CHARACTERIZING DIETARY EFFECTS ON *DROSOPHILA MELANOGASTER* REPRODUCTIVE BEHAVIORS

A Dissertation

by

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ABSTRACT

When choosing a mate, animals evaluate the attractiveness of potential partners. Attractiveness and its perception are condition-dependent traits. Animal condition is affected by complex interactions between genetic and environmental factors. Although the effects of diet on the condition of *Drosophila melanogaster* is well known, dietary effects on mating behavior are not well characterized. *D. melanogaster*, a genetic model organism, has been underutilized in the examination of intraspecific mate discrimination. In my Ph.D. dissertation, I developed behavioral assays to examine how macronutrient enriched diets affect *D. melanogaster* reproductive behaviors, demonstrating how traditional genetic models can advance condition-dependent mate choice studies.

I first examined how flies raised on either a control or a fat, protein, or sugar enriched diet interacted during courtship. Each diet affected the flies differently, and the responses were sexually dimorphic. High sugar diet had negligible effects. High protein diet affected only copulatory and post-copulatory traits, while high fat diet affected all traits with a stronger effect on females. I next examined the post-copulatory effects caused by low to high protein diets. This study confirmed that dietary protein content affects reproductive traits in a sexually dimorphic manner, as male contributed reproductive costs to females decrease with increasing protein content, while female contributions to the cost increase with dietary protein levels. Finally, I examined what female sexual cues could be altered by high fat diet (HFD), and the genetic factors that mediate HFD behavioral responses. I found that HFD alters female pheromone profiles to alter precopulatory behavior. Genetic manipulation of the same conserved metabolic pathways that rescue HFD health defects only rescued HFD male mating defects, further illustrating the sexual dimorphism of conditional responses in *D. melanogaster*. Through these three dissertation chapters I have established a robust method for examining both environmental and genetic effects on a complex behavioral system.

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All work for the dissertation was completed by the student, under the advisement of Dr. Ginger Carney of the Department of Biology. Undergraduate researchers helped to collect portions of the data throughout the dissertation under the advisement of the graduate student.

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CHAPTER I
INTRODUCTION

Intra-species variation in quality, or condition, can be explained by environmental factors, genetics, and the interaction between the two. The contribution of the genotype by environment interaction (G x E) can be greater than either factor individually, as the physiological manner in which an animal responds to environmental pressures is mediated by the alleles it possesses. Diet, an important environmental factor that interacts with genetics, impacts animal condition. Diet alone influences condition, as malnutrition can lead to weak animals that are unable to gain mates or that die prematurely, and excessive consumption of calories or specific macronutrients can lead to metabolic disease (obesity, insulin resistance, cardiac dysfunction) or decreased reproductive output. Both under- and over-consumption of food can therefore decrease fitness. The G x E effects of diet also contribute to animal condition, and can have stronger effects than diet alone (Reed et al., 2014). This interaction effect has been seen across a large range of animal taxa, from insects to humans.

Many animal traits are condition-dependent, meaning that these traits will also be altered by environmental factors affecting condition. Attractiveness, or the subjective evaluation of potential mates, is a condition-dependent trait, and as such, mate choice (the act of choosing a partner from the total pool of available mates based on mate preferences, or the innate inclination towards certain sexual traits) often results in assortative mating between individuals of the same quality or condition. This means that animals in good condition are more attractive while poor condition animals are less attractive. For assortative mating to occur, the perception of attractiveness must also be condition-dependent as good condition individuals will discriminate against poor condition mates while poor condition individuals will not. This process is called condition-dependent mate preference, which likely occurs because the benefits associated with gaining high quality mates (more/better offspring, etc.) do not outweigh the cost of exerting expensive mate preference behaviors (mate searching, fending off advances from undesirable mates, etc.).

Understanding how environmental factors like diet affect both animal condition and mate choice and the fitness consequences for choosing mates of varying condition will provide a more accurate understanding of mate choice as animals do not live in static settings but are constantly adapting to fluctuating environments (Miller and Svensson, 2014).

The goal of my dissertation has been to expand the emerging examination of how mating behaviors respond to variable environmental factors to a powerful model organism, *Drosophila melanogaster*. Identification of genetic pathways that contribute to mate choice has proved difficult, partly because many studies have focused on non-traditional, genetically intractable species with obvious sexual traits. Such flashy traits allow for robust examination of mate choice, but provide few resources for in-depth genetic analysis beyond transcriptomics and proteomics analysis. These "-omic" approaches can provide a broad picture of potential genetic candidates, but confirmation through genetic analysis is limited. Alternatively, fruit flies are genetically tractable, allowing for spatial and temporal genetic manipulation, and their mating behavior in static environments has been thoroughly investigated (Sokolowski, 2001). Many necessary genetic and neural components driving male courtship behaviors and female receptivity have also been characterized. These genetic tools and the extensive characterization of *Drosophila* mating behavior provide the perfect opportunity to examine G x E effects on mate choice.

To date, inquiry into *Drosophila* mate choice has mostly used diet as a blunt tool to drastically alter fly physiology and condition. For example, severely malnourished flies have been found to be less attractive (Mery et al., 2009). The effects of diets completely lacking a major macronutrient have also been characterized, finding that females fed protein devoid diets were unreceptive to male advances but more attractive (McRobert, 1986). A diet with no protein and high sugar could increase insulin signaling, which has been found to increase female attractiveness as discovered by genetic manipulation of insulin signaling levels (Kuo et al., 2012), although a diet low in protein and high in sugar did not affect female attractiveness (Fedina et al., 2012). While dietary protein and carbohydrate levels are important determinants in fly life history traits (Lee et al., 2008; Musselman et al., 2011), these are not the only macronutrients that affect fly condition.

Lipids also greatly contribute to fly health (Birse et al., 2010), although the effect of dietary lipid content on *Drosophila* reproductive behaviors had not yet been examined before my dissertation.

In this dissertation, I have examined the effects of dietary macronutrient levels on *D. melanogaster* pre- and post-copulatory mating behaviors, with specific attention to developmental vs adult exposure to enriched diets and to sex-specific responses. First, I tested diets enriched in either fat, protein, or sugar affected fly mating behavior, attractiveness, or fecundity. Second, I characterized how protein affects post-copulatory behaviors important for *D. melanogaster* fitness. Finally, I looked into which *D. melanogaster* sexual cues were altered by high fat diet and the genetic pathways that mediated high fat diet effects. Through these three dissertation chapters I have established methodology for examining G x E effects on a complex behavioral system.

CHAPTER II

DIET ALTERS *DROSOPHILA MELANOGASTER* MATE PREFERENCE AND ATTRACTIVENESS*

2.1 Introduction

How and why an animal selects a particular mate from among a pool of potential mates is a complex and incompletely understood process that is influenced by the continuously changing environment in which animals live. Animals evolve preferences for certain traits; these preferences fall along a continuum and are influenced by the animal's own condition and genetic make-up (Holveck and Riebel, 2009; Hunt et al., 2005; Lopez, 1999; Penton-Voak et al., 2003; Rodríguez and Greenfield, 2003). Sexual selection theory posits that the most attractive animals gain the largest number of matings and produce the most offspring (Andersson and Simmons, 2006; Jones and Ratterman, 2009). Mate preference can affect allelic distributions in populations via assortative mating that creates barriers to gene flow and is therefore an important force driving evolution and speciation (Arnegard et al., 2010; Mullen et al., 2007; Nosil et al., 2007; Shaw and Lesnick, 2009; Turner and Burrows, 1995). Consequently, understanding how fluctuating environmental factors shape mate preferences is integral to understanding species maintenance and hybrid avoidance (Miller and Svensson, 2014). While females are considered the 'choosy' sex in most cases, males also play a role in determining whether or not a mating occurs (Amundsen and Forsgren, 2001; Edward and Chapman, 2011, 2012, 2013), further complicating the study of mate choice.

Mate preference, which is the attraction of an animal to another with particular phenotypic characteristics, underlies mate choice and is a condition-dependent trait, meaning that discrimination between potential mates depends upon the internal mate preference, which is the attraction of an animal to another with particular phenotypic characteristics, underlies mate choice and is a condition-dependent trait, meaning that

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discrimination between potential mates depends upon the internal physiology of the choosing animal (References above and reviewed by Cotton et al., 2006). Therefore, preferences measured in a static scenario may not always predict mating outcomes as the 'preferred' partner could change depending upon a variety of environmental constraints such as access to mates of differing quality and availability of resources (Borgia, 1980; Chaine and Lyon, 2008; Danielson-Francois et al., 2006; Reviewed by Miller and Svensson, 2014; Svensson and Waller, 2013). However, high-condition animals, those with greater reproductive potential resulting from increased available energy stores, are generally more choosy when picking mates, are preferred by high-quality mates, gain greater numbers of matings and are more fecund (Bakker et al., 1999; Hebets et al., 2008; Hingle et al., 2001; Hunt et al., 2005; Jasienska et al., 2004; Lerch et al., 2011; Lerch et al., 2013; Mazzi, 2004; Moller, 1991; Moore and Moore, 2001; Petrie, 1983; Rintamaki et al., 1998; Rintamäki et al., 1995). While mate choice has the potential to confer fitness benefits in terms of offspring quantity or quality (Bos et al., 2009; Byrne and Rice, 2006), mate discrimination is costly. Energy is spent on increased sampling of the population and fending off courtship advances from undesirable mates, and poor-condition individuals are expected to exhibit lower levels of discrimination because the benefits gained from mate discrimination do not outweigh the costs (Cockburn et al., 2008; Cotton et al., 2006; Hingle et al., 2001; Holveck and Riebel, 2009; Wilgers and Hebets, 2012), resulting in increased rates of pairing between poor-condition individuals (Janicke et al., 2015; Kunz and Uhl, 2015; Xue et al., 2016). Alternatively, if the benefits gained from mating with good-condition animals continue to outweigh the costs of choosiness, poor-condition animals would be expected to continue to prefer good-condition mates, and evidence exists to support this hypothesis (Griggio and Hoi, 2010; Perry and Rowe, 2010).

Unstable environments can lead to fluctuating mate preferences, so it is important to understand how continually changing environmental factors, such as nutrient availability, influence mate choice (Reviewed by Miller and Svensson, 2014). Animal fitness is dependent upon condition and can be influenced by environmental factors such as diet, and nutrient availability has been shown to affect sexual selection and mate choice

(Janicke et al., 2015; Kunz and Uhl, 2015; Xue et al., 2016). The best ratio of macronutrients (fat, protein and sugar) varies by sex and species, but an ideal diet increases lifetime fecundity (Lee et al., 2008; Maklakov et al., 2009; Pirk et al., 2010; Solon-Biet et al., 2015). If imbalanced diets decrease fecundity, we expect that good-condition animals will find these mates less attractive and will modify their behaviour while poor-condition individuals will not.

Given the complexity and number of open questions surrounding mate choice, disentangling these variables is a vexing problem. However, the genetically tractable *Drosophila melanogaster* provides a good animal model for assessing how diet affects mate preference and individual attractiveness. Fruit fly mating behaviour has been intensively studied and described, as have the underlying required genetic and neural circuits (Reviewed by Yamamoto and Koganezawa, 2013). *Drosophila melanogaster* mate preference has been shown to have a genetic basis, as female preferences for male genotype vary by inbred line, while male preference rankings of female genotypes are largely independent of male genotype (Ratterman et al., 2014). *Drosophila melanogaster* mate preferences also can be altered by environmental factors such as temperature fluctuation (Narraway et al., 2010) and diet (Cook and Connolly, 1976; Cook and Cook, 1975; Fedina et al., 2012; Kuo et al., 2012; McRobert, 1986; Mery et al., 2009; Nandy et al., 2012).

In the wild, *D. melanogaster* consume rotting fruit that is colonized by yeast (Broderick and Lemaitre, 2012). Fruit macronutrient content varies based on genetics, environment and season (Arvanitoyannis and Mavromatis, 2009), and as yeast contains protein and lipids, the extent of colonization will also contribute to dietary diversity in a natural landscape. As adult *D. melanogaster* have wide dispersal ability (Coyne et al., 1982), sexually mature adults that have developed on substrates of varying quality likely aggregate and mate on new food sources. In this scenario, condition-dependent discrimination of mates of varying quality could be important for maximizing fitness. While a link between diet and attractiveness has been demonstrated in *D. melanogaster*, a thorough understanding of how specific macronutrients (fat, protein or sugar) affect mate

preference is lacking and could be expanded upon by addressing gaps in previous studies. The effect of dietary fat has not yet been examined, and mate preferences have often been measured indirectly via physical separation of flies (Mery et al., 2009) or through elicitation of courtship by immobilized or decapitated females that cannot perform a full repertoire of mating behaviours (Cook and Connolly, 1976; Cook and Cook, 1975; Fedina et al., 2012; Kuo et al., 2012; McRobert, 1986). Additionally, previous studies examined the effects of diet on one sex at a time. Our study aims to systematically characterize changes in mating behaviour and preference caused by specific increases in each individual macronutrient in intact, freely performing animals (Reed et al., 2014; Reed et al., 2010). We tested for dietary effects on mate preference in each sex by conducting in-depth analyses of mating behaviours and asked whether changes in behaviour corresponded with potential fitness, which we approximated with measures of fecundity for 5 days after mating. We expected that diet would alter the fecundity of the flies either positively or negatively and that we would detect changes in behaviour as a consequence.

2.2 Methods

Fly husbandry

In this study, we used Canton-S (CS) flies that had been isogenized for 10 generations via single-pair sibling matings and maintained continuously on standard laboratory diet (*Drosophila* agar, 10 g/litre; dextrose, 40 g/litre; sucrose, 20 g/litre; nutritional yeast, 12 g/litre; cornmeal, 70 g/litre; 3 ml/litre of 10% Tegosept). We placed five 5–10 day old, nonvirgin female and male CS flies in bottles containing 75 ml of either control or macronutrient-enriched food. The diets used to manipulate macronutrient content were modified from Reed et al. (2014): control (C, 7 g/litre of agar, 65 g/litre of cornmeal, 13 g/litre of inactive yeast, 7.5 g/litre of sucrose); high fat (C + 30 g/litre of coconut oil); high protein (C + 30 g/litre of sodium caseinate); and high sugar (C with 40 g/litre of total sucrose). We used Tegosept as a preservative in all diets. The control diet is similar to diets used in many *D. melanogaster* studies, including in our laboratory. In

the enriched diets, each macronutrient was increased by approximately 3%.

After 5 days, we removed the parents and collected progeny upon eclosion beginning at 1 h after 'lights on' within a 3 h window. Progeny matured in vials containing their respective diet for 5 days, with females in groups of five and males in isolation. We housed males in isolation to minimize the effect of perceived competition, which is known to alter male reproductive behaviours (Bretman et al., 2009). We know of no effect on mating behaviours of aging females in groups, and it is standard laboratory practice to do so (Ejima and Griffith, 2007). We raised all flies in the study in an incubator at 25 °C with a 12:12 h light:dark cycle. We performed all behavioural experiments on 5-day-old flies and did not anaesthetize flies on experimental days. Flies mated in 1 cm diameter and 0.785 cm^3 Plexiglas chambers containing moistened filter paper. We recorded fly interactions using JVC-HDD Everio and Sony HD Handycam cameras and stopped recording after mating was complete.

Single-pair mating assay

To evaluate how diet affects mate preference, we first used single-pair mating assays in which one male and one female were placed together in a courtship chamber and scored for various behavioural parameters. These assays are often referred to as 'no-choice' assays, although animals have a choice between mating and not mating. However, most flies mate during the assay period, and we evaluate their overall preferences using the parameters described below. Single-pair mating assays are ideal for determining preferences without the experimental confound of intrasexual competition. In our experiments, we quantified mating behaviours of animals raised on the control diet that were paired with individuals raised on either the control diet or on each of the enriched diets (high-fat, high-protein or high-sugar diet; Fig. 1a) in a combinatorial manner (control female with control male, control female with high-fat male, high-fat female with control male, high-fat female with high-fat male, control female with high-protein male, high-protein female with control male, high-protein female with high-protein male, control female with high-sugar male, high-sugar female with control male, high-sugar female with

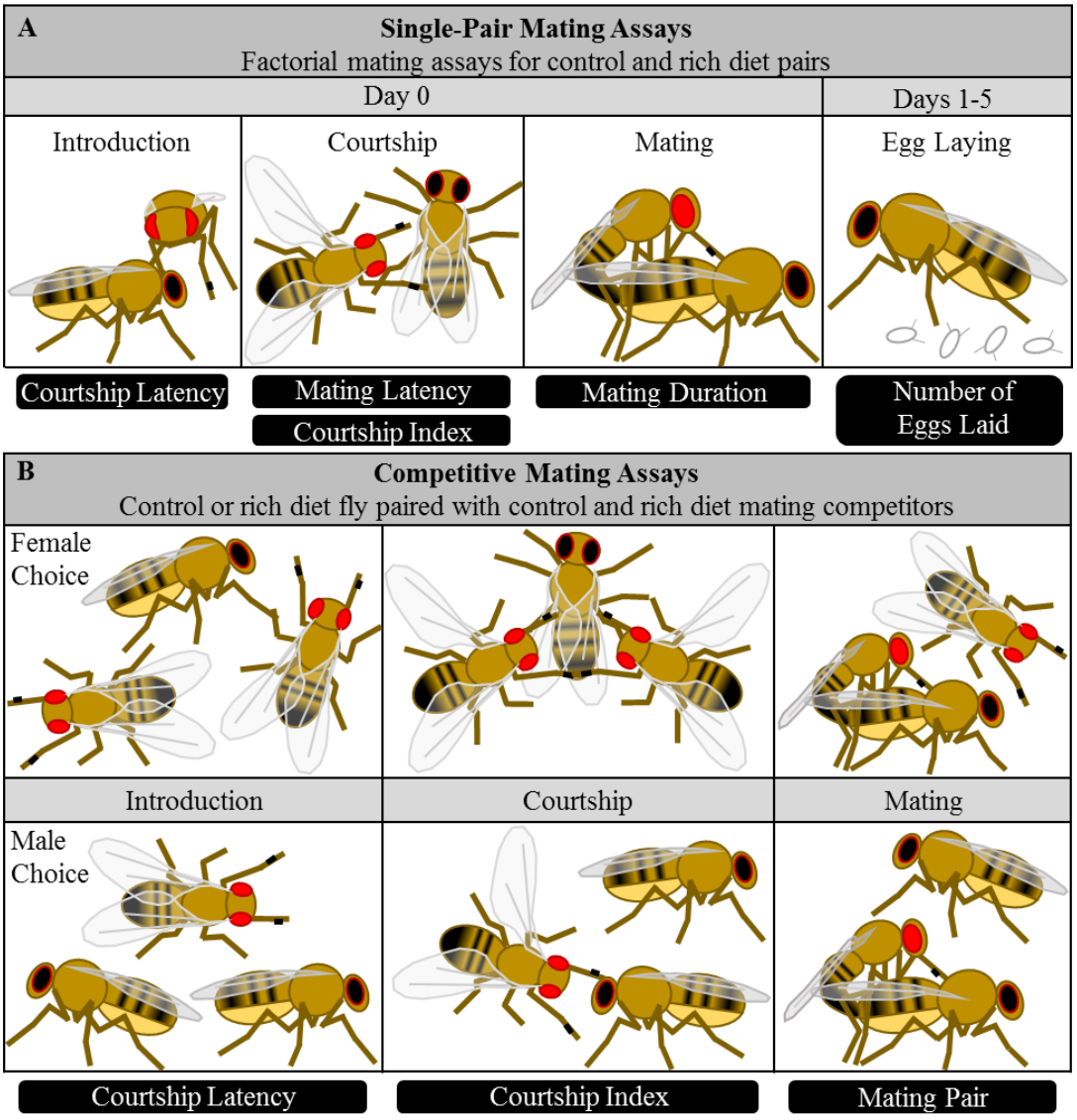

Figure 1. Quantifying the impact of dietary macronutrients on *D. melanogaster* reproductive behaviour. Wild-type Canton-S raised on either control (C) or rich (R; high fat, protein or sugar) diets were paired in a factorial manner (female × male: C × C, C × R, R × C, R × R) and reproductive traits (listed in black boxes) were measured. (a) Reproductive behaviours were quantified in single-pair mating assays to determine how diet affects attractiveness and mate preference. Courtship latency (male's time to initiate courtship) and courtship index (time spent courting the female from courtship initiation until mating) reflect female attractiveness to the male. Mating latency (time from courtship initiation until mating begins) reflects the willingness of the female to mate with that male. Mating duration represents the ejaculatory investment made by males. Eggs were counted for 5 days postmating to characterize which mating pairs produced the most offspring and whether relationships between fecundity and mate preference exist. (b) Competitive mating assays were performed to determine whether mate preferences found in (a) were maintained in competitive settings when direct comparison of mates from control and rich diets was possible. In female choice assays, females were paired with a control male and a rich-diet male, and in male choice assays, males were paired with a control female and a rich-diet female. In female choice assays, differences in courtship latency and courtship index indicate which male found the choosing female more attractive, while in the male choice assay, courtship latency and courtship index indicate which competing female was more attractive to the choosing male. Competition ceased when the choosing fly mated with one of the competing flies, and the resulting pair was noted. Males are pictured with red eyes and females are pictured with black eyes for illustrative purposes, although all flies tested had wild-type, red eye colour.

