The role of macronutrients in the evolution of sex differences in reproduction and lifespan: lessons from novel holidic, medium-based diets

Submitted by Matthew Carey, to the University of Exeter

as a thesis for the degree of

Masters by Research in Biological Sciences

September 2017

MbyRes THESIS

2017

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ABSTRACT

Life-history traits form an integral part of evolutionary biology as they closely relate to an individual's fitness. There is a huge archive of empirical research that has shown diet influences the expression of nearly all life-history traits, ranging from immune response to sperm number. Exactly how diet actually influences the expression of important life-history traits, however, is often poorly understood, as traditionally diet has been viewed in a one-dimensional context, namely energy or calories. More recent research has begun to challenge this notion, by suggesting that it is actually the intake of specific nutrients that influences trait expression and fitness. Clearly, our understanding of the role of diet is shifting, yet more empirical studies are needed, which focus on how the intake of specific nutrients influence key life-history traits, whether these regulate life-history trade-offs and how this may impact an individual's fitness.

In this thesis, I use the Geometric Framework (GF) of nutrition to examine the role nutrition plays in the expression of key life-history traits (reproduction and lifespan) and any trade-offs that may exist between them. Historically, the type of diet used in some nutritional studies has been suboptimal, with some researchers proposing that CAFÉ assays, in particular, effect trait expression in *Drosophila melanogaster*. This is because they are a foreign substrate for the fly to feed from and thus it is difficult for flies to acquire the nutrients they need to function properly. Therefore, I formulated novel diets that represent the smallest deviation from natural feeding conditions, when compared to previous nutritional research, for *D. melanogaster*. I used these diets to focus on the effect two macronutrients, protein and carbohydrate, have on lifespan and reproduction in male and female *D. melanogaster*. I found that both protein and carbohydrates play a role in the expression of these traits, and not calories *per se*, and that there are divergent nutritional demands for reproduction between the sexes. I also found evidence that implies the type of diet (liquid versus medium-based) used to study nutritional effects on life-history traits could influence findings, with flies fed a medium-based diet living longer and having greater reproductive success. This could possibly be due to greater nutrient acquisition by flies fed medium-based diets.

Overall, my thesis highlights that nutrition is multifaceted and complex, which is paramount to understanding life-history trait expression and the trade-offs that may exist between them. My work challenges the central dogma that calories are responsible for changes in trait expression and life-history trade-offs, and advances our understanding of diet composition and also the way that diet is delivered to organisms. In addition, it opens the door to new questions relating to how the type of

diet and nutrient composition effect not only lifespan and reproduction but other life-history and sexually selected traits as well.

ACKNOWLEDGEMENTS

The completion of the work in this thesis would not have been possible without the support of a wide network of people. Firstly, I would like to thank my supervisor John Hunt for his guidance and support throughout this Masters, and most importantly making my time in his lab fun. Secondly, thank you James Rapkin and Meaghan Castledine who provided a hand, or an ear, whenever it was needed during this whole process. I would also like to thank the people who kept me on track namely Sarah Bainbridge, Stefan Store and Greg Wills. Finally to all of the others who have helped me throughout my time completing this thesis: Dave Hosken, Ruth Archer, Kim Jensen, M.D Sharma, Michael Hawkes and Sam Oyesiku-Blakemore.

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AUTHORS DECLARATION

The work that contributes to this thesis was conducted by Matthew Carey. All of the chapters presented in this thesis were written by Matthew Carey, with comments and editing from John Hunt.

CHAPTER 1: GENERAL INTRODUCTION

1.1. Life-history traits and strategies

Life-history traits, broadly defined, are variations in investments in growth, reproduction and survivorship (Roff, 2002). Life-history traits are commonly negatively correlated with one another, with the assumption that there is a finite amount of resources, and traits compete for these limited resources, resulting in trade-offs (Roff, 2002). Without trade-offs, all traits related to fitness would be driven, by selection, to the limit imposed by history and design (Stearns, 1989; Reznick, 1985; Stearns, 2000). Studies show, however, that life-history traits are commonly maintained well within those limits, providing evidence of trade-offs between these traits (Stearns, 1989). The aim of life-history theory is to understand the variation in life-history strategies, and understanding trade-offs is essential, when considering the optimal life-history of an organism in a given environment (Stearns, 1992; Roff, 2002).

Life-history trade-offs are found almost ubiquitously throughout the animal kingdom, including insects (Zhao & Zera, 2002; Hunt et al., 2004), fish (Einum & Fleming, 2004), reptiles (Zera & Harshman, 2001) and mammals (Hill & Kaplan, 1999; Catoni et al., 2008). Interestingly, the correlations between trade-offs are not universally negative and there are examples from nature and laboratory studies of positive correlations between life-history traits (Stearns, 1989; Stearns, 2000; Roff, 2002). The 'acquisition allocation' model or 'Y-model' is the most common model used to explain the variance in the sign of phenotypic correlation between life-history traits (Noordwojk & de Jong, 1986; Roff & Fairbairn, 2007). Whilst the model has been extended, the core principle remains the same, the sign of the covariance between life-history traits, depends on the relative variances in the acquisition and allocation of resources (Noordwojk & de Jong, 1986; Roff & Fairbairn, 2007). In other words, a negative covariance between life-history traits occurs when the sum of the variances in resource allocation to the two life-history traits exceeds the variance in resource acquisition. Alternatively, if the variance in resource acquisition is greater than the sum of the variances in resource allocation to the two life-history traits, a positive covariance will occur (Roff & Fairbairn, 2007).

