaster*, to investigate how male diet choice and female diet choice are impacted by remating, and how these choices are linked to reproductive rate (Chapter 2 and 3). I then studied the impact of male presence and social cues on the diet and oviposition choices made by females when foraging (Chapter 4) and how males and females may adapt to long-term macronutrient consumption (Chapter 5).

Summary and implications of Chapter 2 - Diet choice is insensitive to mating in male fruit flies

In Chapter 2, I investigated how males adapt their diet choice to compensate for ejaculate and energy depletion after ecologically realistic multiple matings. Though it is widely assumed that

sperm and non-sperm components are cheap to produce (Bateman, 1948; Trivers, 1972), more recent work has found ejaculate production may be costly and limiting to male mating rate and fertility (Dewsbury, 1982; Olsson, Madsen and Shine, 1997; Lewis, 2004; Reinhardt, Naylor and Siva-Jothy, 2011; Kant et al., 2012; Perry and Tse, 2013; Perry, Sirot and Wigby, 2013; Macartney et al., 2019; Simmons, Ng and Lovegrove, 2022). Males are also able to mate multiply (Lewis, 2004; Douglas, Anderson and Saltz, 2020). Therefore, I tested whether male *D. melanogaster* that were mated close to their daily reproductive limit (i.e. mating five times in series) would perceive and experience costs of multiple mating and hence replenish reserves by increasing their consumption of protein. Interestingly, I found that males did not alter the amount or ratio of protein and carbohydrate eaten and there were no differences in consumption between unmated, singly mated and five-times mated treatment groups. The inability or reluctance of males to adjust their protein intake was contrary to the predictions and was seen despite severe ejaculate and fertility depletion resulting in severely reduced offspring numbers.

The results of this chapter contributed new knowledge to our understanding of distinct differences in macronutrient preference between the sexes after mating. In contrast to males, females experience a dietary 'switch' post-mating and increase their overall diet consumption. The female diet switch has been observed in multiple insect models (Carvalho *et al.*, 2006; Barnes *et al.*, 2008; Perry, 2011; Pérez-Staples and Abraham, 2023) and is thought to support the frequently observed uptick in costly egg production after mating, in contrast to relatively low-cost sperm production in males. This is supported by evidence on diet choice in males after a single mating in which no change to diet intake is reported (Lee, Kim and Min, 2013; Camus *et al.*, 2018). However, males are in fact able to mate multiply in quick succession and as such, may not perceive or start to incur costs of reproduction until after successive bouts of mating have occurred (Jensen and Silverman, 2018). In addition, recent evidence suggests that sperm

production may indeed be costly and limiting for males (Reinhardt, Naylor and Siva-Jothy, 2011; Macartney *et al*., 2019; Simmons, Ng and Lovegrove, 2022). Despite this, males in our study did not adjust their macronutrient intake to recover from depletion of sperm and non-sperm components of the ejaculate.

Male carbohydrate preference remained stable across the different mating treatments, even directly after mating. This result was surprising considering the energetic requirement of male mating in *D. melanogaster*. Males of this species perform a courtship 'dance' to persuade females to mate, involving singing (through wing vibrations) and repeated movements (Bastock and Manning, 1955; von Schilcher, 1976). Courtship rituals are also common in other insect species (Droney, 2002; Maklakov *et al*., 2008; Cordes *et al*., 2015). Males in the five times mated treatment in our study did appear to experience reduced energy levels, as seen in the increase in mating latency and the requirement to introduce novel females to induce males to mate. In a parallel experiment conducted by an undergraduate (Herrera Grau, Sydney, Perry, unpublished data., 2021) we tested whether males that consecutively courted 5 mating-resistant females in turn, but that did not mate with them (and thus did not transfer any ejaculate) would show increased carbohydrate consumption solely due to energetic expenditure. However, consistent with my findings reported here, the results also did not reveal any changes to male dietary preferences for carbohydrate.

We observed a decrease in successful ejaculate transfer in males that had mated five times, with the number of offspring sired decreasing with each successive mating. However, it would be interesting to build on the results in this study, by assigning the five times mated males to P:C diets of differing ratios (following the mating assays) and measuring offspring production after a second mating round to test whether macronutrient balance impacts ejaculate replenishment. Sperm number could also be measured directly by dissecting testes and

recording mature sperm counts. If it was observed that successful ejaculate regeneration over time is impacted by diet, for example males on a high P:C regime sired more offspring than those on a low P:C diet, it would be even more interesting, and paradoxical, that males in our study did not adapt their diet choice. The effect of macronutrient ratio eaten by males on offspring production has been assessed previously but not in the context of sperm replenishment between bouts of multiple matings (Bunning *et al.*, 2015). Further work would reveal whether the diet chosen by the multiply mated males in our study is the optimum diet for fertility replenishment, or whether males are unable to perceive or translate a nutrition debt into their feeding choices.