Table 1. Description of mating parameters measured in this study

Mating Parameter	Formula	Description
Mating Success (MS)	$MS = \frac{\text{number of successful matings}}{\text{number of total pairings}} \times 100$	Mating propensity[1,2,3]
Courtship Latency (CL)	CL = (courtship start time) – (time of introduction)	Female attractiveness to male[3,4,5,6,7]
Courtship Index (CI)	$CI = \frac{\text{time spent courting}}{ML}$	Female attractiveness to male[3,8]
Mating Latency (ML)	ML = (mating start time) – CL	Male attractiveness to female[3,6,7,9,10]
Activity Levels (AL)	AL = #of times flies cross chamber midline one minute before mating	Male condition; female receptivity[11,12,13]
Mating Duration (MD)	MD = (mating end time) – (mating start time)	Male reproductive investment[3,7,13,14,15]
Fecundity (F)	$\sum$ Number of eggs laid daily for 5 days	Fitness outcome of each pairing

[1](Merrell, 1949);[2](Villella and Hall, 1996); [3](O'Dell, 2003); [4](Eastwood and Burnet, 1977); [5](Tompkins et al., 1982); [6](Rybak et al., 2002); [7](Ratterman et al., 2014); [8](Siegel and Hall, 1979); [9](Connolly and Cook, 1973); [10](O'Dell et al., 1989); [11](McRobert, 1986); [12](McRobert et al., 2003); [13](MacBean and Parsons, 1967); [14](Gilchrist and Partridge, 2000); [15](Bretman et al., 2009)

high-sugar male, for a total of 10 pairings). One 5-day-old male and female were placed in a mating chamber and given 30 min to begin mating. We ran all single-pair mating assays examining all dietary treatments in parallel.

We then analyzed the mating videos to quantify standard behavioural parameters (Table 1), including male courtship latency, male courtship index (a composite measure of male courtship effort), mating latency, mating duration and overall mating success (Fig. 1a, Table 1). Courtship latency, courtship index and mating duration are commonly used measures for male preference, while mating latency, which is controlled by the female's

response to male signals, is a measure of female preference (Bretman et al., 2009; Connolly and Cook, 1973; Gilchrist and Partridge, 2000; Merrell, 1949; O'Dell, 2003; Ratterman et al., 2014). We also measured female and male activity levels by counting the number of times the flies crossed a line drawn through the middle of the courtship chamber in the final minute prior to mating (McRobert et al., 2003). Flies do not continually interact or move during the trials, so we examined activity levels in the final minute before mating when interaction and movement are most likely. Female activity levels prior to mating reflect female receptivity, as females that move less receive more copulation attempts (McRobert, 1986) and are more likely to mate (Tompkins et al., 1982). We quantified male activity as a measure of whether diet negatively affected male ability to keep pace with females during courtship.

We expected that each macronutrient would differentially affect the mating behaviours under examination by differentially affecting fly health and fecundity, which would ultimately lead to altered fitness. Fitness is dependent upon condition, the available resources for reproduction (Bakker et al., 1999; Hebets et al., 2008; Hingle et al., 2001; Hunt et al., 2005; Jasienska et al., 2004; Lerch et al., 2011; Lerch et al., 2013; Mazzi, 2004; Moller, 1991; Moore and Moore, 2001; Petrie, 1983; Rintamaki et al., 1998; Rintamäki et al., 1995). We predicted that mating partners that have higher fitness would be perceived as more attractive potential mates, and we measured fecundity to evaluate dietary effects on fitness. Although males would not be able to identify a priori which females are more fecund, we anticipated that some female attribute, such as body size, which is positively correlated with female fecundity (Lefranc and Bundgaard, 2000) and male preference (Byrne and Rice, 2006), would likely serve as an indirect indicator of her egg-laying potential. If so, more fecund females should be courted faster (reduced male courtship latency) and more vigorously (higher courtship index) by males and mate longer (increased mating duration), while males that are perceived by females to be more attractive will be mated faster (lower mating latency).

Upon completion of mating, we aspirated females into egg-laying vials, which contained 2.5 cm of standard laboratory food topped with 1 ml of 0.7% agar and a 1 cm

solid drop of either control, high-fat, high-protein or high-sugar food. We placed the females in vials with the food on which they had been raised and transferred them into a fresh egg-laying vial every 24 h for 5 days. We counted eggs daily and summed the total number of eggs laid over the 5-day period to measure fecundity.

Competitive mating assay

Effects of dietary manipulations on mate preferences identified through single-pair mating assays may not predict mating propensity in a situation where there is opportunity to choose between multiple potential partners. Animals use a variety of indicators to choose mates, relying upon a complex calculation from valences assigned to the many traits being evaluated (Hill, 2015a). We evaluated animal mate preference using two main experimental designs, a single-pair mating assay (where only one potential mate was available and choice between possible partners could not take place) and competitive mating assays (where animals chose between two potential mates) (Reviewed in Dougherty and Shuker, 2015; Wagner, 1998). In competitive mating assays, the animals being evaluated as potential mates are also competing against each other for the mating opportunity. The design of these tests differs in each animal's perception of risk, competition and mate availability, elements that are known to alter mate preference functions (Reviewed in Jennions and Petrie, 1997). Competitive assays more closely mirror the experiences of animals in natural settings where mate competition is likely high. Knowing that experimental design can influence the strength of preference expression, we used both single-pair mating and competition assays to measure mate preference. The competitive mating assays encompass both choice between two possible mates and competition, depending on whether we examine the preference of the competing or the choosing animal. Comparisons of preferences between these experiments (single-pair and competitive) are valid because they were carried out on animals from the same strain, by the same set of researchers in the same place, over a small window of time (Dougherty and Shuker, 2015).

Therefore, to evaluate how diet affects mate choice when animals can choose

between multiple potential mates, we employed a competitive mating assay design (Saleem et al., 2014) in which one fly (male or female) was placed in a mating chamber with two flies of the opposite sex. In each assay one potential mate was raised on the control diet, while the other was raised on one of the enriched diets (Fig. 1b). For example, we allowed females raised on control diet to choose between two prospective males, one of which was raised on control diet and one on high-fat diet; we also performed the converse experiment (high-fat diet female placed with one control diet male and one high-fat diet male). We used this experimental design to test all three enriched diets. We used the same design to test for dietary effects when a male was given a choice between two females raised on different diets. In all competition assays, flies had 30 min to begin mating. In female choice experiments, we scored each male separately for courtship latency and courtship index to understand how male courtship behaviour influences female mate choice, which we determined by observing which male mated with the female. In male choice experiments, we quantified male courtship latency and courtship index towards each of the two potential mates and determined which female mated with the male.

Statistics

Continuous data (courtship latency, female and male activity, mating latency, mating duration and total fecundity), proportion data (courtship index) and their residuals were first tested for normality (Shapiro–Wilk test), and if the set was not distributed normally, data were transformed (logarithmic or arcsine) and retested. To examine how each enriched diet affected *Drosophila* reproductive behaviours in single-pair mating assays, we performed a one-way MANOVA on all measured mating traits with all pairings between control and enriched diet mates. We next ran multiple univariate one-way ANOVAs on each measured mating trait to identify the traits affected by diet, and then performed Dunnett's tests comparing the pairing with only control mates (control female with control male) to all other pairings to gain a post hoc understanding of effects of specific diets on mating behaviours. Finally, we performed a Bonferroni correction on α

to counteract multiple testing effects.

In single-pair mating assays, mating success was analysed using a chi-square test. To determine which pairings differed significantly in this chi-square test, the difference between expected values and actual values were standardized to expected values, producing a *Z* statistic (Sharpe, 2015). *Z* statistics greater than 1.96 were found to be significantly different at $\alpha = 0.05$.

In competitive assays, we compared mating frequencies using chi-square tests and we compared courtship latency between the two males in female choice tests and between the two females in male choice tests using *t* tests. We compared the courtship indices of the two males in female choice tests or of the single male towards each female in male choice tests using Mann–Whitney *U* tests because the courtship index data did not have a normal distribution.

All individual flies used in this study were only tested once (i.e. all interacting animals were naïve virgins). JMP Pro 12 (SAS Institute, Cary, NC, U.S.A.) was used for all statistical analyses.

Ethical note

Although there are no federal or state of Texas requirements pertaining to wild-type *Drosophila*, we adhered to the ASAB/ABS Guidelines for the use of animals in research to minimize the numbers of animals used in our study. To minimize damage and discomfort to flies from the Canton-S laboratory strain used in this study, we handled flies by using CO_2 anaesthesia or aspiration, and we carried out euthanization by CO_2 anaesthetization, followed by placing flies in ethanol prior to freezing at -20 ºC as per Texas A&M University Institutional Biosafety Committee-approved protocols. Approval by an Institutional Animal Care and Use Committee was not required for this study.

2.3 Results

Single-pair mating assay

Diet significantly affected mating success, the total percentage of pairings that resulted in a mating (chi-square test: $\chi^2_{df=9} = 53.163$, $P < 0.0001$; Fig. 2). The only significant decrease occurred between high-fat diet females and control males ($Z = 5.378$).

A one-way MANOVA on all measured mating traits revealed a significant dietary effect (Wilks' λ: $F_{54,2008.5} = 7.7533$, $P < 0.0001$). Univariate one-way ANOVAs showed that diet significantly affected all measured traits (Bonferroni-corrected $\alpha = 0.00714$; courtship latency: $F_{9,555} = 3.3996$, $P = 0.0004$; courtship index: $F_{9,498} = 5.1318$, $P < 0.0001$; female activity: $F_{9,546} = 21.2995$, $P < 0.0001$; male activity: $F_{9,549} = 12.9494$, $P < 0.0001$; mating latency: $F_{9,547} = 8.7284$, $P < 0.0001$; mating duration: $F_{9,547} = 8.8076$, $P < 0.0001$; fecundity: $F_{9,479} = 49.4759$, $P < 0.0001$; Table 2). We next used Dunnett's tests to compare the control female and control male pairings to all other pairings.

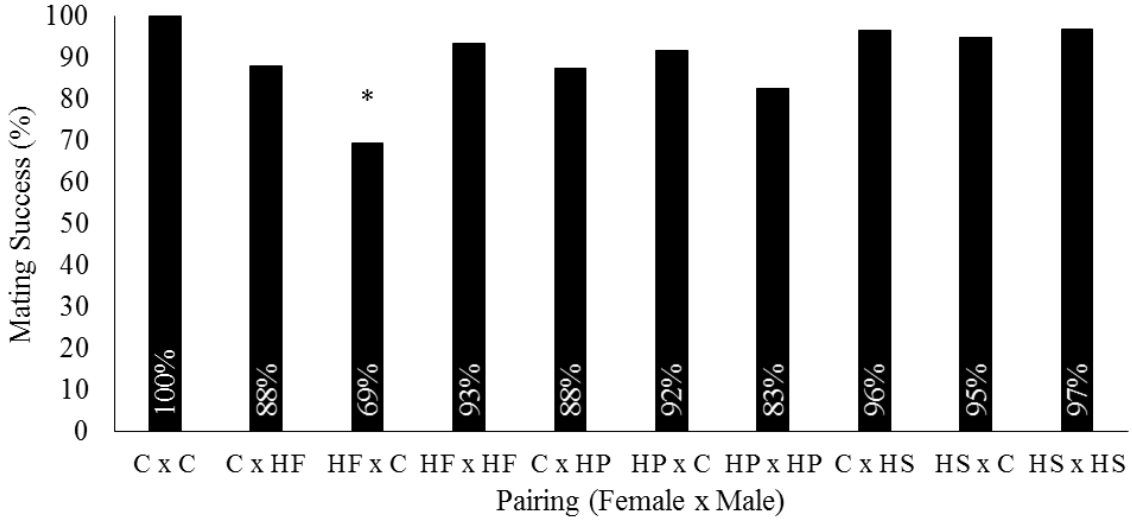

Figure 2. Mating success of control and enriched-diet fly pairings. C = control; HF = high fat; HP = high protein; HS = high sugar. Asterisk denotes significance via *Z* statistic test.

Table 2. The effect of diet on traits measured in single-pair mating assays.

Pairing: Female x Male	Log Courtship Latency	Arcsine Courtship Index	Female Activity	Male Activity	Log Mating Latency	Mating Duration	Log Fecundity
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
C x C	1.81 ± 0.05	1.17 ± 0.04	17.0 ± 0.78	16.2 ± 0.74	2.46 ± 0.048	1231 ± 28.0	1.91 ± 0.023
C x HF	1.76 ± 0.05 *P* = 0.9841	1.12 ± 0.04 *P* = 0.9715	16.0 ± 0.75 *P* = 0.9337	14.3 ± 0.71 *P* = 0.3147	2.62 ± 0.047 *P* = 0.1043	1259 ± 27.2 *P* = 0.9858	1.97 ± 0.023 *P* = 0.4134
HF x C	2.07 ± 0.05 ***P* = 0.0029**	0.90 ± 0.04 ***P* = 0.0001**	8.60 ± 0.80 ***P* < 0.0001**	10.2 ± 0.75 ***P* < 0.0001**	2.28 ± 0.049 *P* = 0.0539	1345 ± 28.6 *P* = 0.0312	1.71 ± 0.023 ***P* < 0.0001**
HF x HF	1.85 ± 0.05 *P* = 0.9992	1.11 ± 0.04 *P* = 0.9080	11.2 ± 0.80 ***P* < 0.0001**	10.3 ± 0.75 ***P* < 0.0001**	2.17 ± 0.049 ***P* = 0.0006**	1274 ± 28.6 *P* = 0.8715	1.65 ± 0.023 ***P* < 0.0001**
C x HP	1.81 ± 0.05 *P* = 1.0000	1.05 ± 0.04 *P* = 0.2192	17.7 ± 0.73 *P* = 0.9942	16.3 ± 0.69 *P* = 1.0000	2.59 ± 0.046 *P* = 0.2781	1087 ± 26.3 ***P* = 0.0016**	1.85 ± 0.022 *P* = 0.2687
HP x C	1.79 ± 0.05 *P* = 1.0000	1.20 ± 0.04 *P* = 0.9999	12.5 ± 0.80 ***P* = 0.0006**	12.3 ± 0.75 ***P* = 0.0018**	2.50 ± 0.049 *P* = 0.9974	1349 ± 29.1 *P* = 0.0249	2.20 ± 0.024 ***P* < 0.0001**
HP x HP	1.79 ± 0.05 *P* = 1.0000	1.09 ± 0.04 *P* = 0.6701	13.2 ± 0.75 ***P* = 0.0039**	12.7 ± 0.71 ***P* = 0.0049**	2.42 ± 0.046 *P* = 0.9930	1150 ± 26.8 *P* = 0.2142	2.11 ± 0.023 ***P* < 0.0001**
C x HS	1.80 ± 0.05 *P* = 1.0000	1.21 ± 0.04 *P* = 0.9922	19.2 ± 0.79 *P* = 0.2469	17.7 ± 0.75 *P* = 0.5860	2.50 ± 0.048 *P* = 0.9989	1250 ± 28.2 *P* = 0.9991	1.95 ± 0.024 *P* = 0.8271
HS x C	1.85 ± 0.05 *P* = 0.9992	1.16 ± 0.04 *P* = 1.0000	18.0 ± 0.78 *P* = 0.9392	16.9 ± 0.74 *P* = 0.9939	2.48 ± 0.048 *P* = 1.0000	1273 ± 28.2 *P* = 0.8792	1.93 ± 0.023 *P* = 0.9983
HS x HS	1.73 ± 0.05 *P* = 0.7493	1.23 ± 0.04 *P* = 0.8975	19.0 ± 0.80 *P* = 0.3640	17.5 ± 0.75 *P* = 0.7484	2.59 ± 0.046 *P* = 0.2983	1292 ± 27.2 *P* = 0.5203	1.92 ± 0.023 *P* = 0.9999
F	$3.3996_{(9,555)}$	$5.1318_{(9,498)}$	$21.2995_{(9,546)}$	$12.9494_{(9,549)}$	$8.7284_{(9,547)}$	$8.8076_{(9,547)}$	$49.4759_{(9,479)}$
P	**0.0004**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

High-fat diet affected all measured traits. Control males paired with high-fat females had a significantly increased courtship latency (P = 0.0029) and decreased courtship index (P = 0.0001). High-fat females were less active when paired with either control (P < 0.0001) or high-fat males (P < 0.0001), and both control (P < 0.0001) and high-fat males (P < 0.0001) were less active when paired with high-fat females. High-fat female mating latency was also decreased when females were paired with high-fat males (P = 0.0006). Finally, high-fat females paired with control (P < 0.0001) or high-fat males (P < 0.0001) had decreased fecundity.