The most notable trade-off involves the cost of reproduction, where the sum of resource allocation is often greater than the variance in resource acquisition (Hunt et al., 2004; Stearns, 1989). The two foremost components driving trade-offs with reproduction are the investments in both survival and future reproductive events (Stearns, 1989; Stearns, 2000; Zera & Harshman, 2001). The risk of investment in future reproduction, e.g. mortality risk, can mean that individuals invest heavily in

early life reproduction but have one or very few reproductive events, possibly as a result of a reduction in lifespan (Hunt et al., 2004; South et al., 2011; Stearns, 1989). Moreover, the cost of increased reproduction can indirectly constrain lifespan, for example there is evidence to show reproduction trades-off with immune function (Reznick, 1985; Stearns, 1989; Roff, 2002) or general somatic maintenance (Fanson et al., 2012; Jensen et al., 2015). The trade-off between lifespan and reproduction forms one of the more interesting questions in life-history theory, as these two traits are fundamental to our understanding of fitness. Clearly, a comprehensive understanding of the basis of this trade-off and the resource(s) that regulate it are crucial to our understanding of an organism's life-history strategy in a given environment.

Males and females often invest different amounts of their resources into reproduction, with males typically investing less (numerous, small, highly motile microgametes: sperm) and females typically investing more (fewer, larger, highly nutritious, macrogametes: eggs) (Trivers, 1972; Andersson, 1994). Therefore, males and females have opposing reproductive roles and interests, with each sex attempting to maximise their individual fitness. The opportunity for sexual selection is greater in males, due to greater variance in reproductive success than females (Trivers, 1972). Thus, males typically have more resources available for behaviours and displays used to compete for access to females including mate guarding (Bateman & MacFadyen, 1999), male coercion (Cluttonbrock & Parker, 1995) and sexual signals (Kavanagh, 1987; Sreng, 1990). Consequently, male fitness is typically more variable than females, which results in different levels of trade-offs between the sexes (Trivers, 1972; Jensen et al., 2015).

1.2. The link between life-history traits and nutrition

As already highlighted, life-history theory dictates that not all traits can be maximised due to a common limited pool of resources. The allocation of these resources gives rise to the variance seen in life-history strategies and by extension, expression of life-history traits. Furthermore, the production and maintenance of certain traits will cost more than others (Stearns, 1992). Costly traits should be more sensitive to the variation in resource acquisition, and thus co-vary positively with the available pool of resources an animal can allocate to fitness related traits (Roff, 2002; Hunt et al., 2004). Whilst resources can come in a variety of different forms, the most common resource manipulated in the literature is energy, normally in the form of food (Simpson & Raubenheimer, 2007; Simpson & Raubenheimer, 2012; Nakagawa et al., 2012; Fanson et al., 2012; Rapkin et al., 2015; Jensen et al., 2015). Indeed, food is fundamental for all organisms, with evidence to suggest

the type, quantity and quality of food all play a role in the expression of a given trait, including but not limited to immunity (Triggs & Knell, 2012), growth (Forsman & Lindell, 1996), reproduction (Hunt et al., 2004; Bunning et al., 2015; Rapkin et al., 2015) and lifespan (McCay et al., 1935; Maklakov et al., 2008; Lee et al., 2008).

Historically, nutritional ecology studies concerned with life-history traits have manipulated the intake of a single resource, food, or more specifically calories (McCay et al., 1935; Masoro, 2005). This was done because research focussed on a quantitative resource constraints paradigm, where an individual maximised their intake of a single resource, which was later allocated to competing traits (Simpson & Raubenheimer, 1993). As a result, there is a large body of research which looked at the effect of manipulating caloric content, also known as caloric restriction, on a variety of condition dependent life-history traits. Caloric restriction has been shown to maintain female reproductive function into advanced age and improve postnatal offspring survival of older females (Selesniemi et al., 2008). However, reducing calories more often has a detrimental effect on reproduction, delaying the age of sexual maturity (McCay et al., 1939) and reducing the fecundity of females (Ball et al., 1947). Interestingly, studies have shown that a reduction in calories almost always results in an increase in lifespan (McCay et al., 1935; Piper et al., 2005; Nakagawa et al., 2012).