Summary and implications of Chapter 3 - Post-mating switch in diet preference and reproductive behaviour in female *D. melanogaster*

In Chapter 3, I aimed to address gaps in our knowledge of post-mating diet changes in females. After a single mating, females increase protein preference and increase consumption of diet overall compared to their virgin counterparts. However, the trajectory of P:C preference of mated females over time had, to our knowledge, not previously been tested alongside ecologically relevant levels of egg laying. Additional data on female diet responses to a second mating are also key to determining whether the post mating response is a switch, triggered singly, or whether it can be further strengthened in a dose-dependent manner with additional matings (Perry, 2011). Therefore, we followed the diet choice and egg laying of unmated, singly mated, and two times mated (mated once at the start and again at the mid-point of the experiment) females, raised on either a high – or low-quality diet, across a 33-day assay. Carbohydrate intake was consistent between treatments, which is surprising considering previous findings of increased total diet consumption by mated females compared to virgin females (Lee, Kim and Min, 2013; Camus *et al.*, 2018). Conversely, I found that protein intake was dependent on mating treatment, increasing upon mating and remating. Egg laying was also

dependent on mating treatment, and was highest in remated flies, then mated flies. Surprisingly, condition did not affect the numbers of eggs laid despite reports of reduced ovariole numbers and egg counts following similar manipulations in previous studies (Duxbury and Chapman, 2020; Bath *et al*., 2023).

Tests on female diet choice after remating have been reported previously in *Adalia bipunctata* (Perry, 2011), though the increase in feeding that was observed after a second mating was not statistically significant. In *D. melanogaster*, intake of yeast-based protein and carbohydrate was measured in virgin, mated and remated flies using a sequence of CAFE assays over 2-weeks (Bowman and Tatar, 2016). Remated flies appeared to increase protein consumption after their second mating (Bowman and Tatar, 2016). The authors compared the number of eggs laid among treatments on the agar-water surface in CAFE vials, in the first three days of the experiment, however, they did not measure differences in egg laying following remating (Bowman and Tatar, 2016). This might have been because females were not consistently laying eggs by the time of the remating assay, at day 7; female *D. melanogaster* require multiple cues of oviposition site quality (such as softness (Zhang *et al*., 2020) and yeast odorants (Becher *et al*., 2012)) to exhibit sustained levels of egg laying, which they do not receive from an agar-water substrate. The issue regarding realistic egg laying and consumption in a CAFE assay is perhaps limited to the fruit fly model, as consumption can easily be separated from egg laying in other species. For example, in laboratory assays, tephritid fruit flies lay their eggs through mesh or film (Meats and Leighton, 2004; Fanson and Taylor, 2012), eggs are held in ootheca in cockroaches (Bunning *et al*., 2015), and crickets oviposit into sand (Maklakov *et al*., 2008).

The results in our study reveal an increase in protein consumption in remated flies, consistent with Bowman and Tatar (2016). Due to the difficulties in measuring protein consumption alongside realistic levels of egg laying in *D. melanogaster* outlined above (Lee, Kim and Min,

2013; Bowman and Tatar, 2016), we used a rotation of both the CAFE assay and provision of vials containing food media. Though females would have been able to compensate their nutrient intake on the solid diet, which may have diluted measurements of P:C intake in the CAFE assay, we still saw treatment differences. This suggests that a rotation of CAFE assay - solid food may be a reliable solution to measuring diet consumption without compromising on reproductive output.

Summary and implications of Chapter 4 - Female feeding and oviposition site preference is influenced by male social cues

In chapter 4 I investigated the impact of male harassment and the perception of male harassment on female feeding and oviposition site choice. Female *D. melanogaster* were placed into observation arenas containing two patches of diet, of distinct P:C ratios. The two diets chosen represented either a high protein diet (4:1 P:C), typically preferred by females, or a lower protein diet (1:4 P:C). In one treatment, females were left in single sex groups to interact and make dietary and oviposition choices between the two diet patches. In another treatment, an equal number of males was added to the middle of the arenas, to directly interact with females (including harassing and mating). Males in the third and fourth treatments were added behind perforated acetate barriers (allowing transfer of auditory, olfactory and visual cues; Bretman *et al.*, 2011) on one or other of the food patches. This allowed females to sense male presence on a food patch and make decisions on foraging and oviposition site, without direct, physical displacement by courting males. Contrary to our predictions, females did not avoid the food patches containing males (and the associated increased harassment risks). Instead, it appears that females gained important social information about the patch quality due to the presence of conspecifics (Lihoreau, Clarke, *et al.*, 2016). Females spent more time, and laid more eggs on patches that contained males, regardless of food quality.