High-protein diet did not affect courtship latency, courtship index or mating latency. High-protein females were less active with both control (P = 0.0006) and high-protein males (P = 0.0039), while control (P = 0.0018) and high-protein males (P = 0.0049) were only less active when paired with high-protein females. Control female and high-protein male pairings had decreased mating duration (P = 0.0006). High-protein females paired with control (P < 0.0001) and high-protein males (P < 0.0001) had increased fecundity.

High-sugar diet did not affect any of these measured traits.

Competitive mating assays

High fat diet

Female choice. When control males and high-fat males competed for control females (Table 3), male diet did not affect courtship latency (*t* test: $t_{df=138.8926}$ = 0.82518, P = 0.4107), courtship index (Mann–Whitney *U* test: Z = 0.48146, N_1 = 72, N_2 = 72, P = 0.6302) or which male mated (chi-square test: $\chi^2_{df=1,\, N\,=\,142}$ = 4.50, P = 0.0339). The results differed when a high-fat female was the courtship object (Table 3). When a control male and a high-fat male competed for a high-fat female, high-fat males courted more intensely overall as indicated by the higher courtship index (Mann–Whitney *U* test: Z = 2.43562 , N_1 = 50, N_2 = 50 P = 0.0149). This extra effort did not correlate with which male mated

because the control and high-fat males were equally likely to achieve matings with high-fat females (chi-square test: $\chi^2_{df=1, N=100} = 0$, $P = 1$).

Male choice. When control males had the opportunity to choose between a control female and a high-fat female (Table 4), control males courted control females more intensely (Mann–Whitney *U* test: $Z = -5.58113$, $N_1 = 50$, $N_2 = 50$, $P < 0.0001$) and mated with control females at significantly higher rates (chi-square test: $\chi^2_{df=1,N=100} = 13.52$, $P = 0.0002$). In contrast, high-fat males did not differ significantly in their courtship efforts towards females raised on either a control or a high-fat diet (courtship latency: *t* test: $t_{df=92.92} = 1.57$, $P = 0.1206$; courtship index: Mann–Whitney *U* test: $Z = 0.36540$, $P = 0.7148$). Despite directing similar effort towards both types of females, high-fat males mated more frequently with high-fat females (chi-square test: $\chi^2_{df=1, N=50} = 9.68$, $P = 0.0019$).

Table 3. The effect of high fat diet on female mate choice.

Female	Males	Behavior	Result	Statistic	P-value
Control	Control and High Fat	Courtship Latency	C = 1.83 ± 0.040 HF = 1.79 ± 0.042	t = 0.82518	0.4107
		Courtship Index	C = 0.678 ± 0.031 HF = 0.691 ± 0.032	$\chi2 = 0.2337$	0.6288
		Mated	C = 62.5% HF = 37.5%	$\chi^2 = 4.50$	0.0339
High Fat	Control and High Fat	Courtship Latency	C = 2.13 ± 0.0649 HF = 1.99 ± 0.0531	t = 1.7554	0.0831
		Courtship Index	C = 0.311 ± 0.0404 HF = 0.451 ± 0.0415	$\chi2 = 5.949$	**0.0147**
		Mated	C = 50% HF = 50%	$\chi^2 = 0$	1

Values in bold are statistically significant at Bonferroni corrected $\alpha = 0.0167$.

Table 4. The effect of high fat diet on male mate choice.

Male	Females	Behavior	Result	Statistic	P-value
Control	Control and High Fat	Courtship Latency	C = 1.97 ± 0.050 HF = 2.07 ± 0.040	t = 1.99	0.0867
		Courtship Index	C = 0.396 ± 0.035 HF = 0.127 ± 0.018	$\chi 2 = 31.19$	**<0.0001**
		Mated	C = 76% HF = 24%	$\chi^2 = 13.52$	**0.0002**
High Fat	Control and High Fat	Courtship Latency	C = 1.95 ± 0.039 HF = 2.04 ± 0.039	t = 1.57	0.1206
		Courtship Index	C = 0.278 ± 0.029 HF = 0.288 ± 0.0265	$\chi 2 = 0.1360$	0.7122
		Mated	C = 28% HF = 72%	$\chi^2 = 9.68$	**0.0019**

Values in bold are statistically significant at Bonferroni corrected $\alpha = 0.0167$.

Table 5. The effect of high protein diet on female mate choice.

Female	Males	Behavior	Result	Statistic	P-value
Control	Control and High Protein	Courtship Latency	C = 1.91 ± 0.0425 HP = 1.90 ± 0.0379	t = 0.22292	0.8240
		Courtship Index	C = 0.671 ± 0.0310 HP = 0.618 ± 0.0374	$\chi 2 = 0.7569$	0.3843
		Mated	C = 46% HP = 54%	$\chi^2 = 0.2857$	0.5930
High Protein	Control and High Protein	Courtship Latency	C = 1.94 ± 0.0595 HP = 1.97 ± 0.0488	t = 0.41040	0.6823
		Courtship Index	C = 0.554 ± 0.0378 HP = 0.518 ± 0.0413	$\chi 2 = 0.0583$	0.8092
		Mated	C = 61% HP = 39%	$\chi^2 = 2.5714$	0.1088

Table 6. The effect of high protein diet on male mate choice.

Male	Females	Behavior	Result	Statistic	P-value
Control	Control and High Protein	Courtship Latency	C = 1.81 ± 0.065 HP = 1.83 ± 0.063	t = 0.2585	0.7965
		Courtship Index	C = 0.350 ± 0.024 HP = 0.317 ± 0.025	$\chi 2 = 1.4504$	0.2285
		Mated	C = 48% HP = 52%	$\chi^2 = 0.0741$	0.7855
High Protein	Control and High Protein	Courtship Latency	C = 1.73 ± 0.055 HP = 1.73 ± 0.060	t = 0.02507	0.9800
		Courtship Index	C = 0.352 ± 0.0249 HP = 0.329 ± 0.031	$\chi 2 = 0.9315$	0.3345
		Mated	C = 58% HP = 42%	$\chi^2 = 1.28$	0.2579

High-protein diet

There were no significant effects of high-protein diet on behaviours in competitive situations (Tables 5-6). When a female had an opportunity to mate with either a control or a high-protein male, diet did not affect male behaviour and had no significant effect on male mating success (Table 5). Males raised on a control or high-protein diet courted and mated with control and high-protein females at similar rates (Table 6).

High-sugar diet

Female choice. High-sugar diet did not significantly affect behaviour or mating success in experiments where control or high-sugar females were paired with control and high-sugar males (Table 7).

Table 7. The effect of high sugar diet on female mate choice.

Female	Males	Behavior	Result	Statistic	P-value
Control	Control and High Sugar	Courtship Latency	C = 1.83 ± 0.0456 HS = 1.79 ± 0.0559	t = 0.58621	0.5592
		Courtship Index	C = 0.686 ± 0.0431 HS = 0.680 ± 0.0413	$\chi 2 = 0.0518$	0.8200
		Mated	C = 46% HS = 54%	$\chi^2 = 0.320$	0.5716
High Sugar	Control and High Sugar	Courtship Latency	C = 1.84 ± 0.0432 HS = 1.78 ± 0.447	t = 0.97109	0.3334
		Courtship Index	C = 0.688 ± 0.0319 HS = 0.781 ± 0.0306	$\chi 2 = 0.3707$	0.0542
		Mated	C = 44% HS = 56%	$\chi^2 = 1.0$	0.3173

Table 8. The effect of high sugar diet on male mate choice.

Male	Females	Behavior	Result	Statistic	P-value
Control	Control and High Sugar	Courtship Latency	C = 1.78 ± 0.069 HS = 1.83 ± 0.063	t = 0.47388	0.6366
		Courtship Index	C = 0.314 ± 0.029 HS = 0.345 ± 0.025	$\chi 2 = 1.2369$	0.2661
		Mated	C = 31% HS = 69%	$\chi 2 = 7.4074$	**0.0065**
High Sugar	Control and High Sugar	Courtship Latency	C = 1.70 ± 0.053 HS = 1.70 ± 0.049	t = 0.07685	0.9389
		Courtship Index	C = 0.336 ± 0.021 HS = 0.370 ± 0.020	$\chi 2 = 0.7775$	0.3779
		Mated	C = 35% HS = 65%	$\chi 2 = 5.4$	0.0201

Values in bold are statistically significant at Bonferroni corrected $\alpha = 0.0167$.

Male choice. Control males had similar courtship latencies (*t* test: $t_{df = 105.0285} = 0.6366$) and courtship indexes (Mann–Whitney *U* test: $Z = 1.1091$, $N_1 = 54$, $N_2 = 54$, $P = 0.2661$) towards control and high-sugar females (Table 6), but they mated (chi-square test: $\chi^2_{df = 1, N = 108} = 7.4074$, $P = 0.0065$) with high-sugar females more frequently. High-sugar males did not preferentially court or mate with either female (Table 8).

2.4 Discussion

Animals base mating decisions on a number of their potential partner's sexual cues that can be affected by environmental factors such as diet. By quantifying mating behaviour as flies raised on diets enriched for specific macronutrients interacted in single-pair and competitive mating assays, we were able to systematically characterize how each macronutrient affected *D. melanogaster* attractiveness and mate preference.

High-sugar diet

We expected to find changes in mate preference for flies with altered fecundity, a proxy for dietary effects on fitness. High-sugar diet did not cause changes in fecundity, and we found no evidence to support the hypothesis that high-sugar diets altered mate preferences in single-pair mating assays. However, examining the behaviours of flies in competitive assays revealed effects of high-sugar diet on mating success. While female diet did not affect measures of male preference (courtship latency or courtship index) in male choice assays, we found that control males mated more frequently with high-sugar females than with control females. We also detected a trend for increased matings between high-sugar males and high-sugar females. Our finding that male behaviour was similar towards control and high-sugar females suggests that males did not find high-sugar females more attractive and supports our observations from single-pair mating assays. The likely explanations for increased matings between males and high-sugar females are that high-sugar females are generally more receptive to mating or that they are less choosy about their partners.

Insulin signaling has been shown to be an important mediator of female

attractiveness through effects on pheromone profiles (Kuo et al., 2012), major sexual cues that males use to identify mates (Ferveur, 2005). Genetically increasing insulin signaling resulted in more attractive females, while genetically decreasing insulin signaling made females less attractive as measured by male courtship effort towards immobilized females (Kuo et al., 2012). Increased sugar intake resulted in increased insulin signaling in *D. melanogaster* (Musselman et al., 2011), and decapitated as well as freely behaving females fed sugar-only diets are more attractive (courted more by males) (Cook and Connolly, 1976; Cook and Cook, 1975). However, high-sugar diets that also contained yeast did not make females more attractive, even though changes in pheromone profiles were detected (Fedina et al., 2012). Our results are similar to those of Fedina et al. (2012) in that we found no evidence that high-sugar females are more attractive to males. Instead, it appears that high-sugar females are generally more receptive to mating, less choosy about their partner, or both. This possibility of high-sugar diet effects on female receptivity is in line with previous results showing that females with genetically decreased insulin signaling have decreased mating rates (Wigby et al., 2011).

High-protein diet

We found that high-protein females were more fecund, but males did not prefer high-protein females in single-pair or competitive mating assays, contrary to our expectations that more fecund females would be more attractive. Even though dietary protein content affects female pheromone profiles, high-protein females have not been found to be more attractive in other studies (Fedina et al., 2012; McRobert, 1986). Females fed a diet that included yeast were shown to be less active and more receptive to mating (McRobert, 1986). In our single-pair mating assays, high-protein females were also less active, which could be an indication that high-protein females were more receptive to mating. Logically, more receptive females were expected to mate more quickly, but we did not detect changes in high-protein female mating latency. Dietary protein is important for establishing female receptivity to mating and for copulatory and postcopulatory processes, such as fecundity and remating rate (Fricke et al., 2008; Fricke et al., 2010),

but increased dietary protein content does not seem to affect female or male attractiveness or mating decisions. Flies given prolonged exposure to increased dietary protein showed increased body mass and lipid content but incurred a cost in developmental survival (Kristensen et al., 2010). Our results suggest that the balance struck between these traits does not alter precopulatory mating behaviours that reflect evaluation of attractiveness in *D. melanogaster*.

High-fat diet

High-fat diet decreased female fecundity, indicating that the high-fat diet negatively affected female fitness. We hypothesized that less fecund flies would be less attractive and less choosy. We found support for this hypothesis in the single-pair mating assays as high-fat females were less active prior to mating and mated faster, indicating that high-fat females either had increased receptivity to mating, or were less choosy, or both. Control males paired with high-fat females also had decreased mating success. This effect is unlikely to be a consequence of decreased mating ability of high-fat females, as mating success between high-fat females and males was unchanged. We attribute this reduced mating success to decreased attractiveness of high-fat females, supported by the finding that control males also reduced courtship effort towards these females. Control males took longer to court high-fat females, and they also courted these females less intensely, providing further evidence that control males found high-fat females less attractive. Control female mating latency did not differ when females were paired with control or high-fat males, implying that the high-fat diet did not affect male attractiveness. Interestingly, mating success and courtship behaviours of high-fat males were similar towards control and high-fat diet females, whereas control males spent significantly less time courting high-fat females and mated with them less frequently. We propose that control males found high-fat diet females less attractive while high-fat males did not, a result that provides evidence for condition-dependent mate preference changes caused by high-fat diet.

High-fat diet also had affected competitive mating outcomes. When examining

high-fat diet effects on female mate choice, we found that control and high-fat males performed courtship similarly towards control females, and females did not show a bias towards either male when choosing a mate, consistent with results from the single-mating pair assays (i.e. that control females did not find high-fat males less attractive). In contrast, high-fat females elicited more robust courtship from high-fat males, but high-fat females were equally likely to mate with either male. Our finding that high-fat males showed increased courtship output towards high-fat females in competitive mating assays provides more evidence that high-fat males were indeed exhibiting condition-dependent changes in mate preference. We found it interesting that despite courting high-fat females more vigorously, high-fat males did not have an advantage in gaining matings with high-fat females. We do not know whether equal mating success for control and high-fat males is a sign that high-fat females are less choosy, resulting in random matings, or whether the low courtship but higher-than-expected matings for control males is a sign that high-fat females preferred control males over high-fat males.

High-fat diet also altered male mate choice outcomes. Control males found high-fat females less attractive in competitive settings, with control males almost exclusively courting and mating with control females when high-fat females were present. High-fat males, on the other hand, expended similar effort on courting control and high-fat females in competitive settings but mated more frequently with high-fat females. This result was unexpected as high-fat males' behaviour and mating success with control and high-fat females were indistinguishable in single-pair mating assays. Significantly higher matings between high-fat females and high-fat males in a competitive setting could be explained either by control female resistance to high-fat male advances, or by increased receptivity to mating by high-fat females. In noncompetitive circumstances, the high-fat male had no other mate to court, and therefore courted the control female until mating occurred. Yet, in a competitive setting, the high-fat male courted both females equally, and increased receptivity of high-fat females may have resulted in more matings. These experiments confirm the negative impact that high-fat diet has on female attractiveness and male discrimination ability. These outcomes lend further support to the idea that high-fat diet

negatively affects condition and fitness in *D. melanogaster*.

Previous studies that tested for the effects of environmental factors on mate choice in *D. melanogaster* examined single variable effects in one sex. One study demonstrated that female preferences change when females are raised under standard laboratory temperature compared to colder rearing conditions (Narraway et al., 2010). Another study assessed whether ejaculate depletion affected adaptive male mate choice (Byrne and Rice, 2006), finding that multiply mated males become more selective for females with higher reproductive potential. Our work is a more comprehensive evaluation of mate choice than these earlier studies since we assessed effects of multiple treatments (i.e. a range of diets with increases in fat, protein or sugar) and determined the effects of the manipulations on preference and attractiveness for both sexes in competitive and noncompetitive mating situations. Our findings demonstrating the effects of high-fat diet on male mate preference along with the previous studies mentioned above support the idea that environmental stressors like diet, temperature and rearing condition can decrease *D. melanogaster* mate discrimination abilities.

Conclusions

We tested the underlying assumption that changes in diet would lead to changes in fitness and attractiveness that would cause flies to alter the way they perform mating behaviours, either in response to their own internal state, or in response to the altered condition of potential mates. Dietary effects on *D. melanogaster* physiology, health, fitness and life span have been studied extensively as the major pathways controlling metabolic homeostasis are conserved between *D. melanogaster* and vertebrates (Reviewed by Padmanabha and Baker, 2014; Smith et al., 2014; Teleman, 2010; Tennessen et al., 2014). High-fat diet leads to a dysregulation of nutrient storage (Carvalho et al., 2012; Heinrichsen et al., 2014), adverse health effects and stress response (Birse et al., 2010; Heinrichsen and Haddad, 2012; Reed et al., 2014; Reed et al., 2010), decreased life span (Driver and Cosopodiotis, 1979; Holmbeck and Rand, 2015) and changes in oviposition site preferences (Flaven-Pouchon et al., 2014). Varying dietary sugar-to-yeast

(S:Y) ratios has been found to influence fecundity and longevity, with higher S:Y extending life span and lower S:Y enhancing fecundity (Chapman and Partridge, 1996; Chippindale et al., 1997; Chippindale et al., 1993; Kristensen et al., 2010; Lee et al., 2008; Skorupa et al., 2008). Dietary sugar and yeast content also affect *D. melanogaster* exercise physiology (Bazzell et al., 2013), health (Morris et al., 2012; Musselman et al., 2011; Na et al., 2013) and postcopulatory processes (Amitin and Pitnick, 2007; Fricke et al., 2008; Fricke et al., 2010; McGraw et al., 2007), and some of these effects have been shown to persist through subsequent generations (Buescher et al., 2013; Öst et al., 2014; Valtonen et al., 2012). Tissues and genes involved in metabolism also have been found to be important for *D. melanogaster* reproductive behaviour. The main nutrient storage organ, the fat body (Fujii and Amrein, 2002; Lazareva et al., 2007), and enzymes involved in lipid metabolism such as *desat1* (Labeur et al., 2002; Ueyama et al., 2005), *desatF* (Chertemps et al., 2006) and *sxe1* (Fujii et al., 2008) are essential for typical courtship behaviour. Neurons that express *fruitless*, one of the best characterized and most implicated genes in male courtship behaviour, also regulate lipid storage in the fat body (Al-Anzi et al., 2009).

In our study, we observed corresponding changes in fecundity and mate preference when flies were treated with high-fat diet, but we did not characterize which mating signals high-fat diet modified. High-fat diet could modify pheromone profiles or cause changes in male courtship song that decrease attractiveness as a reflection of poor fly condition. *Drosophila melanogaster* communicate information about species, sex, mating status and age through pheromones (cuticular hydrocarbons or CHCs), which have fatty acid backbones (Ferveur, 2005). These CHC profiles are also known to vary with dietary carbohydrate-to-protein ratios (Fedina et al., 2012), although such changes did not result in corresponding fluctuations in attractiveness as measured through single-pair mating tests. Mate preference changes were observed when *D. melanogaster* were reared on starch or molasses media, and these changes were mediated through pheromone profile changes via influences from the gut microbiome (Sharon et al., 2010). Similar changes in mate preference and pheromone profiles were detected when *Drosophila serrata* were

raised on varying media (Rundle et al., 2005). Since females reared on high-fat diets are less attractive to males, a high-fat diet may affect female CHC profiles and make these females less appealing mates.