Recently, the relevance of caloric restriction has been questioned (Simpson & Raubenheimer, 2007). There is evidence to suggest that the nutritional composition of food eaten plays a crucial role in the evolution and maintenance of life-history traits (Nakagawa et al., 2012). Indeed, there is a growing amount of research that implicates specific nutrients in not only the expression of life-history traits but also in mediating the trade-offs between them (Lee et al., 2008; Maklakov et al., 2008; Fanson et al., 2009; Jensen et al., 2015). Furthermore, the divergent reproductive tactics between the sexes has resulted in sex-specific nutritional dependant trade-offs between key life-history traits (Lee et al., 2008; Maklakov et al., 2008; Fanson et al., 2009; Jensen et al., 2015). However, there have been difficulties in nutritional ecology, resulting from the lack of a unifying approach and between lab variation in protocol, which need to be addressed to effectively investigate the role of specific nutrients on life-history traits and trade-offs between them. One such approach, the Geometric Framework of Nutrition, outlined by Simpson and Raubenheimer (1993) has proved a powerful tool in disentangling the effects of specific nutrients and calories with many studies successfully applying the principles (Simpson & Raubenheimer, 2007; Simpson & Raubenheimer, 2009; Simpson & Raubenheimer, 2012).

1.3. The Geometric Framework of Nutrition

The core principle which underpins all studies utilizing the Geometric Framework of Nutrition (GF) is that diet is heterogeneous in nature but that animals will consume a diet that maximises their fitness (Simpson & Raubenheimer, 1993; Simpson & Raubenheimer, 2007; Raubenheimer et al., 2016). This is partly due to an animal needing to support a diverse array of biological functions that have a variety of nutritional requirements, which includes macro- and micronutrients (Simpson & Raubenheimer, 1993; South et al., 2011; Simpson & Raubenheimer, 2012). As previous outlined, studies which predate or do not successfully implement the GF, often only manipulate the total energy, or calories, that an animal has access to (Kirkwood & Shanley, 2005; Simpson & Raubenheimer, 2012). Whilst the caloric restriction approach has had success in eliciting a fitness response, it fails to fully capture the diverse nutritional needs and thus foraging an animal undertakes to achieve the complex balance of specific nutrients needed for investment in life-history traits (Simpson & Raubenheimer, 2012). Thus, a model is required to better understand the specific nutrients an individual needs in order to maximise said traits (Simpson & Raubenheimer, 2012). The GF can successfully account for the animal, its environment and the multiple nutritional component basis for the interaction of an individual and its environment (Simpson & Raubenheimer, 2009; Simpson & Raubenheimer, 2012). Moreover, this model is effective as it is applicable to an evolutionary biological framework, so that the significance of an individual's nutritional choices can be examined in detail (Simpson & Raubenheimer, 2012).

The GF enables researchers to investigate the effect of an individual's nutritional intake on their life-history traits, and any measurable trait for that matter, by using a multidimensional nutritional framework able to differentiate between intake and utilization (Lee et al., 2008; Simpson & Raubenheimer, 2012). To apply the GF, one first needs to design a range of diets varying both in their nutritional make up, (e.g. varying protein and carbohydrates) and their total nutritional content (i.e. how much of the diet is digestible) (Simpson & Raubenheimer, 2007; Lee et al., 2008; Simpson & Raubenheimer, 2012). These diets are then used investigate the nutritional optima for a given trait through feeding experiments, which produce a nutritional landscape (a fine-scale response surface) (Archer et al., 2009). To create nutritional landscapes, diets of a known nutritional composition are made. These are diets are then fed to animals in a no-choice feeding trial, where an individual is fed a single diet for the duration of the experiment. The animal's intake of diet is usually measured as well as the expression of the trait of interest. Landscapes are then constructed by mapping traits onto the nutrient intake data (Simpson & Raubenheimer, 2012). In fact, the GF has yielded fruitful

results when used to investigate the effect of specific nutrients on a variety of life-history traits including, lifespan and reproduction (Lee et al., 2008; Maklakov et al., 2008; Fanson et al., 2009; South et al., 2011; Fanson & Taylor, 2012; Rapkin et al., 2015; Jensen et al., 2015).

Choice feeding trials, where two or more diets are provided in a treatment to a given individual, are also a powerful tool in the GF for determining how animals optimally regulate their intake of nutrients to maximise fitness (Simpson & Raubenheimer, 2012). While a no-choice experiment can inform us of the nutritional content and concentration which maximises a given trait, this may differ from the 'intake target' or regulated intake point that an animal eats towards when given a choice (Simpson & Raubenheimer, 2012). In addition, this point can be 'static' (i.e. does not vary with time), or 'dynamic' (i.e. vary through time or development) (Archer et al., 2009; Simpson & Raubenheimer, 2012). The GF has proved revolutionary in the field of nutritional ecology with application from invertebrates to humans and as such will be a central tenant of this thesis.

1.4. Nutritional optima and trade-offs

Currently, empirical support for the role of specific nutrients in the regulation of life-history trade-offs is lacking, because of difficulties in quantifying resource allocation, caused by the variety of different measures used as proxies for resource allocation (Noordwojk & de Jong, 1986; Roff, 2002; Roff & Fairbairn, 2007). Thus, the types of dietary manipulation the GF allows, (i.e. the manipulation of the composition and concentration of resources available to an animal) could prove a vital tool in improving our understanding of trade-offs between life-history traits, an important aspect of life-history research (Simpson & Raubenheimer, 2012). There is evidence across a wide range of taxa, which shows dietary manipulation has a significant effect on life-history trade-offs, ranging from invertebrates (Hunt et al., 2004), reptiles (Van Dyke et al., 2012), fish (Devigili et al., 2013), birds (Robb et al., 2008) and mammals (Hill & Kaplan, 1999). However, a limitation these studies and others like them have is that they use either too few diets, diets which are not chemically defined or a combination of these two factors. Further, some do not measure dietary intake, which makes it difficult to carry out a comprehensive analysis, by which I mean an analysis that is able to partition the effects of specific nutrients and calories, and also show any interaction between specific nutrients and life-history trade-offs (Hunt et al., 2004; Simpson & Raubenheimer, 2012). As such, I find a recent trend in the literature in the application of the GF in studies which aim to accurately quantify the resource allocation to life-history traits, and thus trade-offs between them.