The effect of harassment on female feeding has been observed previously in *Anthophora plumipes* where females chose to feed from more sheltered, low quality flowers during times of day when males were present (Stone, 1995). In addition, the negative relationship between harassment and female feeding has been studied extensively in poecilid fish (Griffiths, 1996; Magurran and Seghers, 1997; Dadda, Pilastro and Bisazza, 2008). Males may also directly disrupt female foraging behaviour in species with mounted mates (Rowe, 1992, 1994) and disrupt learning phases related to foraging on aversive versus non-aversive stimuli in *D. melanogaster* (Teseo *et al.*, 2016).

Despite the negative effects of male harassment on feeding reported in *D. melanogaster* and other species, we did not observe that females compromised on diet quality to avoid males when males were present on the high protein (female-preferred) diet patch. Perhaps in the experimental design used here, females did not perceive a true risk of harassment since males were behind a barrier. In addition, previous work in guppies has established that increasing numbers of female conspecifics dilute the impact of harassment on a focal female – sexual harassment encounters are shared among the females present, rather than concentrated on a single female (Dadda, Pilastro and Bisazza, 2005, 2008). Therefore, females in our study may have experienced a reduced harassment level in their groups of 10, compared to lone females (which we did not test). Varying the number of same-sex conspecifics would be interesting to further investigate harassment risk perception and dilution.

As discussed above, females did not avoid male flies. Surprisingly, they preferred instead to feed and lay eggs on patches where males were confined, regardless of nutrient quality. This result contributes new data to the study of social nutrition. This branch of animal nutrition research has revealed that individuals may make choices on diets depending on information derived from conspecifics, as in *D. melanogaster* (Lihoreau, Clarke, *et al.*, 2016) or make

collective, group decisions based on diets, as in *Blattella germanica* (Lihoreau, Deneubourg and Rivault, 2010) and desert locusts, *Schistocerca gregaria* (Günzel, Oberhauser and Couzin-Fuchs, 2023). It is likely that the effect seen in our study – i.e. the preference of females to feed on patches on which males were present – was a consequence of social cues. Females appeared to choose the most populous patch, regardless of nutrient benefit to themselves or offspring (Rodrigues *et al.*, 2015; Lihoreau, Poissonnier, *et al.*, 2016). It is unclear from our data whether the number of conspecifics would impact these social nutrition decisions in this assay. Perhaps reducing the number of flies making a choice (the females) or those already on a patch (the males) would in turn reduce strength of preference for the populated patch. Therefore, there are multiple avenues of further study to investigate this result.

Summary and implications of Chapter 5 - Differences in nutrient sensing gene expression as a result of diet and sex in two experimentally evolved populations

There is little information on the effects of long-term variation in diet despite the importance of macronutrient composition on life history, which I discussed in Chapter 1. Therefore, in Chapter 5 I investigated the impact of exposure to long-term macronutrient contents. Groups of *D. melanogaster* had been experimentally evolved on either a high P:C diet (1:2) or a low P:C diet (1:8). The relative expression patterns of genes involved in the IIS/TOR nutrient signalling pathways in the two treatments was measured at approximately 78 generations. *dilp2*, *dTOR* and *dfoxo* relative expression changed in the head and thorax samples between the evolutionary diet treatments, with similar changes in males and females. Meanwhile, the expression pattern of *dfoxo* in the abdomen was similar between the sexes in the 1:2 treatment group, but distinctly male-biased in the 1:8 treatment group. Evolutionary diet did not appear to impact relative expression of *dTOR* in the abdomen, as expression was strongly female biased in both treatment groups.

Survival, reproduction and activity were also assessed between the two treatments, and a stock population, after approximately 48 generations. Survival was impacted by evolutionary diet, with 1:8 females living longer that 1:2 females, and 1:8 males living longer than 1:2 males. Survival was especially reduced in males evolved on the 1:2 diet. No difference was found in the number of offspring produced by females (mated with males from the same treatment group) or geotaxis response times (a proxy for health and activity (Gargano *et al.*, 2005)) between the diet treatment groups.