Male courtship song is another important factor for female mate choice (Reviewed by Sokolowski, 2001), but whether song is an indicator of male condition has not been thoroughly explored in *D. melanogaster*. It is unlikely that high-fat diet affected male courtship song since females in our study did not appear to find high-fat males less attractive. However, larger male *D. melanogaster* are more attractive to females (Partridge and Farquhar, 1983) and produce more energetic (Partridge et al., 1987) and attractive songs (Talyn and Dowse, 2004), so additional studies are needed to determine whether song is a conditional cue.

Gaining a holistic understanding of mate choice is an important task that is complicated by the fact that the benefits of mate preferences are not always obvious. In species where mates provide parental care or other services, and better mates can provide better services, the benefits of mate choice are direct (Reviewed by Jones and Ratterman, 2009). Yet in species where no direct benefits exist, indirect benefits are expected to mitigate the costs incurred from expensive mate choice. Theory predicts that animals that reproduce with preferred, more attractive mates will produce more or better-quality offspring, leading to higher fitness (Reviewed by: Andersson and Simmons, 2006; Jones and Ratterman, 2009). While empirical examples describing the benefits of mate choice have been identified (Edward and Chapman, 2012; Promislow et al., 1998), instances where preferred mates are detrimental to individual fitness also exist. For example, female *D. melanogaster* housed with preferred large males had decreased fitness (Friberg and Arnqvist, 2003) and did not recoup these fitness costs through sexy sons (Orteiza et al., 2005). The results of our study contribute to this discussion by highlighting the fact that environmental factors can affect mate preference in ways that are not always predictable. Recent attention has been called to the lack of understanding in how fluctuating environments can affect sexual selection (Janicke et al., 2015; Miller and Svensson, 2014). Model organisms such as *D. melanogaster* are good test subjects for studying mate

preference because of the array of genetic and molecular tools available. While our study focused on one isogenic line, future experiments using genetic tools, such as the Drosophila Genetics Reference Panel, to address how variable environments affect mate preference and sexual selection in multiple genetic backgrounds could identify genetic pathways important for evolutionary processes.

CHAPTER III

DIETARY PROTEIN CONTENT ALTERS BOTH MALE AND FEMALE CONTRIBUTIONS TO *DROSOPHILA MELANOGASTER* FEMALE POST-MATING RESPONSE TRAITS

3.1 Introduction

Male ejaculate transferred during mating induces an array of physiological and behavioral changes in the female (Reviewed by: Avila et al., 2011; Chen, 1984; Gillott, 2003; Perry et al., 2013; Wolfner, 2002). These changes, which are collectively referred to as the female post-mating response (PMR) in insects, have been studied extensively in *Drosophila melanogaster*. The male ejaculate components facilitate female reproductive activity and manipulate female physiology and behavior to simultaneously increase reproductive output (Chapman et al., 2003; Liu and Kubli, 2003) and decrease the opportunity for sperm competition (Bretman et al., 2009; Gilchrist and Partridge, 2000). While increased offspring production and paternity share benefit males, mating and reproduction harm females by decreasing lifespan (Fowler and Partridge, 1989). High fecundity harms females by increasing stress susceptibility (Salmon et al., 2001; Wang et al., 2001) and by shunting finite resources towards reproduction and away from somatic maintenance and processes that promote longevity (Fredriksson et al., 2012; Lee et al., 2008). Seminal fluid proteins and their effects on female physiology are also toxic and harm females (Chapman et al., 1995; Civetta and Clark, 2000; Fowler and Partridge, 1989; Wigby and Chapman, 2005). These opposing interests between the sexes can result in sexual conflict and an evolutionary arms race where males seek to increase female reproductive output while females develop responses to mitigate the costs. Sexual conflict has shaped the reproductive biology of *D. melanogaster*, and the PMR benefits male fitness while potentially harming females (Chapman et al., 1995; Civetta and Clark, 2000),

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yet *Drosophila* females are not passive players and can partially allay mating costs (Holland and Rice, 1999).

The molecular processes underlying the *Drosophila* PMR are well characterized. Males produce seminal fluid peptides (SFPs) (Wolfner, 1997) and other molecules that are transferred, either suspended within the ejaculate mixture, bound to sperm (Peng et al., 2005a), or packaged in exosomes (Corrigan et al., 2014), to females during copulation. The ejaculate triggers the female PMR (Ram and Wolfner, 2007, 2009), which includes sperm storage (Kaufman and Demerec, 1942; Lefevre Jr and Jonsson, 1962; Neubaum and Wolfner, 1999; Qazi and Wolfner, 2003; Tram and Wolfner, 1999), increased ovulation and egg laying (Chapman et al., 2003; Heifetz et al., 2000; Herndon and Wolfner, 1995; Liu and Kubli, 2003; Saudan et al., 2002), increased nutrient intake and preference for protein rich diets (Carvalho et al., 2006), immune responses (Fedorka et al., 2007; Kapelnikov et al., 2008; Peng et al., 2005b; Short and Lazzaro, 2010; Wigby et al., 2008), decreased attractiveness and receptivity to remating (Pitnick, 1991; Tram and Wolfner, 1998), and decreased sleep and increased activity (Isaac et al., 2009).

The PMR is plastic and varies according to fly developmental conditions. High larval rearing densities produce small males that perform poorly during sperm competition, and females that initially mate with these small males remate sooner (McGraw et al., 2007). While small males produce less of two SFPs (sex peptide and ovulin) that promote many aspects of the PMR, they are able to transfer nearly normal amounts during mating, indicating that small males that are reproductively disadvantaged can increase mating investment (Wigby et al., 2015).

Diet is another environmental factor known to affect *D. melanogaster* reproduction. Protein is an important macronutrient that contributes to increased egg production (Bownes and Blair, 1986; Bownes et al., 1988), and female diets that are high in protein increase female fecundity but decrease female mating rate and longevity (Fricke et al., 2010; Lee et al., 2008). Dietary protein also affects male reproductive ability, as males fed high protein diets mate for shorter periods of time (Fricke et al., 2008; Schultzhaus et al., 2017), and males fed intermediate levels of protein sire more offspring

than males fed low or high protein diets (Fricke et al., 2008). While these previous experiments demonstrate that dietary protein can affect postcopulatory reproductive processes, no studies have thoroughly investigated how the dietary protein content of both sexes specifically affects the *D. melanogaster* female PMR.

To test whether the amount of dietary protein available to both males and females affects the magnitude of the female PMR, we fed flies a range of low, moderate, and high protein diets, and males and females from each diet were mated in a fully combinatorial manner. We evaluated effects on PMR by measuring fecundity and remating latency (a measure of when female mating receptivity returns). We expected that the magnitude of the female PMR would decrease with increasing male protein content (decreased fecundity and shorter remating latency) as males fed high protein diets have been shown to mate for shorter periods (Fricke et al., 2010; Schultzhaus et al., 2017) and may transfer less ejaculate to females. If the traits of the PMR are coupled and respond to environmental factors similarly, then we expected that the magnitude of the PMR would increase (increased fecundity and longer remating latency) with higher female protein content because female fecundity is tightly correlated with dietary protein content (Schultzhaus et al., 2017).

3.2 Methods

Fly husbandry

To follow up on our initial observation that a high protein diet decreased male mating duration while increasing female fecundity (Schultzhaus et al. 2017), we examined how a range of dietary protein content would affect postcopulatory processes. The diets used to manipulate protein content were modified from Schultzhaus et al. 2017: 71% protein (7 g/L agar, 65 g/L cornmeal, 6.5 g/L inactive yeast, 7.5 g/L sucrose; half the amount of yeast as the standard protein diet), 100% protein (7 g/L agar, 65 g/L cornmeal, 13 g/L inactive yeast, 7.5 g/L sucrose), and 206% protein (16 g/L agar, 65 g/L cornmeal, 13 g/L inactive yeast, 7.5 g/L sucrose + 12 g/L sodium caseinate; two times the amount of

protein as the standard protein diet). We used Tegosept as a preservative in all diets. The standard protein diet used here is identical to the control diet (Schultzhaus et al. 2016) and the low sugar diet (Reed et al., 2014; Reed et al., 2010) used previously. The protein content of cornmeal is approximately 7%. Inactive yeast is approximately 50% protein, and sodium caseinate is 100% protein.

We tested the effect of these diets on PMR in wild-type *Canton-S* (*CS*) flies that had been isogenized for 10 generations via single pair sibling matings and maintained on standard lab diet (Drosophila agar 10 g/L, dextrose 40 g/L, sucrose 20 g/L, nutritional yeast 12 g/L, cornmeal 70 g/L, 3 ml/L of 10% Tegosept). Bottles containing 75 ml of the 70%, 100%, and 200% diets were seeded with five 5-10 day old, non-virgin female and male *CS* flies. We removed the parents after 5 days and collected progeny upon eclosion beginning at 1 hr after lights "on" within a 3 hr window. We allowed progeny to mature in vials containing their respective diet for 5 days, with females in groups of 5 and males in isolation. Perceived male competition affects male reproductive behaviors (Bretman et al., 2009) and ejaculate composition (Fedorka et al. 2010), so we housed males individually prior to testing to reduce these effects. Since there is no evidence that group housing affects female behaviors, we kept females in groups prior to testing as is standard practice (Ejima and Griffith, 2007). We raised all flies in an incubator at 25°C with 12 hr light/dark cycles.

Initial mating

We initially mated flies when they were 5 days old. We did not anesthetize flies on experimental days. Flies mated in 1 cm diameter and 0.785 cm^3 Plexiglass chambers that contained water-moistened filter paper. We paired males and females raised on the full range of protein diets (70%, 100%, and 200%) in a fully combinatorial manner (9 different pairings: 70% female x 70% male; 70% female x 100% male; 70% female x 200% male; 100% female x 70% male; 100% female x 100% male; 100% female x 200% male; 200% female x 70% male; 200% female x 100% male; 200% female x 200% male). We recorded matings using JVC-HDD Everio and Sony HD Handycam cameras. Upon

completion of mating, we stopped video recording and removed females to fresh vials containing the same diet on which they had been raised. We measured mating duration from the recorded videos. All assays were run in parallel over a period of 3 months.

Remating

Every 24 hrs, beginning the day after the initial mating, we introduced a novel virgin male to each mated female in a courtship chamber and recorded their interactions. We raised the males used for remating assays on the 100% diet and reared them in groups of 10 for 4-6 days before experiments. Males for remating tests were not kept singly because we were less concerned about group rearing effects on mating behaviors and ejaculate composition since we were not evaluating second mating effects on female PMR. We monitored pairs for mating, and if no mating occurred we placed females in fresh vials containing the same food on which they had been raised. We repeated this procedure daily until the female remated. The number of days from the initial mating until remating is the remating latency (Manning, 1962; Pitnick, 1991).

Fecundity measurements

We counted the number of eggs each female had laid the previous day and summed the total number of eggs laid by each female prior to remating to measure fecundity in response to the original mating. We used this measure of fecundity to examine how the original male mate affected the female PMR.

Statistics

We verified that mating duration, fecundity, and remating latency data were distributed normally (Shapiro-Wilk test). We then performed multiple regression tests to analyze mating duration (y = female diet + male diet). Fecundity and remating latency were analyzed using two-way ANOVAs (y = female diet + male diet + female diet*male diet) because the data for these traits significantly violated the lack of fit test for the

multiple regression model (Fecundity: $F_{6,445} = 5.3845$, $P < 0.0001$; Remating latency: $F_{6,449} = 3.1534$, $P = 0.0049$).

3.3 Results

Effect of dietary protein on mating duration

We quantified the effects of adult dietary protein content on male mating duration, since we previously demonstrated that males fed high protein diet throughout development had shorter mating durations than males raised on standard food (Schultzhaus et al., 2017). Mating duration is an important indicator of male reproductive investment (Gilchrist and Partridge, 2000; MacBean and Parsons, 1967; O'Dell, 2003; Ratterman et al., 2014), and although males can adjust ejaculate composition independent of mating time (Sirot et al., 2011), differences in mating duration are indicative of differential ejaculate transfer and affect aspects of the female PMR (Bretman et al., 2009). We detected a statistically significant effect of diet on mating duration (Multiple regression, $F_{2,451} = 3.3892$, $P = 0.0346$, Table 9; Figure 3).

Table 9. Effect of dietary protein on mating duration.

Sex	Diet	N	Mating Duration	*F*	*P*
Female Diet	71%	149	1482 ± 21s	0.0700	0.7915
	100%	150	1469 ± 17s		
	206%	153	1483 ± 19s		
Male Diet	71%	160	1511 ± 19s	6.7355	**0.0098**
	100%	152	1481 ± 21s		
	206%	140	1436 ± 18s		

Multiple Regression, $F_{2, 451} = 3.3892$, $P = 0.0346$

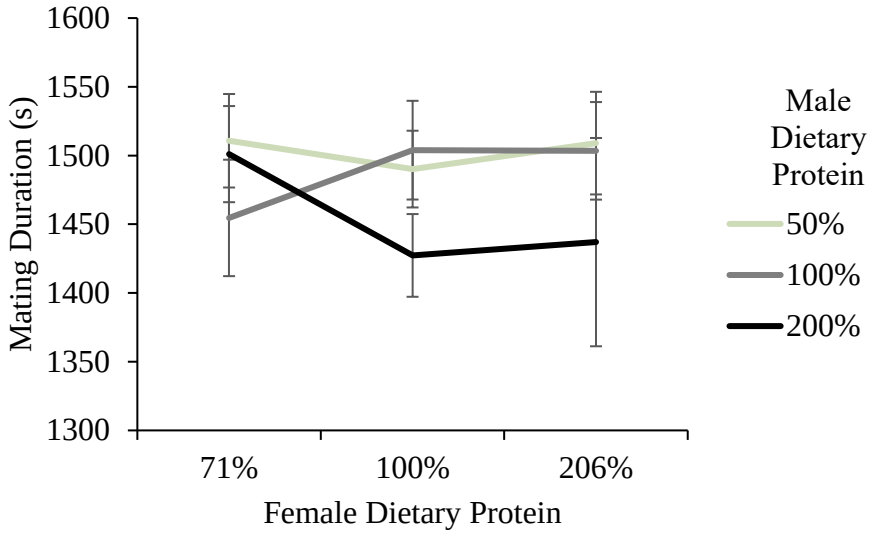

Figure 3. Male dietary protein content affects mating duration. Each data point represents the mean ± SE of N = 50.

Only male diet contributed to the effect (Male diet: $F_{1,451} = 6.7355$, $P = 0.0098$; Female diet: $F_{1,451} = 0.07$, $P = 0.7915$), with mating duration decreasing with increasing male dietary protein.

Effect of dietary protein on fecundity

To examine how male and female dietary protein content affected female fecundity, we measured fecundity after the initial mating until the second mating occurred. During this period, females are unreceptive to mating with other males, and we wanted to measure female fecundity during this time frame. We found that diet strongly affected fecundity (ANOVA, $F_{8,447} = 24.5061$, $P < 0.0001$, Table 10; Figure 4). Both female diet ($F_{2,447} = 89.6418$, $P < 0.0001$) and male diet ($F_{2,447} = 4.6644$, $P = 0.0099$) contributed significantly to this effect, but there was no significant interaction between male and female diet ($F_{4,447} = 1.9632$, $P = 0.0991$). Female dietary protein had a strong effect on fecundity, with increasing protein leading to higher levels of fecundity. In contrast, females had lower fecundity when they mated with males fed diets with higher protein content.

Table 10. Effect of dietary protein on fecundity.

Sex	Diet	N	Fecundity	*F*	*P*	Tukey's HSD
Female Diet	71%	149	146 ± 4.6 eggs	89.6418	**<0.0001**	C
	100%	148	198 ± 5.1 eggs			B
	206%	151	243 ± 5.7 eggs			A
Male Diet	71%	159	206 ± 6.5 eggs	4.6644	**0.0099**	A
	100%	150	194 ± 5.4 eggs			AB
	206%	139	187 ± 6.3 eggs			B
Female Diet x Male Diet	71 x 71	55	147 ± 7.7 eggs	1.9632	0.0991	
	71 x 100	49	158 ± 7.9 eggs			
	71 x 206	45	131 ± 8.0 eggs			
	100 x 71	53	213 ± 8.7 eggs			
	100 x 100	49	185 ± 7.7 eggs			
	100 x 206	46	195 ± 9.9 eggs			
	206 x 76	51	261 ± 11.3 eggs			
	206 x 100	52	236 ± 8.6 eggs			
	206 x 206	48	233 ± 9.4 eggs			

Two-way ANOVA, $F_{8, 447} = 24.5061$, $P < 0.0001$

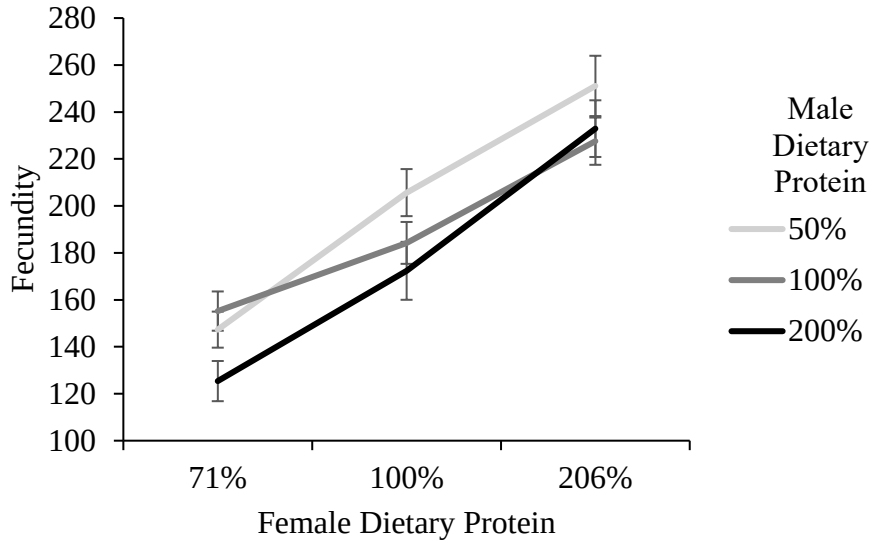

Figure 4. Female and male dietary protein content affects fecundity. Each data point represents the mean ± SE of N = 50.

Effect of dietary protein on remating latency

The final parameter we examined was remating latency. Since males fed higher amounts of protein mated for shorter periods and their female mates laid fewer eggs, we expected that the females that mated with high protein males would have shorter remating latencies. While we found that diet significantly affected remating latency (ANOVA, $F_{8,451} = 16.497$, $P < 0.0001$, Table 11; Figure 5), only female diet had a significant effect ($F_{2,451} = 58.6257$, $P < 0.0001$), while male diet ($F_{2,451} = 2.9551$, $P = 0.0531$) and the interaction between male and female diet ($F_{4,451} = 1.5191$, $P = 0.1955$) did not. Remating latency significantly decreased only in response to increasing female dietary protein, although there was a non-significant trend for increasing male dietary protein to decrease female remating latency ($P = 0.0531$).