However, it is important to note that the term trade-off is used in more than one way throughout the literature and as such clarification of its use in this thesis is worthwhile. Most commonly in nutritional ecology, and this thesis, the type of trade-off being referenced is a multiple trait trade-off. Where two or more traits, that share a limited amount of resources, are both under selection to increase but selection is constrained by the other trait (Argawal et al., 2010). A more accurate label for this occurrence is perhaps 'metabolic trade-offs', because this represents a better description of the underlying process of the trade-off occurring. Consider the manner of selection taking place, in essence selection is freeing resources for a given trait by facilitating the reduction of a different trait, thus the trade-off is metabolic in nature. Alternatively, we see references in the literature to trade-offs involving only one trait, sometimes called 'contrasting selection' (Bailey, 2008). Argawal et al., (2010) define 'contrasting selection' as selection that occurs when there is opposing selection on a single trait by different selective agents or through different components of fitness. Alternatively, a phenotype that is optimal in one given setting or life-history function is suboptimal, and therefore decreases fitness, in a different context or life-history function. Here, although a trade-off occurs it is not explicitly due to resource competition and is, therefore, not metabolic in nature and thus is discussed less within the nutritional ecology literature (Argawal et al., 2010). Finally, a similar clarification of 'cost' is also valuable. I draw attention to two types of costs, which are analogous with the trade-offs discussed. Firstly, metabolic cost, which occurs when the opportunity of potential investment is lost due to the allocation of resources to one trait (Argawal et al., 2010). Alternatively, there is the cost which we observe as a detrimental trait expression in one fitness context, when the trait was beneficial in a different fitness context (Argawal et al., 2010).

1.4.1 Trait-specific nutritional optima

Classical optimal foraging models, which posited that fitness increases with total energy intake, overlooked that animals must regulate not only their energy intake but also their intake of specific nutrients, a concept that has recently been shown through the use of the GF (Stephens & Krebs, 1986; Simpson & Raubenheimer, 2012). For example, work by Lee *et al.* (2008) found that in female *Drosophila melanogaster*, lifespan was maximised at a more carbohydrate biased protein to carbohydrate ratio (P:C) of 1:16 than reproduction which was maximised at a P:C ratio of 1:2. This finding highlights how fitness related traits can be maximised at different amounts and combinations of specific nutrients. This work goes some way to suggesting possible metabolic trade-offs between

fitness enhancing traits, as a result of competitive allocation of limited resources (Zera & Harshman, 2001).

Subsequent work has added weight to the notion of trait-specific nutritional optima driving metabolic trade-offs between life-history traits. For instance, female Queensland fruit flies, *Bactrocera tryoni*, were found to have their lifespan maximised on an even more carbohydrate rich diet than that of *D. melanogaster*, with a P:C ratio of 1:21, whilst egg production was maximised at a P:C of 1:3 (Fanson et al., 2009). Although it should be noted that the 1:16 P:C ratio was the most carbohydrate rich diet in the Lee *et al.* study (2008). Studies have not only found different nutritional optima between lifespan and egg production, but trait-specific nutritional optima have resulted in metabolic trade-offs between other key life-history traits including between sexually selected traits, for example sperm number and fertility (Bunning et al., 2015), immunity and reproduction (Schwenke et al., 2016) and lifespan and reproduction (Jensen et al., 2015).

1.4.2 Sex-specific nutritional optima

With evidence of trait-specific nutritional optima arising from divergent nutritional needs between traits shown in the previous section, one would expect sex-specific nutritional optima as a result of the divergent reproductive tactics between males and females (Simpson & Raubenheimer, 2012; Jensen et al., 2015). Indeed, when I look at the literature, I find that this is the case in the limited number of studies that have investigated this topic. Maklakov *et al.* (2008) found that in the field cricket, *Teleogryllus commodus*, male and female reproductive effort was maximised at different P:C ratios. Male reproductive effort, which was assessed through calling effort, shown to be metabolically costly and a good proxy for mating success (Kavanagh, 1987), was maximised on carbohydrate rich diets, whereas female reproductive effort, assessed by number of eggs laid, was maximised on more protein rich diets (Maklakov et al., 2008). A similar result was also found in *D. melanogaster* where male reproductive effort, assessed through number of offspring sired when in competition with a marker male, was maximised on carbohydrate rich diets, P:C of 1:16 (Jensen et al., 2015). In contrast, female reproductive effort, assessed through egg production, was maximised at P:C 1:2 again a more protein biased diet (Jensen et al., 2015). Interestingly, when both these experiments provided individuals from both sexes with a choice, there was little difference in the regulated intake point between males and females (Maklakov et al., 2008; Jensen et al., 2015). This perhaps suggests that individuals were constrained by the opposite sex from reaching their sex-specific nutritional optima to maximise fitness (Jensen et al., 2015).