Males and females tend to choose different macronutrient optima to maximise their reproduction, and this has been underpinned by sex-biases in the IIS/TOR pathways in *D. melanogaster* (Templeman and Murphy, 2017; Bennett-Keki *et al.*, 2023). We saw a strong females bias of *dTOR* expression in the abdomen samples, which is consistent with previous work (Bennett-Keki *et al.*, 2023). A change in sex-biased gene expression occurred as a result of evolutionary diet in one of the genes tested: *dfoxo* expression in the abdomen was female-biased in the 1:2 group but strongly male biased in the 1:8 group. This is interesting considering that the diets chosen typically maximised female (1:2) or male (1:8) reproduction and might suggest that adaptation of IIS/TOR pathways is sex specific. Interestingly, evolutionary diet altered expression patterns of *dilp2*, *dTOR and dfoxo* in the head and thorax of adult flies similarly between males and females. Overall, the nutrient sensing pathways are important in aiding adaptation to long term macronutrient consumption and additional genes could be tested to further investigate expression patterns under experimental evolution.

The 1:8 evolutionary diet appeared to benefit survival in both sexes. Males provided the 1:2 evolutionary diet exhibited the lowest survival, when comparing the diet evolved treatments. However, the difference between 1:2 male survival versus 1:8 male survival was larger than the difference between 1:2 versus 1:8 female survival. The greater cost of 1:2 for male survival

is interesting considering that the 1:2 diet would typically benefit female reproduction. Therefore, it is possible that females were under stronger selection to adapt to the 1:2 diet than males, considering the benefits of high P:C to female egg laying and offspring.

Though offspring production and geotaxis responses were consistent between the two diet treatments, additional tests are required to definitively establish life history traits under each regime. For example, though offspring numbers were comparable between the 1:2 and 1:8 groups, differences in development times may have evolved to ensure viability of larvae developing on 1:8 diets. Larvae on short-term low protein diets (commonly low-yeast diets) show increased development times (Duxbury and Chapman, 2020). 1:8 larvae may therefore have evolved longer development times to ensure uptake of protein quantity necessary for successful development. In addition, it would be interesting to know whether females evolved on 1:8 P:C would have reduced ovariole numbers, as in females raised on low P:C yeast-based diets for a single generation (Bath *et al.*, 2023). If 1:8 females have fewer ovarioles than 1:2 females, it would be surprising for both populations to have comparable levels of reproduction.

In further work, the reproductive success of treatment groups could be tested separately for each sex, by mating a focal treatment fly with a stock, wild-type fly of the opposite sex. In this chapter, reproductive output was instead calculated after mating females and males from the same treatment group. Since female reproduction is typically maximised on high P:C while male reproduction is maximised on low P:C, it may be than any positive impact of diet on reproduction in one sex would have been masked by a negative impact of that diet in the opposite sex. Separating tests of reproductive output by sex would allow us to further confirm sex differences in adaptation to long-term macronutrient manipulation.

Wider significance and conclusions

The work in this thesis revealed important differences between the sexes in response to the socio-sexual environment. For example, we found that females show a dynamic change in feeding in response to mating, consistent with previous studies. It is widely thought that increases in diet and protein consumption in mated females serve to support the production of nutrient costly, large gametes, as discussed above. It is therefore likely that the socio-sexual environment is fundamental in determining female diet choice due to this close relationship of mating, egg production and feeding.

In contrast, the socio-sexual environment appeared to have little impact on diet choice of multiply mated males. However, it remains to be tested whether the lack of male response observed occurred because males did not sense a nutrient debt or did not need to adapt their feeding because the high carbohydrate diet was sufficient. Perhaps the lack of increased intake may also be observed because males do not adjust their dietary consumption rates until sperm reserves have been fully depleted. As discussed above, further tests to manipulate male ejaculate investment, sperm depletion, sperm replenishment and diet choice would be useful to test the role of mating in male versus female diet choice in more depth.