Table 11. Effect of dietary protein on remating latency.

Sex	Diet	N	Remating Latency	*F*	*P*	Tukey's HSD
Female Diet	71%	146	12.1 ± 0.36 days	58.6257	**<0.0001**	A
	100%	152	10.1 ± 0.26 days			B
	206%	154	7.8 ± 0.21 days			C
Male Diet	71%	157	10.5 ± 0.31 days	2.9551	0.0531	
	100%	153	9.6 ± 0.30 days			
	206%	142	9.6 ± 0.34 days			
Female Diet x Male Diet	71 x 71	52	13.2 ± 0.54 days	1.5191	0.1955	
	71 x 100	49	11.9 ± 0.57 days			
	71 x 206	45	11.2 ± 0.76 days			
	100 x 71	54	10.6 ± 0.37 days			
	100 x 100	50	9.4 ± 0.50 days			
	100 x 206	48	10.1 ± 0.48 days			
	206 x 76	51	7.7 ± 0.39 days			
	206 x 100	54	7.8 ± 0.35 days			
	206 x 206	49	7.8 ± 0.39 days			

Two-way ANOVA, $F_{8, 451} = 16.4970$, $P < 0.0001$

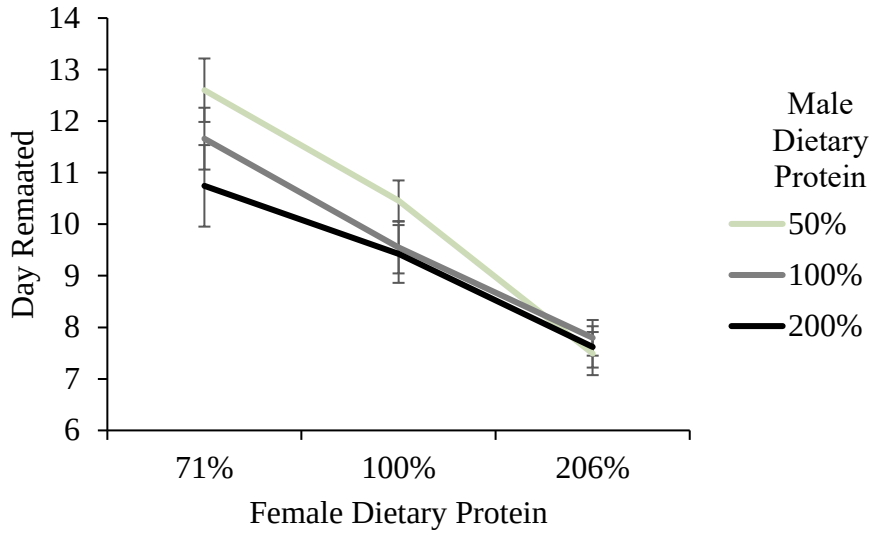

Figure 5. Female dietary protein content affects remating latency. Each data point represents the mean ± SE of N = 50.

3.4 Discussion

We tested whether dietary protein affected the magnitude of the PMR in *D. melanogaster*. We initially hypothesized that the PMR would increase (higher fecundity and longer remating latency) with increasing female dietary protein and decrease (lower fecundity and shorter remating latency) with increasing male dietary protein levels. As predicted, we found that males on high protein diets mated for shorter time periods and that their female mates had decreased fecundity. Unexpectedly, we also found that male diet had no effect on female remating latency. Female diet had no effect on mating duration, but increasing female dietary protein caused females to lay more eggs and remate faster. Contrary to our expectations, female dietary protein content did not alter the measured aspects of the PMR in the same direction, though these results do support the hypothesis that increasing dietary protein differentially affects male and female contributions to the female PMR.

Effects of female high protein diet on female PMR

It has long been known that female fecundity is coupled with dietary protein content (Bodenstein, 1947). Sugar-only diets inhibit yolk formation in oocytes and reduce yolk-protein transcript levels to decrease female fecundity (Bownes and Blair, 1986), but the addition of yeast, a protein source, reverses these effects (Bownes et al., 1988). Not only is dietary protein necessary for egg formation, but fecundity also increases with the amount of protein available (Chapman and Partridge, 1996; Fricke et al., 2010; Lee et al., 2008). High protein diet previously has been shown to decrease female lifetime mating rates (Fricke et al., 2010), and we had hypothesized that high protein females would have stronger induction of the PMR, i.e. that they would take a longer time to remate, but we found that high protein females remated more quickly. Our data agree with previous studies showing that female remating rate and fecundity both increase with total nutritional content, i.e. yeast and sugar (Chapman and Partridge, 1996), indicating that this response is attributable to dietary yeast rather than sugar content. While we had expected fecundity would increase with higher female protein diet, we were intrigued to find that high protein females lay more eggs before remating even though the remating interval was shortened. Females provided with the lowest protein diet had an average of 4.3 additional days to lay eggs (about 1.5 times longer) before remating, but their total egg counts were ~40% lower than those of females provided the highest protein diet. Whether the PMR is a concerted physiological response by females to mating has not been specifically examined, so it is interesting to discover that distinct aspects of the PMR can vary independently with female exposure to environmental factors.

There are several possibilities that could explain the observation that high protein females regain mating receptivity more quickly than females with lower protein diets. After mating, sperm are stored and used sparingly for several days (Reviewed in Qazi and Wolfner, 2003). Sex Peptide (SP), a male ACP that is essential for the induction of certain aspects of the female PMR, including decreased receptivity (Aigaki et al., 1991; Chapman et al., 2003; Chen et al., 1988; Liu and Kubli, 2003), binds to sperm and is slowly released upon cleavage while sperm are stored after mating (Peng et al., 2005a). As high protein

females lay more eggs, the accelerated depletion of stored sperm and SP could lead to faster recovery of female receptivity. However, females often remate before sperm from the first mating are completely exhausted (Manier et al., 2010), so this theory may not fully explain how high protein diet affects female PMR. Also, female dietary protein content did not affect mating duration, so males likely transferred similar amounts of sperm to all females regardless of their diet.

A second possibility is that the physiology of high protein females somehow supports increased cleavage of SP from sperm so that females become SP, rather than sperm, depleted. Examining sperm storage and SP dynamics in mated high protein females could identify whether female condition differentially affects the use of stored sperm and SFP. Finally, high protein females may be more receptive overall, as has been suggested for virgin high protein females (McRobert, 1986). Increased remating rate in highly fecund, high protein females and decreased remating rate in low fecundity, low protein females could then be explained by effects of internal nutrient reserves on female sexual receptivity.

SP binds to the SP Receptor (SPR) (Yapici et al., 2008), which is expressed in neurons that line the female reproductive tract (Häsemeyer et al., 2009; Rezával et al., 2012; Yang et al., 2009) and repress ascending neurons that project to the CNS to inhibit female receptivity (Feng et al., 2014). Examination of the responsiveness of these sets of neurons, as well as others known to regulate female receptivity (Bussell et al., 2014; Demir and Dickson, 2005; Kvitsiani and Dickson, 2006; Zhou et al., 2014), to nutrient storage levels could provide insights into how female behavior and condition are coupled.

Effects of male high protein diet on female PMR

Male dietary protein content affected the extent to which female PMR was induced. Male diet significantly decreased female fecundity, and although the effect of male diet on female remating latency was not significant ($P = 0.06$), there was a trend for male diet to also decrease female remating latency. We previously observed that high protein diet males mated for shorter periods (Schultzhaus et al. 2017), and this effect was

also detected in the current study even though the high protein diet used here (200%) did not contain as much protein as the previous study (~370%). Because high protein males mate for shorter periods, we expected that they transfer less ejaculate to females and therefore trigger a diminished PMR. The ejaculate contains both sperm and SFPs, and during copulation, males only transfer sperm for approximately the first 10 minutes while SFPs transfer likely occurs the entire time (Gilchrist and Partridge, 2000). Males can alter mating duration and the composition of the transferred ejaculate based on social experience (Bretman et al., 2009; Fedorka et al., 2011; Garbaczewska et al., 2013; Wigby et al., 2009), condition (McGraw et al., 2007; Wigby et al., 2015), and female mating status (Friberg, 2006; Lüpold et al., 2010; Sirot et al., 2011). The effect of dietary protein on male ejaculate transfer has not been examined, so we do not know whether high protein males transfer sperm for a similar amount of time, which would result in a similar number of sperm transferred across male diets but could drastically reduce the amount of SFPs transferred, or if the sperm transfer period decreases proportionally with mating duration, which would result in a less drastic change in the amount of time where only SFPs are transferred. Investigation of these theories would shed light on how males allocate ejaculate in response to costs imposed by condition.

Conclusion

Sexual conflict arises when one sex benefits while another is harmed. While studies have sought to identify genetic regions associated with sexual conflict (Innocenti and Morrow, 2010), less work has been done to examine how environmental factors can affect animal condition and the dynamics of sexual conflict, although this topic is beginning to get more attention in the more generalized field of sexual selection (Miller and Svensson, 2014). We have shown here that the *D. melanogaster* female PMR, a suite of traits that set the stage for sexual conflict, is affected differentially by the dietary protein content of both sexes. These results suggest that when males and females experience the same change in environmental conditions (in this case dietary protein availability), the ability of males to inflict harm and of females to mitigate mating costs can change in

potentially opposite qualitative directions. Female dietary protein increases the costs associated with mating (Chapman and Partridge, 1996; Fricke et al., 2010; Lee et al., 2008). After being reared on high protein diets for 17 generations, *D. melanogaster* experienced decreased reproductive success (Kristensen et al., 2010). Yet, in our study, increased male dietary protein seemed to decrease mating costs for females. Low protein diet females had long remating latencies and low fecundity. These PMRs would decrease mating costs for low protein females, while low protein males mated longer and induced higher fecundity in the females they mated, indicating that mating with low protein males would be more costly. So although mating costs continue to rise with dietary protein content, reduced costs received from high protein males could help to slow cost acceleration. Our results suggest that the ability of animals to induce or mitigate mating costs is responsive to environmental conditions in a sexually dimorphic manner, balancing sexual antagonism across fluctuating environments (Figure 6).

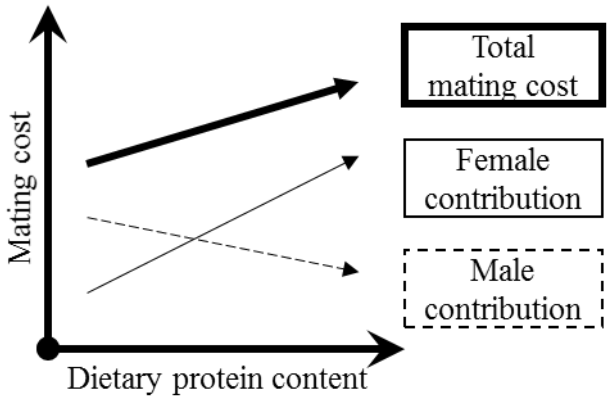

Figure 6. Predicted cost of mating and the contribution from each sex with increasing dietary protein content.

CHAPTER IV
HIGH FAT DIET EFFECTS *DROSOPHILA MELANOGASTER* MATING TRAITS AND BEHAVIOR

4.1 Introduction

To produce the best quality or greatest number of offspring, animals discriminate between potential mates to identify those in the best condition (Reviewed in Andersson and Simmons, 2006; Cotton et al., 2006; Edward and Chapman, 2011; Rosenthal, 2017). Condition, which is influenced by environmental and genetic factors, is a term commonly employed to describe the internal physiology or overall quality of an animal (Hill, 2011) and reflects the animal's fitness potential (Jasienska et al., 2004; Monaghan et al., 1996; Moore and Moore, 2001). Mating decisions depend upon the condition of the potential partner and the choosing animal. Sexual traits, which are thought to convey information about individual fitness potential, and the ability to accurately perceive this information are condition-dependent traits (Hill, 2015b; Holveck and Riebel, 2009; Hunt et al., 2005; Lopez, 1999; Penton-Voak et al., 2003). Good condition individuals can then discriminate between potential mates of varying quality during mate searching, but searching requires energy and time and therefore can be costly. Good condition mates may confer benefits such as better or more offspring, yet these benefits may not be recouped by poor condition animals if the costs of securing good condition mates are too high (Cockburn et al., 2008; Hingle et al., 2001; Wilgers and Hebets, 2012). This tradeoff results in condition-dependent mate preference, where poor condition animals exhibit decreased preference for good condition mates, resulting in assortative matings between poor condition pairs.

To identify good condition mates, animals examine their potential partner's sexual traits, which are responsive to environmental and genetic variability (i.e., sexual traits are condition-dependent) (Hill, 2015a). Insect sexual traits include body size, sexual ornament size (mandibles and horns), courtship song, and pheromone profiles (Ingleby, 2015; Prakash and Monteiro, 2016). Dietary influences during development can have particularly strong effects on insect sexual traits because insulin signaling controls growth

during this critical period (Brogiolo et al., 2001; Colombani et al., 2005; Ikeya et al., 2002; Rulifson et al., 2002). Factors that enhance insulin signaling during development will result in larger adult insects, while the reverse is true for factors that reduce insulin signaling, such as reduced nutrient availability (Byrne and Rice, 2006; Lefranc and Bundgaard, 2000) or diets high in fat (Reed et al., 2010). *Drosophila melanogaster* females prefer large males (Partridge and Farquhar, 1983; Pitnick and García–González, 2002) that are not nutritionally deprived (Mery et al., 2009) and that produce energetic courtship songs (Partridge et al., 1987; Talyn and Dowse, 2004). *D. melanogaster* males prefer females that are large (Byrne and Rice, 2006) and have altered pheromone profiles due to elevated insulin signaling (Kuo et al., 2012).

In ecological studies, "good condition" individuals are often identified as those with high total body lipid stores (Moya-Laraño et al., 2008), yet lipid overconsumption often causes metabolic diseases (Birse et al., 2010; Buettner et al., 2006; Riccardi et al., 2004), leading to impaired states of health and lowered life expectancy. The physiological response to dietary lipids is mediated by highly conserved metabolic pathways, such as insulin/TOR and fat lipase signaling. In *D. melanogaster*, an emerging model for metabolic studies (Bharucha, 2009; Padmanabha and Baker, 2014; Tennessen et al., 2014), diets that are high in fat lead to increased total body lipids (Birse et al., 2010), accumulation of lipids in multiple tissues (including the fat body, gut and heart (Birse et al., 2010)), insulin resistance (Birse et al., 2010), and decreased heart function (Birse et al., 2010; Diop et al., 2015), lifespan (Driver and Cosopodiotis, 1979), and fecundity (Schultzhaus et al., 2017). Additionally, HFD females are less attractive to males raised on a control diet, and HFD males exhibit condition-dependent mate preference as they do not discriminate between unattractive HFD females and attractive females raised on a control diet (Schultzhaus et al., 2017). Therefore, exposure to high levels of dietary fat negatively affects fruit fly health and behavior while simultaneously increasing internal fat content, indicating that individuals with high lipid stores cannot always be labeled as "good condition," especially when considering the effects of dietary imbalances.

Understanding how dietary lipids affect mate choice will be informative for sexual selection studies, as lipid reserves are important determinants of condition and as animals live in fluctuating, complex environments which are rarely static (Miller and Svensson, 2014). We previously noted that HFD altered *D. melanogaster* reproductive behavior by causing decreases in female attractiveness and fecundity and male mate discrimination ability (Schultzhaus et al., 2017). We hypothesize that HFD alters sexual traits, such as body size, courtship behavior courtship song or pheromones, to decrease female attractiveness via conserved metabolic signaling pathways. Here, we provide support for this hypothesis with behavioral experiments and direct quantification of *D. melanogaster* pheromone profiles, followed by an investigation of whether manipulation of the conserved metabolic genetic pathways (insulin, TOR, and Brummer) that rescue physiological defects caused by HFD (Birse et al., 2010) also rescue mating behavior defects caused by HFD.

4.2 Methods

Fly stocks

All Canton-S (CS) wild-type flies used in this study were isogenized by backcrossing sibling pairs for 10 generations. *arm-Gal4* (w[*]; P[w[+mW.hs]=GAL4-arm.S]11), *UAS-TOR^{DN}* (y1 w*; P[UAS-Tor.TED]II), and *UAS-FOXO* (w[1118]; P[[w+mC]=UASp-foxo.S]3) (Birse et al., 2010) were obtained from the Bloomington Drosophila Stock Center, and *UAS-Bmm* was a gift from Dr. Rolf Bodmer at the Sanford/Burnham Medical Research Institute. Each stock was outcrossed 6x into the *CS* background. All stocks were maintained on standard lab food (Drosophila agar 10 g/L, dextrose 40 g/L, sucrose 20 g/L, nutritional yeast 12 g/L, cornmeal 70 g/L, 3 ml/L of 10% Tegosept), and flies used in the behavioral experiments were raised in bottles containing either control (C, 7 g/L agar, 65 g/L cornmeal, 13 g/L inactive yeast, 7.5 g/L sucrose) or high fat diet (3%: C + 30 g/L coconut oil; 7%: C + 70 g/L coconut oil; 15%: C + 150 g/L coconut oil; 30%: C + 300 g/L coconut oil) (Birse et al., 2010; Reed et al., 2014; Reed et

al., 2010; Schultzhaus et al., 2017) and maintained in food vials (5 females/vial; 1 male/vial) as virgins until testing (Schultzhaus et al., 2017).

Body length measurements

The movements of pairs of *CS* male and female flies raised on control or 3% HFD (N = 100 for each sex on each diet) were video recorded in 1 cm diameter courtship chambers. Three separate still frames were captured from the videos during periods when the flies were walking with straight abdomens in a non-angled orientation. Body length was measured from the tip of the head to the tip of the abdomen in ImageJ, and the three measurements per fly were averaged to give a body length measurement.

Behavioral testing: General protocol

For single-pair mating assays, one virgin female and male were placed in a 1 cm diameter courtship chamber with a moistened filter paper. Pairings consisted of either two control flies, one control female and one HFD male, one HFD female and one control male, or two HFD flies. For male competition assays, one control female and two males (one control and one HFD) were placed in the 1 cm diameter courtship chambers. Interactions were recorded for 1 hr with high definition video cameras.

Videos were later analyzed by an observer who was blind to the fly diets. Mating success (the proportion of successful matings out of the total possible number of matings (Merrell, 1949; O'Dell, 2003; Villella and Hall, 1996)), courtship latency (the amount of time from introduction until males begin courting; indicates assessment of female attractiveness (Eastwood and Burnet, 1977; O'Dell, 2003; Ratterman et al., 2014; Rybak et al., 2002; Tompkins et al., 1982)), courtship index (the proportion of time spent courting until the beginning of mating; also indicates assessment of female attractiveness (O'Dell, 2003; Siegel and Hall, 1979)), activity levels in the minute prior to mating (indicative of male condition and female receptivity (McRobert, 1986; McRobert et al., 2003; Tompkins et al., 1982)), and mating latency (the amount of time from the beginning of courtship until mating begins; indicates male attractiveness (Connolly and Cook, 1973; O'Dell et al.,

1989; O'Dell, 2003; Ratterman et al., 2014; Rybak et al., 2002)) were quantified as described previously (Schultzhaus et al., 2017).