1.5. Outline and Objectives

Currently there is gap in our understanding of how the type of food (liquid or medium-based), as well as the nutritional content and concentration, impacts on life-history traits. This is particularly apparent in lab-based animals, which often see large deviations from the food, and feeding conditions, they would typically encounter in the wild. For example, liquid-based diets have been utilised extensively in nutritional research that uses *Drosophila melanogaster* because they can provide an accurate measure of what an individual is eating. However, it has been proposed that liquid-based diets negatively impact trait expression, as liquid-based diets are too large a change of dietary type for individuals to manage. This means *D. melanogaster* cannot feed properly and as a result are not able to get the nutrients they require for proper bodily function and trait expression, a problem which medium-based diets could be able to nullify. Thus, there is a clear need for nutritional research which uses medium-based diets. The primary objective of this thesis is to examine the role of nutrition on the expression of life-history traits and any subsequent role in mediating the trade-offs between traits. I present this thesis with a discrete research paper, which contains its own literature review, methodology, results and discussion, for which I plan to formulate new diets and examine the effect of protein and carbohydrates on lifespan and reproduction on male and female *Drosophila melanogaster*. Furthermore, I will look for trade-offs between these traits and also between the sexes to look for trait- and sex-specific nutritional optima. This chapter and its focus on two key life-history traits, lifespan and reproduction, is in keeping with the wider theme of the thesis, the role of nutrition on life-history traits and the trade-offs between them.

CHAPTER 2: GENERAL METHODS

Diet Manufacture according to the Geometric Framework

The diets used in this thesis needed to vary in both the combination and concentration of nutrients, in order to meet the requirements of the GF and effectively disentangle the effects of specific nutrients and calories on life-history traits. I created 40 artificial diets, which varied in P:C ratio and absolute amount of protein and carbohydrates (P+C), using the established protocol outlined in Simpson and Abisgold (1985). This was a two-stage process, the first generated a 'powdered' form of each diet, with the protein in each of these consisting of casein, albumen and peptone in a 3:1:1 ratio. The digestible carbohydrates in each diet were sucrose and dextrin in a 1:1 ratio. All diets contained the following in equal amounts; Wesson's salts (2.5%), ascorbic acid (0.28%), cholesterol (0.55%) and vitamin mix (0.18%). The diet mixture of proteins, carbohydrates and micronutrients was diluted to the necessary amount through the addition of crystalline cellulose which is indigestible to the majority of insects (Martin et al., 1991). See figure 2.1 for visual representation of each diets place in nutritional space.

To make the vitamin mix, each individual component was weighed out separately, using a micro-spatula and microbalance, these were then put into a pestle and mortar for mixing. This mixture was then stored in an airtight container at -20^{o}C until needed. To make the main body of each diet the required amounts of cellulose and casein were added to a large glass beaker. In a separate smaller beaker, the constant specified amount of cholesterol was added, followed by linoleic acid which was added to the cholesterol using a pipette. This cholesterol/linoleic acid mixture was dissolved thoroughly in chloroform and then added to the dry cellulose/casein mix. The wet diet mixture was left in a fume hood for 24 hours and stirred regularly to allow the chloroform to evaporate. After 24 hours had passed, the required amounts of Wesson salt's, sucrose, dextrin, peptone, albumin and ascorbic acid were added. The specified amount of vitamin mix was then added to small beaker and dissolved in 20% pure ethanol, before being added to large glass beaker. Clean spatulas and weighing boats were used to weigh out each new ingredient, and diets were stirred thoroughly upon the addition of each ingredient.

The wet diet mix was then blended in a domestic kitchen food processor for approximately 2 minutes, before being dispensed into a Pyrex baking tray and placed in a drying oven at 30^{o}C. The diets were then blended every 24 hours until dry, upon which they were ground using centrifugal mill into a homogenous fine powder and stored at -20^{o}C in air tight containers until needed.

The second stage of the process turned the powdered diet into an agar based medium upon which the flies could spend their entire life cycle. Here, the powdered diet was combined with water, agar and Nipagin in a 10:10:1:0.1 ratio. The agar was first added to the water and boiled, to activate the agar, the agar/water solution was then cooled to between 80-60^{o}C. The Nipagin was added once this temperature range had been reached and the solution was stirred thoroughly to ensure all the Nipagin had dissolved. Once this had been achieved the required amount of powdered diet was added and the solution was again stirred thoroughly. Food colouring was added as required to provide contrast to count female eggs. The still hot diet solution was then distributed into 'vial caps' and stirred once again to ensure the even distribution of powdered diet in the medium. These caps were then stored at 4^{o}C until needed, and were at no stage stored longer than a week.