Interactions between the sexes in the socio-sexual environment are also important for determining diet choice: our finding that females in fact preferred to feed from diet patches containing males, reveals an interesting effect of social interactions in *D. melanogaster* and highlights the importance of contacts between conspecifics in a species not considered to be typically social (Lihoreau *et al.*, 2018). Additional tests altering the sex ratio, harassment pressure (using focal versus non-focal females) and group size of foraging social groups would be required to investigate the reasons for the social preferences observed (Lihoreau, Clarke, *et al.*, 2016), while manipulating male pheromonal cues (Dweck *et al.*, 2015) may also help

uncover the mechanisms behind this effect. It would be interesting to know whether social preference consistently overrides diet quality and if the case is similar between males and females. Investigating the links between social cues and foraging are key to understanding why animals choose the diets that they eat under ecologically realistic social scenarios.

The relationship between the socio-sexual environment and diet preferences are also important for subsequent generations, as larvae will develop on the food that the eggs were laid into. In *D. melanogaster* P:C ratio can alter larvae-adult viability, development time, weight and female ovariole number (Rodrigues *et al.*, 2015) and there is evidence that females trade off larval development and offspring life history traits against their own macronutrient intake (Rodrigues *et al.*, 2015; Lihoreau, Poissonnier, *et al.*, 2016). Therefore, female preference for diets containing conspecifics (i.e. movement towards the most populous patch) regardless of nutrient quality would have important implications for offspring.

Differences between the sexes were present in response to evolutionary diet in *D. melanogaster*, with evidence for differential relative expression of genes involved in the IIS/TOR nutrient sensing pathways and survival following long term macronutrient exposure. Additional genes in the IIS/TOR pathways not tested in this thesis also show patterns of sex-biased gene expression in *D. melanogaster* (e.g. female biased expression of *Lnk*, *MEK* and *Myc*; Bennett-Keki *et al.*, 2023). Hence, it would be interesting to know whether the patterns of sex-bias of other genes are sustained or altered under the evolutionary diet regimes.

Though it was not tested in this thesis, it may be that evolutionary diet also differentially affected reproductive output of the males and females of each diet treatment, since short-term exposure to the 1:2 diet would typically benefit female reproduction, while short-term exposure to the 1:8 diet would typically benefit male reproduction (discussed in Chapter 5;

Carey *et al*., 2022). We also observed different levels of survival between the sexes of the two diet treatments, which could impact lifetime reproduction. Therefore, further tests separating reproduction of experimentally evolved males from reproduction of experimentally evolved females, such as by pairing individuals with stock, wild-type flies of the opposite sex, would be needed to discern sex differences in reproduction as a result of evolutionary diet. This would also help build a fuller picture of adaptation to macronutrient content as a whole.

Different assays for measuring diet choice in *Drosophila* were employed in the experimental work presented in this thesis. To account for egg production in female *D. melanogaster* related to diet consumption, we tested a rotation of CAFE assays and food media, and found that alternating CAFE assays and solid food media was a useful method to more accurately measure precise female diet choice. Alternatively, solid casein: sucrose diets were used to compare female foraging and oviposition choice. Though precise consumption quantity is not possible with a solid patch choice assay, unlike the CAFE assay, behavioural observations provided a robust measure of diet preference and collection of patches allowed for simple measures of oviposition diet preference. We also confirmed that females were eating the foods they were present on and were not influenced by colour of the food patch, by relating behaviour assays to the colour of fly abdomens after eating food dyed either red or blue. Therefore, assays using either liquid or solid diets, or a combination, can be useful and reliable to answer questions regarding the dietary preferences of *D. melanogaster*.

In this thesis, I report on the impact of the socio-sexual environment on diet choice in males (Chapter 2), and females (Chapter 3), how interactions between the two sexes alter dietary decisions in females (Chapter 4), and how males and females adapt to long-term macronutrient exposure (Chapter 5). Overall, the work presented furthers understanding of the importance of animal nutrition, specifically the P:C ratio, to fitness related traits such as egg laying,

offspring production and survival. The results outline how the socio-sexual environment may differentially alter the dietary decisions of each sex, and hence, underline the importance of investigating diet choice under ecologically realistic scenarios.

References

Barnes, A.I. *et al.* (2008) 'Feeding, fecundity and lifespan in female *Drosophila melanogaster*', *Proceedings of the Royal Society B: Biological Sciences*, 275(1643), pp. 1675–1683. Available at: https://doi.org/10.1098/rspb.2008.0139.

Bastock, M. and Manning, A. (1955) 'The Courtship of *Drosophila melanogaster*', *Behaviour*, 8(1), pp. 85–110. Available at: https://doi.org/10.1163/156853955X00184.