Behavioral testing: Developmental diet treatment

CS flies were raised and maintained post-eclosion on control or 3% HFD and paired in a fully combinatorial manner as described above. Male courtship and mating behaviors were observed in both light and dark conditions (chambers were illuminated with red light in order to record behaviors) with intact, freely behaving females or with decapitated females. Decapitation occurred directly before the mating assays, which began once females recovered from CO_2 anesthesia.

Behavioral testing: Adult-only diet treatment

Wild-type *CS* flies were raised on control diets and transferred upon eclosion to vials containing either control food or diets with a range of increased fat content (3%, 7%, 15%, and 30%). For single-pair mating assays, separate experimental blocks for each percentage of HFD were performed in a fully combinatorial manner, except for experiments with the 30% HFD because high female mortality necessitated testing males only (two pairings: control female with control male, control female with 30% HFD male). Competition assays were performed in which a control male and a HFD male (15% or 30%) competed for matings with a control female. In a separate experiment, control diet males were provided a choice between a control female and a 15% HFD female.

Cuticular hydrocarbon collection and gas chromatography mass spectrometry

Male and female flies were provided control or HFD (3% developmental diet, 3% adult-only diet, 15% adult-only diet, or 30% adult-only (males only)) and handled as described above. Cuticular hydrocarbons (CHCs) were extracted from five-day-old as described previously (Fedina et al., 2012). Briefly, three replicates of eight flies from each treatment group were incubated in 120 μL hexane spiked with 10 μg/mL hexacosane (Sigma-Aldrich; St. Louis, Missouri, USA) for 10 min at room temperature after brief

vortexing. 100 µL of hexane were removed and put in a new vial, and the hexane was allowed to evaporate for 4-6 hours. Vials were stored at -20C until analysis via GCMS at the University of Hawaii at Manoa.

Gas chromatography mass spectrometry (GCMS) analysis was performed with an 7820A GC system (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a HP-5ms column (5%-Phenyl-methylpolysiloxane column; 30 m length, 250 µm ID, 0.25 µm film thickness; Agilent Technologies, Inc.). Ionization was achieved by electron ionization (EI) at 70 eV. One µL of the sample was injected using a splitless injector. The helium flow was set at 1.0 mL/min. The column temperature program began at 40 °C for 3 min, increased to 200 °C at a rate of 35 °C /min, then increased to 280 °C at a rate of 20 °C/min and held for 15 min. A mass spectrometer was set to unit mass resolution and 4 scans/ sec, from m/z 33 to 500. Chromatograms and mass spectra were analysed using MSD Chem Station software (Agilent Technologies, Inc.).

The relative abundance of each CHC is calculated by dividing the area under the peak by the total area of all CHC peaks detected in the chromatogram. The normalized intensity of each CHC is calculated by dividing the area under the peak by the area of the spiked hexacosane standard. Statistical analysis was performed using a multi-factor analysis of variance (MANOVA) (GraphPad Prism 5, Graph Pad Software Inc., CA, USA). For total CHC levels, the area under each of the CHC peaks were summed and normalized to the area under the peak for the hexacosane standard.

Behavioral testing: Metabolic rescue

UAS-TOR^{DN}, *UAS-Bmm,* and *UAS-FOXO* were expressed ubiquitously with *arm-Gal4* (Birse et al., 2010). The experimental rescue flies containing *arm-Gal4* together with a UAS expression construct were compared to their genetic controls for effects on their ability to rescue HFD behavioral defects. To test for rescue of developmental diet effects, flies were raised on 3% HFD or control food as before, and to test for rescue of adult-only HFD treatment effects, flies were raised on control diet and transferred to control or 15% HFD upon eclosion as before.

Control or HFD (3% developmental diet or 15% adult-only diet) experimental rescue and genetic control females were placed in single-pair mating assays with *CS* males raised on control diet. Mating latency and male courtship behavior towards these females were measured.

To test for rescue of male discriminatory ability, HFD (3% developmental treatment or 15% adult-only treatment) experimental rescue and genetic control males were placed in single-pair mating assays with control and HFD (3% developmental treatment or 15% adult-only treatment) *CS* females. Male courtship behavior towards these females was then quantified.

Statistics

Normal distribution of logarithmic and arcsine transformed data was tested with the Shapiro-Wilk test.

To determine the necessity of visual assessment or female behavior for HFD developmental treatment effects, courtship latency and index for each experiment (light condition assays with intact females, dark condition assays with intact females, light condition assays with decapitated females, and dark condition assays with decapitated females) were examined with a two-way ANOVA (y = female diet + male diet + female*male diet). A Bonferroni correction of $\alpha = 0.025$ was applied to control for testing dietary effects on two behavioral traits.

To examine the effects of adult-only HFD treatment, statistical analysis followed the procedures described in Schultzhaus et al. (2017). A one-way MANOVA was performed with all behavioral parameters for each HFD dosage (3%, 7%, and 15%) to first determine whether diet had any effects on the traits. Each dietary level was then tested with a two-way ANOVA for each behavioral trait (y = female diet + male diet + female*male diet) for post-hoc analysis of significance. Finally, a Bonferroni correction of $\alpha = 0.01$ was applied to control for multiple testing. Mating success was analyzed via chi-square tests.

Competition assay data were analyzed as described previously (Schultzhaus et al., 2017). The proportion of control or HFD males that gained matings was analyzed with a chi-square test, while courtship latency and courtship index were analyzed with a *t*-test. A Bonferroni correction of $\alpha = 0.0167$ was applied to control for testing dietary effects on three behavioral traits.

Cuticular hydrocarbon amounts were compared using one-way ANOVAs for the total amount and two-way ANOVAs for individual cuticular hydrocarbons.

For metabolic rescue experiments, *t*-tests were performed on the behavioral data from the control and the HFD treatment of each genotype for the examination of female attractiveness, and on the behavior of the HFD male towards control versus HFD wild-type females for the examination of male mate assessment. A Bonferroni correction of $\alpha = 0.025$ was applied to control for testing developmental versus adult-only effects.

4.3 Results

We previously found that rearing flies on 3% HFD throughout development and adulthood ("developmental diet") decreased female attractiveness as indicated by reductions in male courtship and mating behavior toward HFD females (Schultzhaus et al., 2017). Males use a variety of sensory cues to judge sexual traits of potential mates, and HFD could influence multiple female traits to cause this decrease in attractiveness. We investigated three possible female sexual cues that could be modified by HFD: body size, behavioral responses to courtship, and pheromone profiles. Body size is an important sexual cue, as Drosophila males prefer larger females (Byrne and Rice, 2006). A previous study found that animals raised on a HFD are smaller (Reed et al., 2010), and we confirmed that raising flies on 3% HFD decreases female body size (Figure 7). Female acceptance or rejection responses also provide feedback to courting males, leading them to increase or decrease their courtship efforts (Greenspan and Ferveur, 2000). We previously demonstrated that HFD decreased female activity prior to mating (Schultzhaus et al., 2017) which may be an indication of increased receptivity (McRobert, 1986). Another possibility is that less mobile females are judged by males as being less attractive.

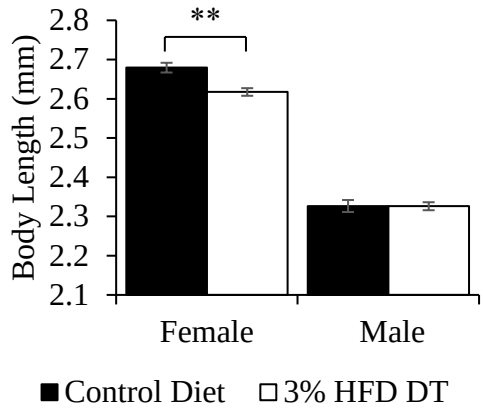

Figure 7. HFD developmental treatment decreases female body length. Each bar represents the mean ± SE of N = 100. Means with ** were significantly different (P = 0.0019).

Pheromones are another important sexual cue that convey information about species, sex, age and mating status (Ferveur, 2005). Drosophila pheromone profiles may also be affected by HFD since profiles are responsive to changes in diet (Fedina et al., 2012) and genetic manipulation of metabolic signaling pathways (Kuo et al., 2012).

Males discriminate against high fat diet females in light and dark conditions

We performed multiple experiments to determine which of the three sexual cues HFD acts on to decrease female attractiveness. First, we compared the behavior of control flies to that of flies raised on 3% HFD (developmental treatment) in light and dark conditions. In the dark, males are unable to see and therefore largely judge females based on pheromonal profiles rather than visual cues such as body size. However, even in the dark, males can detect female movement patterns and their behavioral responses to courtship.

In assays with intact females that were fed a 3% HFD , male courtship latency was affected in both light and dark conditions (Two-way ANOVA, courtship latency in light: $F_{3,121} = 12.9183$, $P < 0.0001$; Figure 8A; courtship latency in dark: $F_{3,118} = 5.2346$, $P = 0.002$; Figure 8B) but only affected courtship index in the light (Two-way ANOVA,

courtship index in light: $F_{3,120} = 4.5272$, $P = 0.0048$; Figure 8E;, courtship index in dark: $F_{3,117} = 0.1168$, $P = 0.9501$; Figure 8F). Control males took longer to begin courting intact females in both light and dark conditions and decreased courtship towards the HFD females only in the light. Conversely, males fed the 3% HFD developmental diet courted control and HFD females similarly across all visual conditions. In the dark, courtship indices for all pairings were drastically reduced (Figure 8F). During video analysis, it was clear that males could not accurately track females in the dark, resulting in sporadic courtship bursts as flies randomly came in contact in the courtship chambers. The general difficulty in finding mates in the dark may explain why there was no decrease in control male courtship towards 3% HFD females. Yet, in both light and dark conditions, we observed control males discriminating against HFD females by delaying the start of courtship, indicating that nonvisual sexual cues are important for male judgement of female quality.

To further parse apart contributions of behavioral and pheromonal cues to female attractiveness, these light or dark assays also were performed with decapitated females. Decapitation removes the female's ability to respond to and influence male courtship, and therefore the male can only use pheromonal information to judge a female. If HFD alters nonvisual cues (such as pheromones), we expected to find that control males would increase courtship latency and decrease courtship index towards decapitated HFD females in the dark, indicating that males continue to find these females unattractive.

The 3% HFD also affected male courtship towards decapitated females. Only courtship index was affected in the light (Two-way ANOVA, courtship latency: $F_{3,115} = 3.0558$, $P = 0.0313$, Figure 8C; courtship index: $F_{3,124} = 6.3870$, $P = 0.0005$, Figure 8G), yet post-hoc Tukey's tests did not reveal differences in either control or HFD male behavior. However, control males significantly decreased their courtship behavior towards decapitated 3% HFD females in the dark condition while HFD males did not (Two-way ANOVA, courtship latency: $F_{3,98} = 3.8501$, $P = 0.0120$, Figure 8D; courtship index: $F_{3,109} = 3.9099$, $P = 0.0108$, Figure 8H). In the light condition, males can visually examine the decapitated females, meaning that, although they stand upright, the females may have

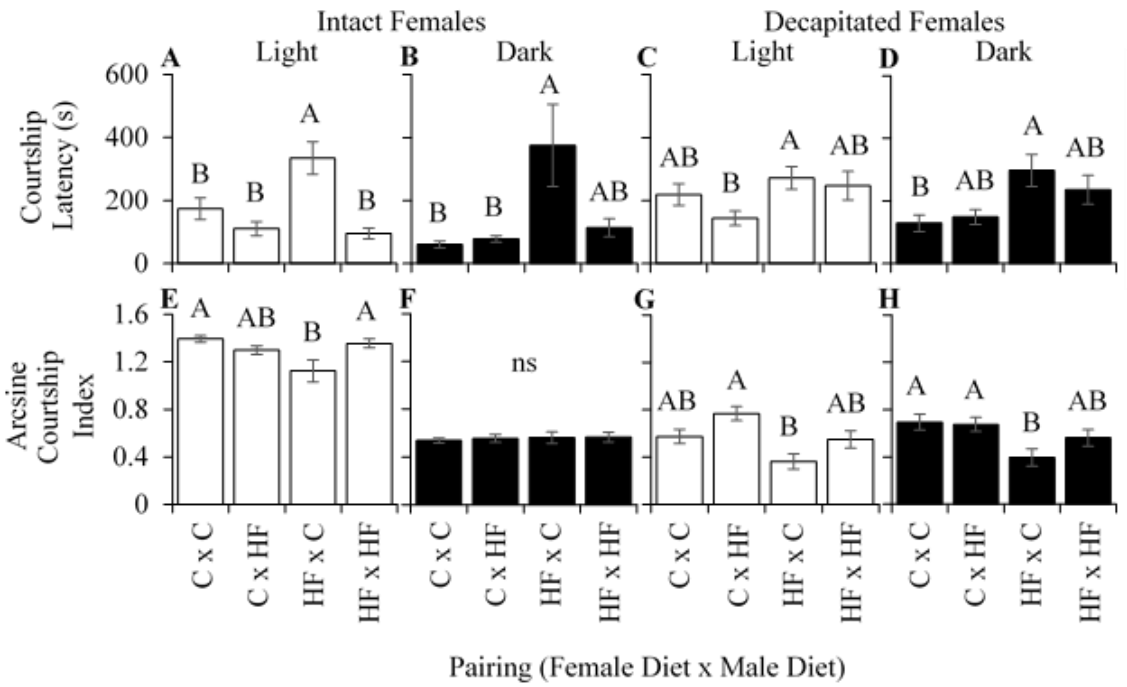

Figure 8. Males continue to find HFD unattractive without visual assessment. The effect of 3% HFD developmental treatment on male courtship latency and index towards intact females in (A, E) light and (B, F) dark conditions and decapitated females in (C, G) light and (D, H) dark conditions. Each bar represents the mean ± SE of N = 25. The letters above the bars represent a post-hoc Tukey's HSD connecting letters report where different letters are different above $\alpha = 0.05$.

appeared injured or the males may have noted their inactivity, and these judgements cannot be made in dark conditions. These negative visual assessments could have then impacted male behavior in the light condition so that differences in control male behavior were only seen in the dark. Nevertheless, control males discriminated against HFD females without visual cues as to body size or behavioral feedback, indicating that changes in pheromonal profiles alone are likely to be the signal control males use to discriminate against 3% HFD females.

High fat diet adult treatment affects mating behavior in a similar manner as high fat diet developmental treatment

Exposure to HFD during development decreases female size and attractiveness (Figure 7 and Figure 8), and control males continued to discriminate against HFD females in the absence of visual and behavioral information (Figure 8). To confirm that HFD affects other visual cues, we removed the developmental influence of HFD and examined only the adult effects by collecting flies that had developed on a common diet (control) upon eclosion and then transferring the flies to diets containing a range of fat (3%, 7%, 15% and 30% adult-only diet) or to the control diet. The effects of the 30% HFD could only be examined on males, as this exposure caused extremely high mortality to females. The effects of the adult-only diets on mating success (the proportion of successful mating pairs out of all total possible pairings), courtship latency, courtship index, activity levels (number of times the male and female cross the chamber midline in the minute prior to mating; indicative of either condition as better condition flies could be more active or female receptivity as more receptive females could be less active), and mating latency (measurement of time from the beginning of courtship until mating; indicative of female receptivity or attraction to the male) were examined. We expected that the developmental diet would have a strong effect on fly physiology and mating behavior and that an increased HFD dosage would be necessary to phenocopy the developmental diet effects. Observation of similar phenotypes in the adult treatment will indicate that HFD likely affects non-visual sexual cues like pheromone profiles.

Mating behaviors between intact male and female pairs were examined in light conditions only (Figure 9). Each adult-only diet treatment affected *D. melanogaster* mating behavior (One-way MANOVA: 3% HFD, Wilks' Lambda = 2.2117, $P = 0.0119$; 7% HFD, Wilks' Lambda = 5.2753, $P < 0.0001$; 15% HFD, Wilks' Lambda = 5.4934, $P < 0.0001$; 30% HFD, Wilks' Lambda = 6.8127, $P = 0.0002$).

The 3% and 7% adult-only treatments had similar effects on *Drosophila* mating behaviors. Mating success was altered with 3% and 7% HFD (Chi squared test: 3% HFD, χ^2 (3, $N = 100$) = 9.506, $P = 0.0278$, Figure 9A; 7% HFD, χ^2 (3, $N = 100$) = 9.777, $P =$

0.0206, Figure 9F). 3% and 7% HFD affected activity levels (Two-way ANOVA: 3% HFD: Female activity, $F_{3,98}$ = 5.6555, P = 0.0013; Male activity, $F_{3,98}$ = 4.5576, P = 0.0050, Figure 9D; 7% HFD: Female activity, $F_{3,99}$ = 17.6635, P < 0.0001; Male activity, $F_{3,99}$ = 8.8919, P < 0.0001, Figure 9I) and mating latency (3% HFD: ANOVA, $F_{3,98}$ = 5.1040, P = 0.0026, Figure 9E), where HFD females were less active and mated faster than control females (3% HFD: Female activity, female diet term, F = 15.5924, P = 0.0002; Male activity, female diet term, F = 11.3461, P = 0.0011; 7% HFD: Female activity, female diet term, F = 48.4933, P < 0.0001; Male activity, female diet term, F = 24.5406, P < 0.0001; Mating latency, 3% HFD: female diet term, F = 10.4559, P = 0.0017; P > 0.01 for male diet and female diet*male diet terms). 3% and 7% HFD adult treatments did not affect courtship latency (Figure 9B and G) or courtship index (Figure 9C and H). These results indicate that although the adult-only 3% and 7% HFD affected female mating receptivity (decreased activity and mating latency), these diets did not decrease female attractiveness.