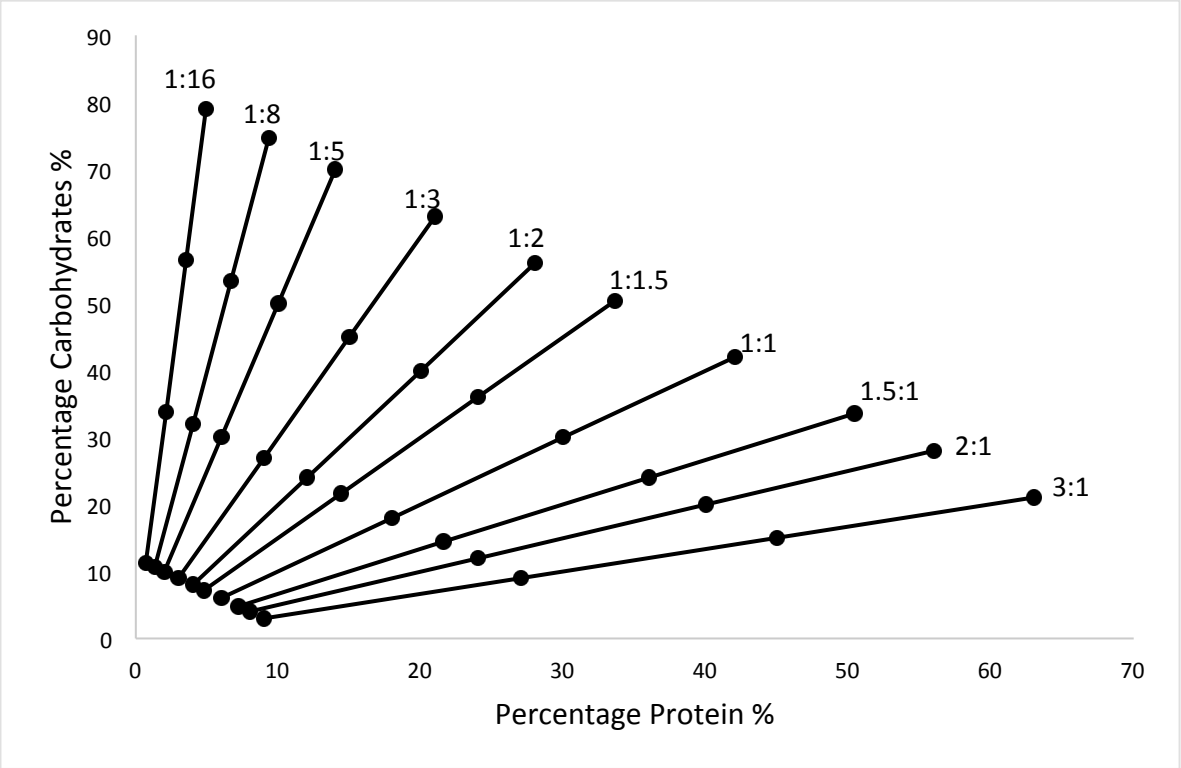

FIGURE 2.1. Location of the 40 diets that define the nutritional space

CHAPTER 3: EVOLVING FROM CAFÉ ASSAYS: SEX-SPECIFIC EFFECTS OF PROTEIN AND CARBOHYDRATE INTAKE ON REPRODUCTION BUT NOT LIFESPAN IN *DROSOPHILA MELANOGASTER* USING A HOLIDIC, MEDIUM-BASED DIET

3.1. Abstract:

There is a large body of research, which suggests that modest dietary restriction extends lifespan in a large range of taxa. This effect is usually greater in females compared to males, with this being attributed to stronger trade-offs between lifespan and reproduction in females, and calories are believed to mediate this trade-off. However, recent research challenges this hypothesis by suggesting that specific nutrients mediate this trade-off and thereby influence lifespan and reproduction. Yet, there is evidence that the type of diet could also be influencing the phenotypic expression of these traits, perhaps even supressing expression, particularly in *Drosophila melanogaster*. I used a revolutionary technique called the Geometric Framework, a state-space modelling approach, to formulate a new type of diet, which is medium-based and aims to alleviate possible trait suppression. Here, I use my novel diets to investigate the effects of protein and carbohydrate on lifespan, reproduction and any trade-off between these traits in male and female *D. melanogaster*. I found that male and female lifespan was maximised at P:C ratios of around 1:16, and that male daily reproductive effort was maximised at a very similar P:C ratio, whereas female daily reproductive effort was maximised at a P:C ratio of 1:1.21. This resulted in larger differences in nutritional optima between lifespan and reproduction for females compared to males, in addition to nutritional optima for lifetime reproductive effort that differed between the sexes. Thus, my work shows that it is specific nutrients, not calories, that mediates the trade-off between lifespan and reproduction, and that there are sex-specific nutritional optima for reproduction but not lifespan between the sexes. Furthermore, I provide the first, promising, evidence that the type of diet used in nutritional studies impacts trait expression. However, despite the differences in lifespan and reproduction caused by diet-type, the effects of protein and carbohydrates on these traits are surprisingly similar and so, my work adds to the growing body of research that challenges the role of caloric restriction in extending lifespan and subsequently the expression of other life-history traits.