Bateman, A.J. (1948) 'Intra-sexual selection in *Drosophila*', *Heredity*, 2(3), pp. 349–368. Available at: https://doi.org/10.1038/hdy.1948.21.

Bath, E. *et al.* (2023) 'Sexual selection and the evolution of condition-dependence: an experimental test at two resource levels', *Evolution*, 77(3), pp. 776–788. Available at: https://doi.org/10.1093/evolut/qpac066.

Becher, P.G. *et al.* (2012) 'Yeast, not fruit volatiles mediate *Drosophila melanogaster* attraction, oviposition and development', *Functional Ecology*, 26(4), pp. 822–828. Available at: https://doi.org/10.1111/j.1365-2435.2012.02006.x.

Bennett-Keki, S. *et al.* (2023) 'Sex-biased gene expression in nutrient-sensing pathways', *Proceedings of the Royal Society B: Biological Sciences*, 290(1994), p. 20222086. Available at: https://doi.org/10.1098/rspb.2022.2086.

Bowman, E. and Tatar, M. (2016) 'Reproduction regulates *Drosophila* nutrient intake through independent effects of egg production and sex peptide: Implications for aging', *Nutrition and Healthy Aging*, 4(1), pp. 55–61. Available at: https://doi.org/10.3233/NHA-1613.

Bretman, A. *et al.* (2011) 'Males use multiple, redundant cues to detect mating rivals', *Current biology: CB*, 21(7), pp. 617–622. Available at: https://doi.org/10.1016/j.cub.2011.03.008.

Bunning, H. *et al.* (2015) 'Protein and carbohydrate intake influence sperm number and fertility in male cockroaches, but not sperm viability', *Proceedings of the Royal Society B: Biological Sciences*, 282(1802), p. 20142144. Available at: https://doi.org/10.1098/rspb.2014.2144.

Camus, M.F. *et al.* (2018) 'Dietary choices are influenced by genotype, mating status, and sex in *Drosophila melanogaster*', *Ecology and Evolution*, 8(11), pp. 5385–5393. Available at: https://doi.org/10.1002/ece3.4055.

Carey, M.R. *et al.* (2022) 'Mapping sex differences in the effects of protein and carbohydrates on lifespan and reproduction in *Drosophila melanogaster*: is measuring nutrient intake essential?', *Biogerontology*, 23(1), pp. 129–144. Available at: https://doi.org/10.1007/s10522-022-09953-2.

Carvalho, G.B. *et al.* (2006) 'Allocrine modulation of feeding behavior by the Sex Peptide of *Drosophila*', *Current biology: CB*, 16(7), pp. 692–696. Available at: https://doi.org/10.1016/j.cub.2006.02.064.

Cordes, N. *et al.* (2015) 'Larval food composition affects courtship song and sperm expenditure in a lekking moth', *Ecological Entomology*, 40(1), pp. 34–41. Available at: https://doi.org/10.1111/een.12156.

Dadda, M., Pilastro, A. and Bisazza, A. (2005) 'Male sexual harassment and female schooling behaviour in the eastern mosquitofish', *Animal Behaviour*, 70(2), pp. 463–471. Available at: https://doi.org/10.1016/j.anbehav.2004.12.010.

Dadda, M., Pilastro, A. and Bisazza, A. (2008) 'Innate responses to male sexual harassment in female mosquitofish', *Behavioral Ecology and Sociobiology*, 63(1), pp. 53–62. Available at: https://doi.org/10.1007/s00265-008-0635-z.

Dewsbury, D.A. (1982) 'Ejaculate Cost and Male Choice', *The American Naturalist*, 119(5), pp. 601–610.

Douglas, T., Anderson, R. and Saltz, J.B. (2020) 'Limits to male reproductive potential across mating bouts in *Drosophila melanogaster*', *Animal Behaviour*, 160, pp. 25–33. Available at: https://doi.org/10.1016/j.anbehav.2019.11.009.

Droney, D.C. (2002) 'The influence of the nutritional content of the adult male diet on testis mass, body condition and courtship vigour in a Hawaiian *Drosophila*', *Functional Ecology*, 12(6), pp. 920–928. Available at: https://doi.org/10.1046/j.1365-2435.1998.00266.x.

Duxbury, E.M.L. and Chapman, T. (2020) 'Sex-Specific Responses of Life Span and Fitness to Variation in Developmental Versus Adult Diets in *Drosophila melanogaster*', *The Journals of Gerontology: Series A*, 75(8), pp. 1431–1438. Available at: https://doi.org/10.1093/gerona/glz175.