The mating behaviors of flies subjected to 15% HFD only during adulthood phenocopied the 3% HFD administered throughout development and adulthood. Mating success was decreased (Chi square test, χ^2 (3, N = 104) = 28.437, P < 0.0001, Figure 9K). 15% adult-only HFD affected courtship latency (Two-way ANOVA, $F_{3,103}$ = 4.1775, P = 0.0079; Figure 2L) but not courtship index (Figure 2M). Only the interaction term between female and male diet was significant (F = 7.1324, P = 0.0088) for courtship latency, and the Tukey's post-hoc test revealed that control males took longer to begin courting HFD females, while HFD males did not. In other words, 15% HFD females are less attractive to control males, while 15% HFD males do not discriminate between unattractive HFD females and attractive control females, indicating that 15% HFD causes condition-dependent mate preference changes in males and decreases female attractiveness and condition. These results are similar to those of the 3% HFD, although control male courtship index was not decreased as we had seen with 3% HFD. Similarly to all other HFD treatments, activity levels (Two-way ANOVA: Female activity, $F_{3,101}$ = 8.89851, P < 0.0001; Male activity, $F_{3,101}$ = 5.2609, P = 0.0021; Figure 9N) and mating latency (Two-

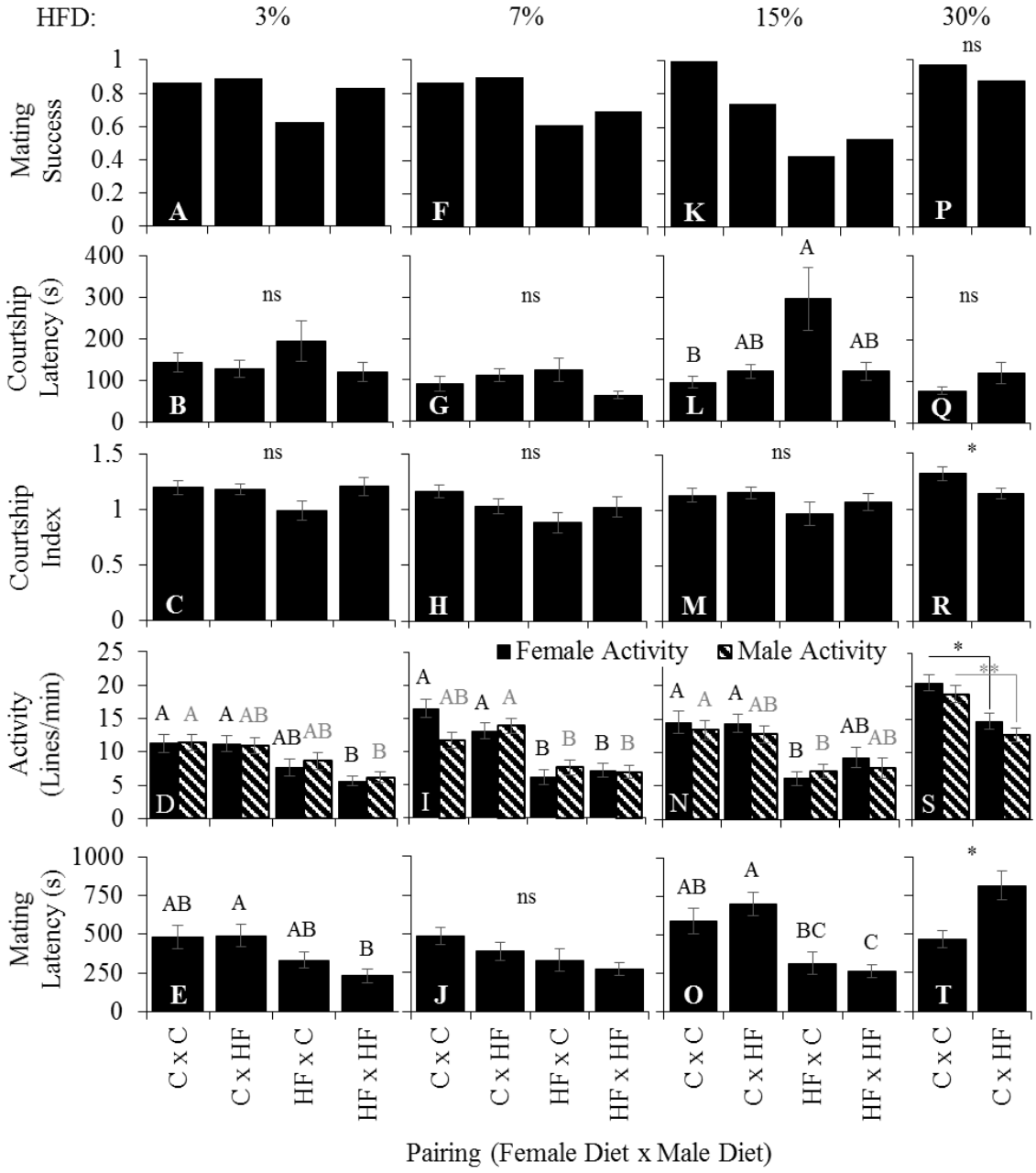

Figure 9. HFD adult treatment alters *D. melanogaster* mating behavior. The effect of 3% (A-E), 7% (F-J), 15% (K-O), and 30% (P-T) HFD adult treatment on *D. melanogaster* mating behavior. Each bar represents the mean ± SE of N = 25. The letters above the bars represent a post-hoc Tukey's HSD connecting letters report where different letters are different above Bonferroni correct $\alpha = 0.01$. Means with * and ** were significantly different ($P = 0.01$ and 0.001, respectively).

way ANOVA, $F_{3,98} = 10.8396$, $P < 0.0001$; Figure 9O) were affected by 15% HFD, where the only the female diet term was significant (Female activity: $F = 24.0652$, $P < 0.0001$; Male activity: $F = 15.2366$, $P = 0.0002$; Mating latency: $F = 31.2037$, $P < 0.0001$). Overall, 15% HFD females were less active, the males courting these HFD females also decreased their activity, and HFD females mated more quickly.

The effect of 30% HFD was tested only on male mating behavior as the few females that survived the treatment did not mate. 30% HFD did not affect male mating success (Figure 9P) or courtship latency (Figure 9Q), but courtship index (*t*-test, $t_{43.5566} = -2.7007$, $P = 0.0096$, Bonferroni corrected $\alpha = 0.01$; Figure 9R), activity (Female activity: *t*-test, $t_{51.7931} = -3.4085$, $P = 0.0013$; Male activity: *t*-test, $t_{52.0143} = -3.7063$, $P = 0.0005$; Figure 9S), and mating latency (*t*-test, $t_{52.3464} = 2.9586$, $P = 0.0046$; Figure 9T) were altered. 30% HFD courted females less and were less active than control males. Control females paired with these HFD males were less active than when paired with control males, and took longer to mate with 30% HFD males. These results indicate that 30% HFD males are worse at courting and are less attractive.

To confirm that 30% HFD decreases male attractiveness, a phenotype not observed with 3% HFD provided throughout development, the competitive ability of both 15% HFD and 30% HFD males was examined (Table 12). When competing with a control male for a control female, 15% HFD males performed courtship behaviors similarly to control males and gained a similar number of matings. The results of this competition assay match the results of the single-pair mating assays where the mating behavior of control females did not indicate aversion to 15% HFD males. Yet when 30% HFD males competed against control males for control females, the HFD males gained fewer matings (Chi-square test, χ^2 (1, $N = 50$) = 11.796, $P = 0.0006$) despite courting similarly. These competition results provide further evidence that control females find 30% HFD males less attractive.

Table 12. Control and adult treatment high fat (15% and 30%) male competition.

Female	Males	Behavior	Result	Statistic	*P*
Control	Control vs 15% AT High Fat	Courtship Latency	C = 1.88 ± 0.04 HF = 1.93 ± 0.05	t = 0.7497	0.4558
		Courtship Index	C = 0.76 ± 0.04 HF = 0.70 ± 0.04	t = 0.9423	0.3491
		Mated	C = 60% HF = 40%	χ^2 = 3.20	0.0736
Control	Control vs 30% AT High Fat	Courtship Latency	C = 1.79 ± 0.05 HF = 1.70 ± 0.05	t = 1.4483	0.1508
		Courtship Index	C = 0.82 ± 0.03 HF = 0.89 ± 0.03	t = 1.6623	0.0999
		Mated	C = 67.3% HF = 32.7%	χ^2 = 11.796	**0.0006**

Values in bold are significant at Bonferroni corrected $\alpha = 0.0167$.

High fat diet affects CHC profiles

All of the experiments described above indicate that HFD alters pheromone profiles to decrease female, and potentially male, attractiveness. To verify that this is indeed happening, we quantified individual CHCs of female and male flies subjected to control, 3% developmental or adult-only (3%, 15%, and 30% (males only)) HFD.

Both adult-only diet treatments decreased total female CHCs while the developmental treatment had no effect (One-way ANOVA, $F_{3,16} = 12.85$, $P = 0.0002$; Dunnett's post-hoc tests: 3% adult-only, $P = 0.004$; 15% adult-only, $P = 0.001$; 3% developmental diet, $P = 0.055$; Figure 10A). The HFD adult-only treatment significantly decreased (Z,Z)-7.11-Pentacosadiene (7.11-PD), 2-Methylhexacosane (27-Br), (Z)-7-Heptacosene (7-H), and (Z,Z)-7.11-nonacosadiene (7.11-ND), and all HFD treatments (both adult and developmental) significantly decreased (Z)-9-pentacosine (9-P), (Z)-7-Pentacosene (7-P), and (Z,Z)-7.11-heptacosadiene (7.11-HD) (Figure 10B). Each of these CHCs have been shown to stimulate male courtship behavior (Antony et al., 1985; Savarit et al., 1999),

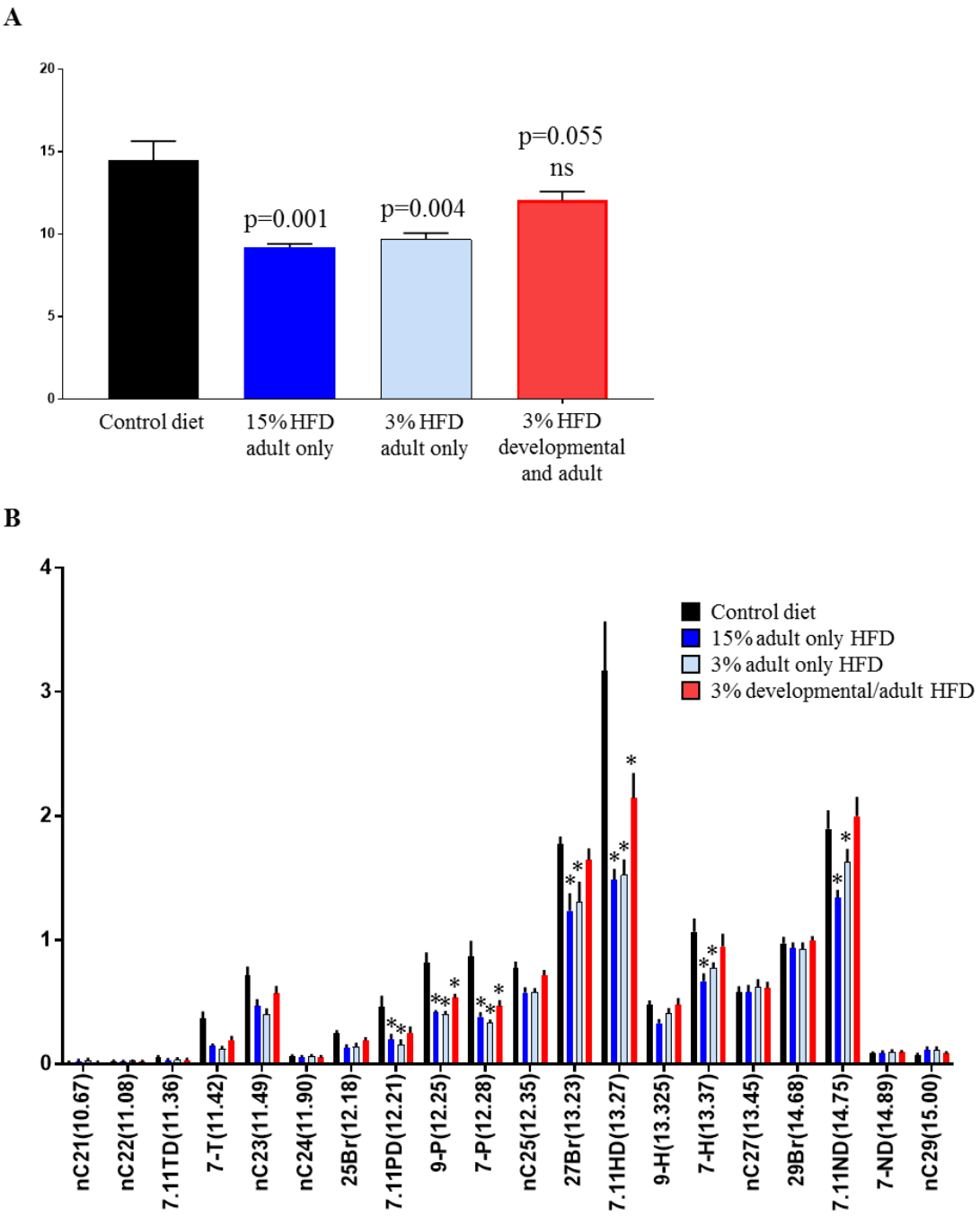

Figure 10. HFD affects female CHC profiles. Total (A) and individual (B) CHCs are affected by each HFD treatment. Each bar represents mean of 3 groups of 8 flies ± SE. *P* values in (A) and **P* < 0.05 in (B) are the results of post-hoc Dunnett's tests compared to control diet (two-way ANOVA).

suggesting that the collective decrease in these excitatory pheromones by HFD is driving the decrease in female attractiveness.

While HFD treatment had no effect on total male CHC amounts (One-way ANOVA, $F_{4,20} = 1.230$, $P = 0.330$; Figure 11A), levels of individual CHCs were altered. All adult-only diet treatments decreased male (Z)-11-Vaccenyl acetate (cVA), while cVA levels of animals raised on the 3% HFD were unchanged (Figure 11B). cVA, a volatile pheromone only produced by males (Everaerts et al., 2010), stimulates female receptivity (Kurtovic et al., 2007). Males fed 3% HFD, both throughout development and only in adulthood, and 15% HFD only in adulthood had increased levels of n-Tricosane (nC23) while 30% HFD males did not (Figure 11B). The production of nC23 increases in *desaturase 1* mutants (Marcillac et al., 2005), indicating that HFD may affect the expression or function of genes necessary for CHC production. (Z)-7-Tricosene (7-T) was increased in males administered 3% HFD during development and decreased in 30% HFD males (Figure 11B). 7-T has also been shown to increase female receptivity, as females mated more readily with males perfumed with extra 7-T (Grillet et al., 2006), so it is interesting that 7-T, the most abundant male CHC, was decreased only in 30% HFD males, the one group of males that females obviously discriminate against.

Metabolic rescue

A number of physiological defects caused by HFD, including heart dysfunction, lipid accumulation, and insulin resistance, are mediated by highly conserved metabolic signaling pathways (Birse et al., 2010; Diop et al., 2015). Immediate exposure to HFD in wild-type flies increases insulin and TOR signaling. Blocking this initial induction or driving overexpression of a fat lipase, Brummer (Bmm), rescues HFD health defects. Insulin signaling results in the repression of *foxo* expression and derepresses TOR signaling by deactivating TOR inhibitors. Overexpression of FOXO and a dominant-negative version of TOR (TOR^{DN}) therefore serve to decrease the downstream components of insulin signaling. Increased Bmm expression leads to increased breakdown of stored lipids, negating the effects caused by lipid accumulation. Because we have noted

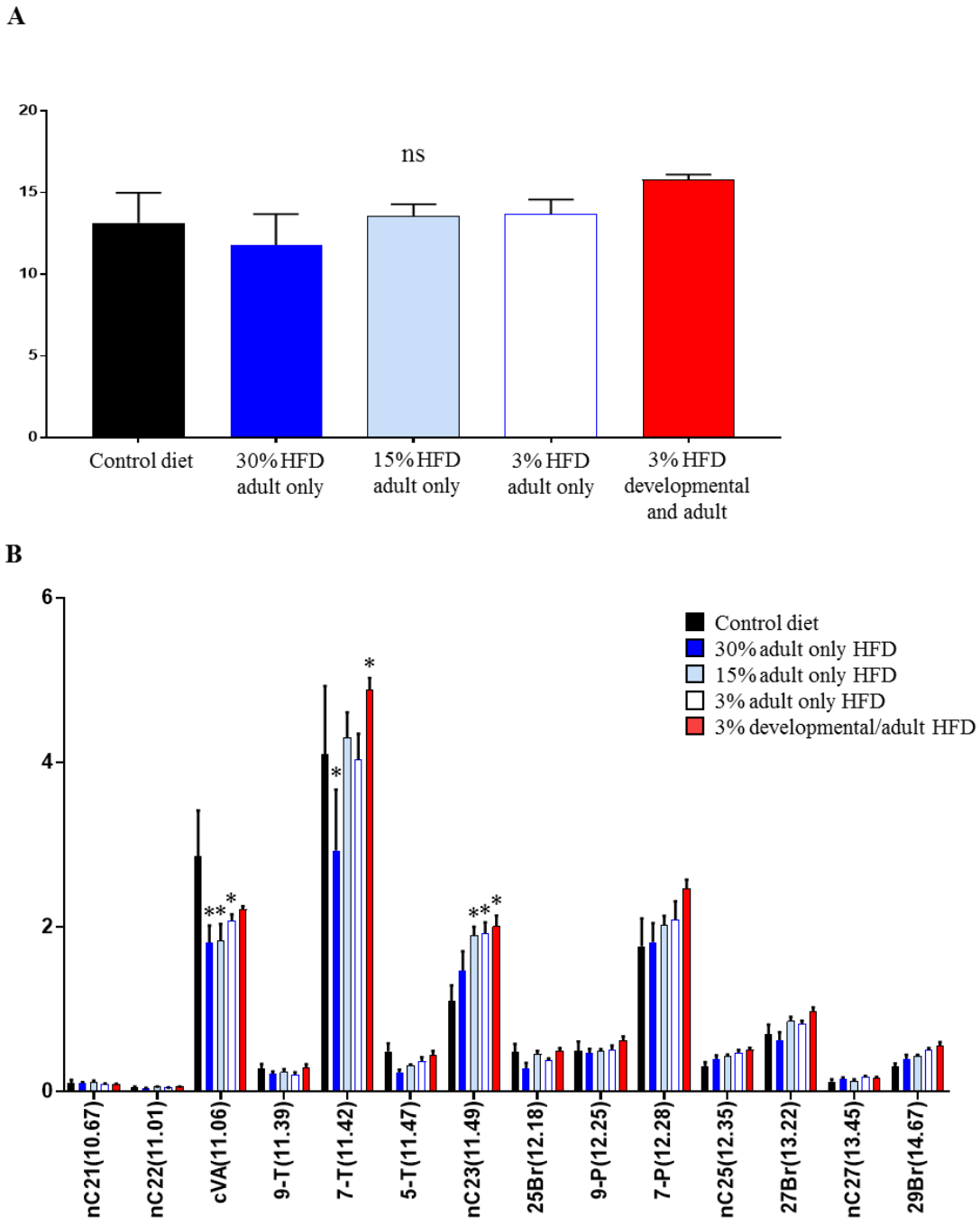

Figure 11. HFD affects male CHC profiles. Total (A) and individual (B) CHCs are affected by each HFD treatment. Each bar represents mean of 3 groups of 8 flies ± SE. *P* values in (A) and **P* < 0.05 in (B) are the results of post-hoc Dunnett's tests compared to control diet (two-way ANOVA).

that the HFD effects on fly health result in altered behavioral interactions during courtship, we hypothesized that genetic rescue of these health defects could also rescue the behavioral defects. We examined three different genetic manipulations: overexpression of FOXO and Bmm and expression of TOR^{DN}.