Key words: *Drosophila melanogaster*, nutrition, macronutrients, fitness, Geometric Framework, lifespan, reproduction, café assays

3.2. Introduction:

An extension of lifespan (LS) as a result of dietary restriction, is a well-documented phenomenon in nutritional research, and has been shown in a diverse range of taxa (McCay et al., 1935; Nakagawa et al., 2012; Raubenheimer et al., 2016). The extension of LS in these studies was typically attributed to a reduction in caloric intake without malnutrition, also known as caloric restriction (Masoro, 2005; Piper et al., 2005). Usually, the effects of caloric restriction on LS are more profound in females, which has been attributed to divergent reproductive tactics between the sexes, with evidence to suggest that a reduction in female fecundity increases available resources for somatic maintenance (Barnes & Partridge, 2003; Bonduriansky et al., 2008; Chapman & Partridge, 1996; Piper et al., 2005). However, there is now a growing body of work challenging the central dogma of nutritional research, that dietary restriction extends LS (Simpson & Raubenheimer, 2007; Lee et al., 2008; Fanson et al., 2009; Jensen et al., 2015). Recent research suggests that it's not calories *per se* that mediates the trade-off between LS and reproduction, rather the effect of specific nutrients, namely protein and carbohydrate (Lee et al., 2008; Fanson et al., 2009; Jensen et al., 2015). While there is research which looks to disentangle the effects of specific nutrients and calories on LS and reproduction, it is still the subject of much debate (Piper et al., 2005; Tatar, 2011; Tatar et al., 2014; Speakman et al., 2016).

Contemporary research utilises the Geometric Framework (GF) for nutrition as a means of investigating the effects of specific nutrients and separating these from calories (Simpson & Raubenheimer, 2007). The GF is a state-space modeling approach which investigates how animals balance multiple nutritional needs in a multidimensional and changing nutritional environment (Simpson & Raubenheimer, 2007; Simpson & Raubenheimer, 2012). This approach allows for the effects of specific combinations of nutrients (*n*) to be separated in an *n*-dimensional nutritional space by limiting experimental organisms to holidic (chemically defined) diets that differ in nutrient composition (Simpson & Raubenheimer, 2012; Jensen et al., 2015). Thus, the GF arguably nullifies the biggest issue faced by ageing research by successfully separating the effects of caloric restriction from that of specific nutrients on LS and reproduction (Simpson & Raubenheimer, 2007; Lee et al., 2008). In this approach, diets are grouped along several 'nutritional rails', with each rail representing a fixed nutrient ratio, most commonly a protein:carbohydrate (P:C) ratio (Simpson & Raubenheimer, 2012).

The GF has been utilized in broad array of species including insects, fish and mice (Nakagawa et al., 2012; Fontana & Partridge, 2015), with a common finding that LS is extended when protein is

restricted, an alternative explanation to caloric restriction. This suggests that previous studies have confounded the effect of caloric restriction with that of protein restriction (Piper et al., 2005; Lee et al., 2008; Fanson et al., 2009; Fanson et al., 2012; Jensen et al., 2015). Further, several studies have suggested that it is actually the balanced intake of protein and carbohydrates that regulates LS and reproduction as opposed to caloric restriction (Lee et al., 2008; Maklakov et al., 2008; Fanson et al., 2009; Jensen et al., 2015). One of the earliest studies by Lee et at. (2008), used female vinegar flies, *Drosophila melanogaster*, and found LS was maximized at a P:C ratio of 1:16. A carbohydrate biased diet increasing LS has since been replicated in several study species including *Teleogryllus commodus* (Maklakov et al., 2008), *Bactocera tryoni* (Fanson et al., 2009) and mice (Solon-Biet et al., 2014). However, there has been limited research using the GF with holidic diets, to compare the effects of specific nutrients on LS and reproduction between the sexes (Jensen et al., 2015). To date only three studies have taken this approach, two used the field cricket, *T. commodus* (Maklakov et al., 2008; Rapkin et al., 2017), the other on vinegar flies, *D. melanogaster* (Jensen et al., 2015). Work on both showed that LS and reproduction was regulated by the balanced intake of protein and carbohydrate, and that there were sex-specific nutritional optima. Although there were some similarities between the species, namely that LS was maximised on low P:C ratio diets and that there were sex-specific nutritional optima for reproduction between the sexes in both species, there were also some key differences as well. Most notably that male and female *T. commodus* have different nutritional optima for LS (Maklakov et al., 2008), which is in stark contrast to the findings of Jensen et al. (2015), who found no difference in nutritional optima between the sexes for LS in *D. melanogaster.*