Dweck, H.K.M. *et al.* (2015) 'Pheromones mediating copulation and attraction in *Drosophila*', *Proceedings of the National Academy of Sciences*, 112(21), pp. E2829–E2835. Available at: https://doi.org/10.1073/pnas.1504527112.

Fanson, B.G. and Taylor, P.W. (2012) 'Protein:carbohydrate ratios explain life span patterns found in Queensland fruit fly on diets varying in yeast:sugar ratios', *AGE*, 34(6), pp. 1361–1368. Available at: https://doi.org/10.1007/s11357-011-9308-3.

Gargano, J.W. *et al.* (2005) 'Rapid iterative negative geotaxis (RING): a new method for assessing age-related locomotor decline in *Drosophila*', *Experimental Gerontology*, 40(5), pp. 386–395. Available at: https://doi.org/10.1016/j.exger.2005.02.005.

Griffiths, S.W. (1996) 'Sex differences in the trade-off between feeding and mating in the guppy', *Journal of Fish Biology*, 48(5), pp. 891–898. Available at: https://doi.org/10.1111/j.1095-8649.1996.tb01484.x.

Günzel, Y., Oberhauser, F.B. and Couzin-Fuchs, E. (2023) 'Information integration for decision-making in desert locusts', *iScience*, 26(4). Available at: https://doi.org/10.1016/j.isci.2023.106388.

Jensen, K. and Silverman, J. (2018) 'Frequently mated males have higher protein preference in German cockroaches', *Behavioral Ecology*, 29(6), pp. 1453–1461. Available at: https://doi.org/10.1093/beheco/ary104.

Kant, R. *et al.* (2012) 'Effects of multiple matings on reproductive fitness of male and female *Diaeretiella rapae*', *Entomologia Experimentalis et Applicata*, 145(3), pp. 215–221. Available at: https://doi.org/10.1111/eea.12007.

Lee, K.P., Kim, J.-S. and Min, K.-J. (2013) 'Sexual dimorphism in nutrient intake and life span is mediated by mating in *Drosophila melanogaster*', *Animal Behaviour*, 86(5), pp. 987–992. Available at: https://doi.org/10.1016/j.anbehav.2013.08.018.

Lewis, S.M. (2004) 'Multiple mating and repeated copulations: effects on male reproductive success in red flour beetles', *Animal Behaviour*, 67(4), pp. 799–804. Available at: https://doi.org/10.1016/j.anbehav.2003.05.013.

Lihoreau, M., Clarke, I.M., *et al.* (2016) 'Collective selection of food patches in *Drosophila*', *Journal of Experimental Biology*, 219(5), pp. 668–675. Available at: https://doi.org/10.1242/jeb.127431.

Lihoreau, M., Poissonnier, L.-A., *et al.* (2016) '*Drosophila* females trade off good nutrition with high-quality oviposition sites when choosing foods', *Journal of Experimental Biology*, 219(16), pp. 2514–2524. Available at: https://doi.org/10.1242/jeb.142257.

Lihoreau, M. *et al.* (2018) 'Social nutrition: an emerging field in insect science', *Current Opinion in Insect Science*, 28, pp. 73–80. Available at: https://doi.org/10.1016/j.cois.2018.05.003.

Lihoreau, M., Deneubourg, J.-L. and Rivault, C. (2010) 'Collective foraging decision in a gregarious insect', *Behavioral Ecology and Sociobiology*, 64(10), pp. 1577–1587. Available at: https://doi.org/10.1007/s00265-010-0971-7.

Macartney, E.L. *et al.* (2019) 'Effects of nutrient limitation on sperm and seminal fluid: a systematic review and meta-analysis', *Biological Reviews*, 94(5), pp. 1722–1739. Available at: https://doi.org/10.1111/brv.12524.

Magurran, A.E. and Seghers, B.H. (1997) 'A cost of sexual harassment in the guppy, *Poecilia reticulata*', *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 258(1351), pp. 89–92. Available at: https://doi.org/10.1098/rspb.1994.0147.

Maklakov, A.A. *et al.* (2008) 'Sex-Specific Fitness Effects of Nutrient Intake on Reproduction and Lifespan', *Current Biology*, 18(14), pp. 1062–1066. Available at: https://doi.org/10.1016/j.cub.2008.06.059.