We tested the ability of metabolic manipulations in females to rescue female attractiveness. We expected wild-type males raised on control diet to have increased courtship latencies toward control genotype (*arm-Gal4, UAS-Bmm, UAS-foxo, UAS-*TOR^{DN}) females on HFD. However, if female overexpression of FOXO, Bmm, or TOR^{DN} rescues female attractiveness, male courtship latencies should not differ significantly toward control diet or HFD females of the rescue genotypes (*arm-Gal4/UAS-Bmm, arm-Gal4/UAS-foxo,* and *arm-Gal4/*TOR^{DN}). We found that control males court control diet females of all genotypes significantly faster than females from both high fat developmental diet (Figure 12A) and adult-only diet (Figure 12B) treatments, indicating that these genetic manipulations failed to rescue female attractiveness.

We next asked whether these same genetic manipulations could rescue the ability of HFD males to discriminate between control and HFD females. If so, we expected that HFD males with rescue genotype (*arm-Gal4/UAS-Bmm, arm-Gal4/UAS-foxo,* and *arm-Gal4/*TOR^{DN}) would have increased courtship latencies toward wild-type HFD females. While all genotypes of 3% developmental diet males had similar courtship latencies toward control and HFD females (Figure 12C), we found that 15% adult-only diet *arm-Gal4/UAS-Bmm* (Two-tailed student's *t*-test: $t = -5.32692$, $P < 0.0001$) and *arm-Gal4/UAS-foxo* (Two-tailed student's *t*-test: $t = -2.68428$, $P = 0.0097$) males took longer to begin courting HFD females, while their genetic controls (*arm-Gal4/+, UAS-Bmm/+,* and *UAS-foxo/+*) had similar courtship latencies toward control and HFD females (Figure 12D). Overexpression of Bmm and FOXO are therefore able to rescue adult-specific HFD effects on male mate judgement.

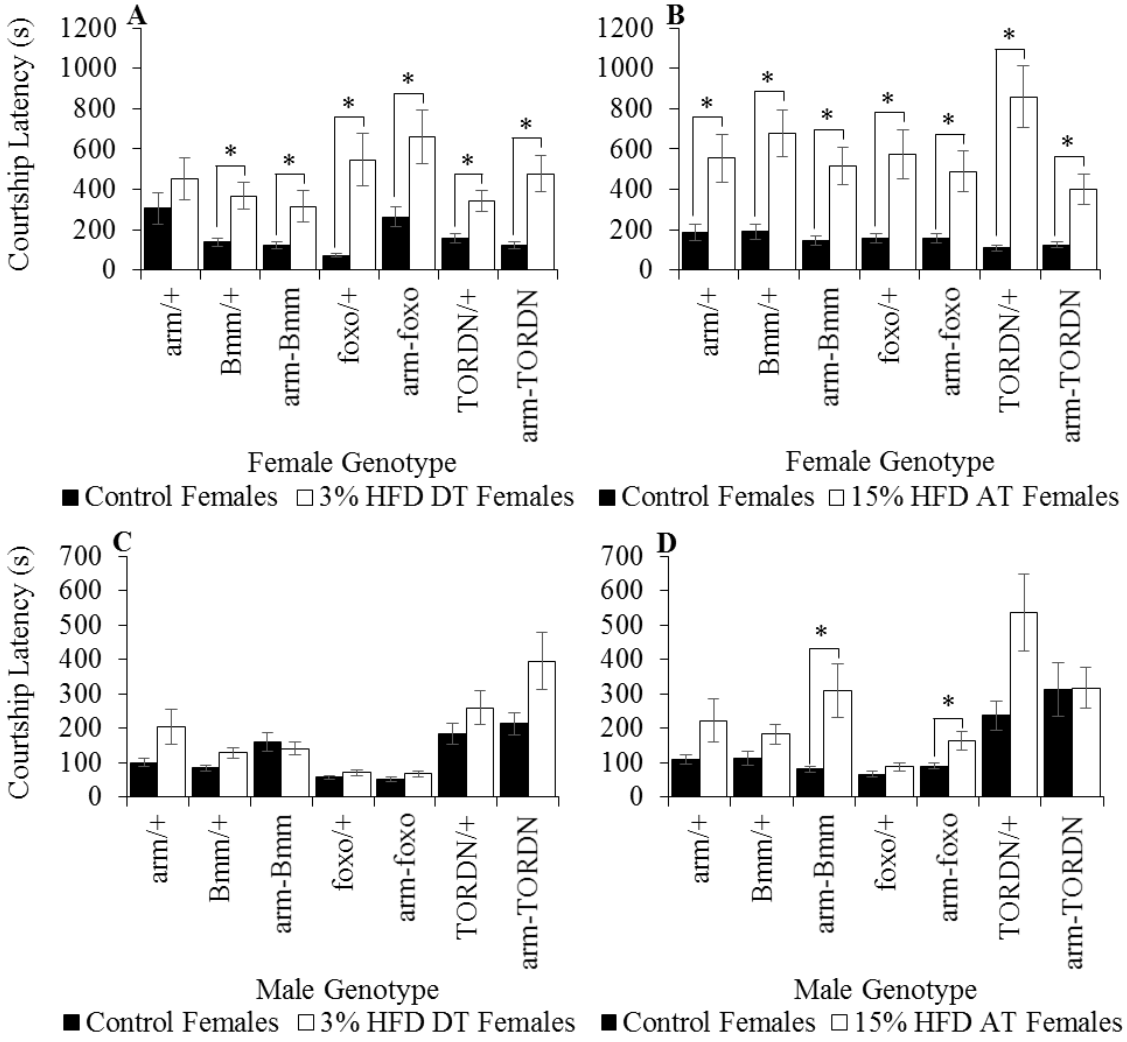

Figure 12. The effect of metabolic rescue on HFD mating behavior defects. The ability of *arm-Bmm*, *arm-foxo*, and *arm-TOR$_{DN}$* to rescue HFD developmental or adult effects on female attractiveness (A, B) and male mate assessment (C, D). Each bar represents the mean ± SE of N = 30. Means with * were significantly different with a Bonferroni corrected $\alpha = 0.025$. DT = developmental + adult treatment; AT = adult only treatment.

4.4 Discussion

Overconsumption of lipids causes severe health defects in *D. melanogaster* (Birse et al., 2010; Diop et al., 2015), and this decrease in fly health is correlated with alterations in mating behavior (Schultzhaus et al., 2017). In females, HFD leads to decreased activity

levels, body size, and fecundity, all of which reflect the worsened condition of the fly, and ultimately lead to decreased female attractiveness. Female sexual traits evaluated by males during courtship must then also be altered by HFD. The major sexual traits that signal female attractiveness are body size, female behavioral responses to male courtship, and pheromone profiles (Greenspan and Ferveur, 2000). Dietary sugar and protein levels are known to alter *D. melanogaster* pheromone profiles (Fedina et al., 2012), so it is likely that dietary lipids also affect pheromones, especially as pheromones are derived from fatty acids (Ueyama et al., 2005). To demonstrate that HFD alters female pheromone profiles in a way that decreases attractiveness, we examined males as they courted HFD females without the confounding effects of female body size and behavior. We found that males continued to behaviorally discriminate against HFD females, indicating that HFD indeed alters pheromone profiles.

First, we examined whether female visual and behavioral cues were necessary for male discrimination when flies were administered HFD during development and adulthood (developmental treatment). Developmental treatment decreased female body size, and so by examining male behavior toward HFD females in the dark where visual assessment is not possible, we could examine whether decreased body size was the major HFD altered sexual trait that males were cuing in on. We found that control males continued to court HFD females differently in the dark, indicating that males were using either female behavioral or pheromonal changes to discriminate against HFD females. To make a final distinction between these two factors, we examined male behavior towards decapitated females in the light and the dark. Decapitated females cannot perform behavioral responses to male courtship, and as such, males can only assess females through their pheromonal profiles in the dark. Control males did not court decapitated HFD and control females differently in the light, possibly because visual assessment of the decapitated females negatively impacted their judgment of the females. In the dark, though, when the only sexual trait males could assess was the pheromone profile, control males strongly modified their courtship behavior towards HFD females. Overall, this experiment provides strong evidence that HFD altered pheromones are sufficient for male

discrimination of HFD females. Indeed, CHC quantification confirmed that developmental HFD affected the pheromone profile. 9-pentacosene, 7-pentacosene, and 7.11-heptacosadiene were all significantly decreased in females. Each of these CHCs have been shown to stimulate male courtship behavior (Antony et al., 1985; Siwicki et al., 2005), and 9-pentacosene and 7.11-heptacosadiene do so in a dose dependent manner. Decreases in these CHCs are therefore sufficient to decrease female attractiveness to good condition males.

Developmental exposure to environmental factors can have dramatic, long-lasting effects on fly health. Larval crowding or malnutrition can cause numerous alterations in fly traits, including body size (Arendt, 2007; Lefranc and Bundgaard, 2000; McGraw et al., 2007; Valtonen et al., 2012; Vijendravarma et al., 2009). To fully remove the potential confounding effect of HFD on fly body size, we raised the flies on a similar diet during development and only exposed the adults to increasing amounts of HFD. We found that all levels of HFD caused changes in female activity levels, but the behavioral defects seen in the developmental treatment were only fully recapitulated with the 15% adult treatment. At this level, HFD females mated faster, indicating that they are less choosy or more receptive to mating, and control males took longer to begin courtship, indicating that the 15% HFD females are less attractive due to changes in pheromone profiles and not decreased body size. Interestingly, though, control males did not decrease their courtship towards the adult treatment HFD females as they had towards the developmental treatment. It is therefore possible that common changes in pheromones caused by the developmental and adult treatment are important for initial male assessment of the female, and influence his decision to begin courtship behavior, i.e. to enter an arousal state (Clowney et al., 2015). Once in this state, visual assessment of smaller developmental HFD females may have led males to decrease courtship, which would not happen with the adult treatment females where body size is unaltered. Yet, we saw that with the developmental treatment of decapitated HFD females, males decreased courtship in the dark when visual assessment was not possible, indicating that differences in pheromonal profiles may underlie this difference in male courtship behavior. While both the

developmental and adult only treatment of HFD caused similar decreases in multiple CHCs, adult only treatment decreased several CHCs (7.11 PD, 27Br, 7-H, and 7.11 ND) while the developmental treatment did not. All the CHCs affected by the developmental treatment were also decreased in the adult only treatment. Therefore in adult only HFD treated females, more individual CHCs were decreased, as was the overall amount of CHCs produced by these females. Continued research into these effects could shed light on why a greater number of affected CHCs would continue to affect male courtship latency but not courtship index.

Neural networks necessary for *D. melanogaster* mating behavior are also established during development. For example, the neural expression of the gene *fruitless* is necessary for normal male courtship behavior. Disruption of *fru* during adulthood has little to no effect on male courtship behavior, but disruption during development drastically reduces male ability to perform courtship (Demir and Dickson, 2005; Kimura et al., 2005; Manoli et al., 2005). We previously observed that males exposed to HFD during development did not find HFD females unattractive, as control males had, and indeed did not appear to distinguish control and HFD females to any degree (Schultzhaus et al., 2017). HFD therefore appears to alter male neural function that is important for mate discrimination, resulting in less choosy males. Whether exposure to HFD during development is necessary for this alteration to occur, or whether the change could happen after neural network establishment was unknown. We addressed this question by raising flies on a common, control diet, and then exposing the flies to increasing amounts of HFD in adulthood. Courtship behavior of 15% HFD males towards 15% HFD and control females did not differ, even though control males found 15% HFD females unattractive. This then indicates that HFD does not need to affect neural development to alter male discrimination ability, but that HFD may affect neural functionality instead. This possibility could be addressed by examining the activity of pheromone responsive neural elements (Clowney et al., 2015; Yamamoto and Koganezawa, 2013), where these neurons would be expected to have a lower threshold of activation towards certain pheromones or neural activity would not be decreased by inhibitory pheromones.

While the 3% developmental and 15% adult HFD treatments had altered female attractiveness, these same treatments had no effect on male attractiveness. It was only when males were fed 30% adult treatment that male attractiveness was affected. In non-competitive assays, these 30% males were slower and had reduced courtship output which resulted in increased female mating latency, which indicates that females were less willing to mate with these males. Competition assays confirmed this finding, where 30% males, but not 15%, gained fewer matings when competing with control males. Males appear to be far more resistant to dietary lipids effects than females, as only changes in male mate perception occurred while a multitude of behavioral defects were seen in females treated with the same dose. Sex specific physiology and responses to environmental factors are well documented in *D. melanogaster*. For example, females live (Magwere et al., 2004) and withstand starvation longer (Chippindale et al., 1996), and exercise only benefits male flies (Sujkowski et al., 2015). 30% HFD males in noncompetitive assays were less active and courted females less, and although HFD males matched control male courtship performance in competitive assays, females preferentially mated with control males. This implies that HFD could be affecting two male sexual traits, pheromones or courtship song. Changes in either trait could allow females to discriminate against 30% HFD males. The CHC quantification results point to 7-tricosene as the prime candidate underlying 30% HFD male unattractiveness. 7-tricosene has been shown to increase female receptivity to male courtship advances (Grillet et al., 2006), so decreased production of this CHC could hinder the male's ability to gain mates.

Our understanding of how HFD affects *D. melanogaster* mating behavior centers on the idea that HFD alters fly physiology and health, which affects sexual traits, leading to changes in mating behavior. This argument would be strengthened by blocking the HFD effects on fly health and seeing a rescue of mating behavior defects. Genetic manipulation of conserved metabolic signaling pathways (insulin, TOR, and Brummer) have been shown to rescue certain HFD physiological defects, like heart dysfunction, lipid accumulation, and insulin resistance (Birse et al., 2010; Diop et al., 2015), and female pheromone profiles and attractiveness are also regulated by insulin and TOR signaling

(Kuo et al., 2012). We performed the same genetic manipulations on both development and adult HFD treatments, using the same Gal4 driver, to determine whether behavioral defects could be rescued. No HFD developmental treatment defects were rescued, which may not be surprising as the only adult specific defects were examined and rescued by Birse et al. (2010). Yet when looking at adult specific behavioral defects, we found that female attractiveness was not rescued and that only FOXO and Brummer overexpression rescued male discrimination ability. These results provide evidence that insulin signaling and fat accumulation can rescue male health sufficiently to rescue male specific HFD behavioral defects but not female specific effects. Yet these results do not necessarily demonstrate that conserved metabolic signaling pathways are not involved in mediating the female response to HFD. HFD affects females more strongly than males, and the female specific effects may be too severe to rescue with these genetic manipulations. Even though certain physiological defects caused by HFD can be rescued, female activity levels were not (Birse et al., 2010), and whether these manipulations rescue other, more major defects, like mortality, has not yet been examined. It is possible that the driver used in these studies (*armadillo-Gal4*) does not provide high enough expression to rescue drastic effects of HFD on behavior.

In conclusion, our study shows that HFD affects *D. melanogaster* behavioral interactions through alteration of non-visual sexual traits, and that females are more susceptible to HFD caused defects. The less severe HFD male defects can be rescued by genetic manipulation of conserved metabolic signaling pathways. Further characterization of HFD impacts on *D. melanogaster* behavior could advance the understanding of how genetic and environmental factors interact to affect animal health and sexual selection (Miller and Svensson, 2014), drawing together two often disparate and unconnected fields of research (Andersson and Simmons, 2006).

CHAPTER V
CONCLUSION

Through this dissertation, I established methodology for the study of genetic by environmental (G x E) effects on *Drosophila melanogaster* mating behaviors. These studies reveal that individual dietary macronutrients affect *D. melanogaster* reproductive behaviors in drastically different ways, with highly sexually dimorphic responses.

I first examined how each major macronutrient (fat, protein, and sugar) affects *D. melanogaster* reproductive behavior by looking at effects on fly condition, interactions during courtship, and fecundity (Chapter II). I found that a sugar enriched diet had no effect. A protein enriched diet increased female body length and fecundity, yet had no effect on how males assessment of female attractiveness. The protein enriched diet also negatively impacted male mating times, resulting in their mates having decreased fecundity. The fat enriched diet affected every female trait examined while male traits were almost unaltered. HFD females were less active, less attractive, less choosy, and less fecund – indicating that increased dietary lipids decrease female condition, mate decision processes, and fitness. HFD males were not less attractive, yet these males did not discriminate against unattractive HFD females, which indicates that their ability to assess mates had been negatively affected. HFD males also fared worse in competition assays with control males for control females, showing that HFD males are perceptively less attractive but only when direct mate comparison was possible. These experiments demonstrate that dietary background is an important determinant of fly condition, attractiveness, and fitness.

Next, I examined the copulatory and post-copulatory effects of dietary protein by feeding flies low, moderate, and high protein diets, and examining how *D. melanogaster* male and female protein dietary levels affected mating duration, fecundity, and female remating latency (a measure of female unwillingness to remate or the return of receptivity) (Chapter III). Mating duration was not affected by female diet, but was negatively correlated with male diet. Increased female dietary protein increased fecundity, yet

females mated to high protein diet males had decreased fecundity. Female remating latency was unaffected by male dietary protein, but increased with female protein. So, males fed higher amounts of protein copulate for shorter periods and their mates have reduced fecundity, and females fed higher amounts of protein produce more eggs and regain mating receptivity faster. This experiment demonstrates that female mating costs decreased with increasing male dietary protein, but increased with increasing female dietary protein.

Finally, I examined which sexual traits were being altered by HFD and whether activity levels of conserved metabolic pathways could mitigate deleterious HFDeffects (Chapter IV). I examined whether visual assessment of or behavioral responses by HFD females were necessary for male discrimination by examining courtship behavior in the dark towards intact and decapitated females. Males continued to discriminate against both intact and decapitated females in the dark, indicating that males use non-visual sexual traits, such as pheromones, to discern that HFD females are unattractive. I next administered HFD to flies during adulthood only (previous treatments were administered during development and adulthood) to remove all potential developmental effects of HFD that could affect mate judgment by males (i.e. changes in body size). Control males continued to discriminate against HFD adult only females while HFD males did not, indicating that HFD developmental effects are not necessary to alter mating behavior, again implicating changes in pheromone profiles. Males fed an extreme level of HFD had decreased attractiveness and were less preferred in competitive settings. I then quantified pheromone profiles to discover which pheromones were altered by HFD, finding decreases in both male and female pheromones that are important in sexual communication. I attempted to rescue male- and female-specific HFD defects by genetically manipulating metabolic signaling pathways that rescue other HFD physiological defects. I found that female attractiveness was not rescued by these manipulations, but that adult treatment HFD male discrimination against HFD females was rescued. These experiments provide evidence that HFD alters female pheromone

profiles, and that conserved metabolic pathways mediate male mate judgement plasticity in response to environmental factors.

This dissertation demonstrates that not all environmental factors affect *D. melanogaster* mating traits similarly, and that responses are often sexually dimorphic in nature. This information should be considered when designing experiments examining environmental effects on condition. In the future, genetic elements that mediate the behavioral response to HFD could be identified using the *Drosophila* Genetic Reference Panel. This experiment could examine whether lines of flies that are more susceptible to HFD in terms of condition (effects on mortality or other physiological changes) also have greater decreases in attractiveness and/or mate preference as would be expected if these behavioral changes are indeed condition-dependent.

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