Meats, A. and Leighton, S.M. (2004) 'Protein consumption by mated, unmated, sterile and fertile adults of the Queensland fruit fly, *Bactrocera tryoni* and its relation to egg production', *Physiological Entomology*, 29(2), pp. 176–182. Available at: https://doi.org/10.1111/j.1365-3032.2004.00383.x.

Olsson, M., Madsen, T. and Shine, R. (1997) 'Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*', *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 264(1380), pp. 455–459. Available at: https://doi.org/10.1098/rspb.1997.0065.

Pérez-Staples, D. and Abraham, S. (2023) 'Postcopulatory Behavior of Tephritid Flies', *Annual Review of Entomology*, 68(1), pp. 89–108. Available at: https://doi.org/10.1146/annurev-ento-120220-113618.

Perry, J.C. (2011) 'Mating stimulates female feeding: testing the implications for the evolution of nuptial gifts', *Journal of Evolutionary Biology*, 24(8), pp. 1727–1736. Available at: https://doi.org/10.1111/j.1420-9101.2011.02299.x.

Perry, J.C., Sirot, L. and Wigby, S. (2013) 'The seminal symphony: how to compose an ejaculate', *Trends in Ecology & Evolution*, 28(7), pp. 414–422. Available at: https://doi.org/10.1016/j.tree.2013.03.005.

Perry, J.C. and Tse, C.T. (2013) 'Extreme Costs of Mating for Male Two-Spot Ladybird Beetles', *PLOS ONE*, 8(12), p. e81934. Available at: https://doi.org/10.1371/journal.pone.0081934.

Reinhardt, K., Naylor, R. and Siva-Jothy, M.T. (2011) 'Male Mating Rate Is Constrained by Seminal Fluid Availability in Bedbugs, *Cimex lectularius*', *PLOS ONE*, 6(7), p. e22082. Available at: https://doi.org/10.1371/journal.pone.0022082.

Rodrigues, M.A. *et al.* (2015) '*Drosophila melanogaster* larvae make nutritional choices that minimize developmental time', *Journal of Insect Physiology*, 81, pp. 69–80. Available at: https://doi.org/10.1016/j.jinsphys.2015.07.002.

Rowe, L. (1992) 'Convenience polyandry in a water strider: foraging conflicts and female control of copulation frequency and guarding duration', *Animal Behaviour*, 44, pp. 189–202. Available at: https://doi.org/10.1016/0003-3472(92)90025-5.

Rowe, L. (1994) 'The costs of mating and mate choice in water striders', *Animal Behaviour*, 48(5), pp. 1049–1056. Available at: https://doi.org/10.1006/anbe.1994.1338.

von Schilcher, F. (1976) 'The role of auditory stimuli in the courtship of *Drosophila melanogaster*', *Animal Behaviour*, 24(1), pp. 18–26. Available at: https://doi.org/10.1016/S0003-3472(76)80095-4.

Simmons, L.W., Ng, S.H. and Lovegrove, M. (2022) 'Condition-dependent seminal fluid gene expression and intergenerational paternal effects on ejaculate quality', *Functional Ecology*, 36(4), pp. 798–811. Available at: https://doi.org/10.1111/1365-2435.13987.

Stone, G.N. (1995) 'Female foraging responses to sexual harassment in the solitary bee *Anthophora plumipes*', *Animal Behaviour*, 50(2), pp. 405–412. Available at: https://doi.org/10.1006/anbe.1995.0255.

Templeman, N.M. and Murphy, C.T. (2017) 'Regulation of reproduction and longevity by nutrient-sensing pathways', *Journal of Cell Biology*, 217(1), pp. 93–106. Available at: https://doi.org/10.1083/jcb.201707168.

Teseo, S. *et al.* (2016) 'Sexual harassment induces a temporary fitness cost but does not constrain the acquisition of environmental information in fruit flies', *Biology Letters*, 12(1), p. 20150917. Available at: https://doi.org/10.1098/rsbl.2015.0917.

Trivers, R.L. (1972) 'Parental Investment and Sexual Selection', in B. Campbell (ed.) *Sexual Selection and the Descent of Man: The Darwinian Pivot*. Somerset, UNITED STATES: Taylor & Francis Group, p. 137. Available at: http://ebookcentral.proquest.com/lib/uea/detail.action?docID=4926119 (Accessed: 26 May 2022).

Zhang, L. *et al.* (2020) 'Parallel Mechanosensory Pathways Direct Oviposition Decision-Making in *Drosophila*', *Current Biology*, 30(16), pp. 3075-3088.e4. Available at: https://doi.org/10.1016/j.cub.2020.05.076.