D. melanogaster have proven a useful model organism in the study of nutrition on life-history traits, as they are easily propagated and have an abundance of related genetic information. However, the type of diets used in these studies has been highlighted as sub-optimal (Piper et al., 2011; Jensen et al., 2015; Wong et al., 2009). Moreover, the lack of a consistent diet type across studies could account for some of the variation found between results from different studies (Piper et al., 2014). Early studies, such as Lee et al. (2008), used liquid CAFÉ assay diets and yeast as a source of protein. Whilst protein is the most abundant macronutrient in yeast, it also contains a variety of micronutrients, essential lipids, sterols and carbohydrates (Simpson & Raubenheimer, 2009; Tatar, 2011; Jensen et al., 2015). As a result, any conclusions drawn from protein restriction using yeast have the possibility of being confounded by the other constituents. Another study, utilized both liquid and medium diets with both yeast and casein as a source of protein, however the small number of diets used did not constitute a full geometric array (Bruce et al., 2013). Most recently, a study by Jensen et al. (2015), was the first to fully integrate the GF with holidic diets to partition the

effects of specific nutrients and caloric intake on LS and reproduction in *D. melanogaster*, with this study using liquid CAFÉ assay diets. These diets, where food delivered in liquid form by a capillary feeder, have the ability to accurately measure intake (Deshpande et al., 2014). However, it has been suggested that CAFÉ feeding substantially reduces LS and egg laying compared to flies fed the standard agar-gelled medium (Lee et al., 2008; Wong et al., 2009; Deshpande et al., 2014; Tennessen et al., 2014).

There are different possible explanations as to why CAFÉ assays restrict phenotypic expression. One of which is the CAFÉ assay restricts *D. melangaster's* ability to feed and as such individuals can't get the nutrients they need for normal bodily function. In other words, the food is not easily accessed, particularly in aged flies, as the fly is feeding from a small tube rather than the surface of a given food source (Wong et al., 2009). Alternatively, CAFÉ assays could be too large a deviation from natural feeding conditions, as *D. melanogaster* typically feed on microorganisms, on the surface of fruit (Werger, 1977; Kimura et al., 1977; Wong et al., 2009). One way to address this phenotypic suppression is to use a medium substrate, in conjunction with the GF, to closer replicate natural feeding conditions. In nature, *D. melanogaster* explore their environment and are constantly sampling this environment using the taste hairs on their tarsi. Once a fly has found a sufficient food source they extend their proboscis and feed (Edgecomb, Harth and Schneiderman, 1994). Therefore, a medium-based substrate should present a more 'natural' feeding condition when compared with capillary feeders, as the fly can 'explore' the surface of the substrate. It is noteworthy however, that all that has been discussed so far is concerned with adult flies. Female *D. melanogaster* lay their eggs on a nutritious substrate which when their eggs hatch, the larva are able to feed on before they enter the pupa stage of their life cycle (Werger, 1977). Females are unable to lay eggs on to the capillary feeders and as such we miss any possible diet mediated effects on development during the larval and pupa stages of the *D. melanogaster* life cycle (Werger, 1977; Kimura et al., 1977; Wong et al., 2009). A problem that a well designed medium-based diet would possibly be able to alleviate, allowing both the study of dietary effects on early life development and also experimental evolution on a given diet for numerous generations.

Indeed, there has been a trend towards using holidic agar based medium to study fly phenotypes in the recent literature (Piper et al., 2014; Bonduriansky et al., 2016; Hawley et al., 2016), yet there is also a high prevalence of studies still using the CAFÉ assay (Jensen et al., 2015; Bowman & Tatar, 2016; Reis 2016; Morimoto & Wigby, 2016). Piper et al. (2014), created a comprehensive holidic medium for *D. melanogaster*, although this was perhaps not designed with the GF in mind, and is

difficult to adapt to nutritional rails due to cost. The protein component of Piper et al.'s (2014) diet consists of different amino acids, which when brought in the quantities required for a study concerned with LS and reproduction, exceeds realistic budgets. Other studies which have used medium diets, have often used 6 rails, leaving large gaps in the nutritional landscape created using the GF (Bonduriansky et al., 2016). As such, there is not yet a study that has fully integrated a holidic medium with the GF to investigate the effects of specific nutrients on LS and reproduction on *D. melanogaster* determining whether medium-based diets effect phenotypic expression and as a result the trade-off between LS and reproduction.

Here, I use the GF approach, in conjunction with holidic medium diets, to investigate the effects of specific nutrients on LS and reproduction in male and female *D. melanogaster*. I used a no choice experiment, with a greater coverage of nutritional space than seen before, and fed 800 males and 800 females on one of 40 holidic medium diets, that varied systematically in protein and carbohydrate content as well as total nutrition. From this experiment, detailed nutritional landscapes were produced, which showed the optimal dietary protein and carbohydrate content for LS and reproduction in males and females and allowed for a detailed comparison of nutritional optima both between traits and the sexes.

3.3. Methods and Materials:

Fly Stock and Maintenance

Dahomey *Drosophila melanogaster* stocks (supplied by Nick Priest, University of Bath) and Krüppel mutation stocks (Bloomington Stock Centre, received September 2015) were maintained in an identical manner. Populations were housed in two large population cages ($1m^3$) with overlapping generations at 25^oC under a 12:12 light:dark cycle. Stocks were maintained at around 2000 individuals and mixed panmictically to avoid inbreeding. Flies were reared on 'Jazz mix' (Fisher Scientific, Loughborough, UK), using wide neck 1000ml jars. Stock cultures were maintained using this protocol for 9 months prior to use in my experiment.

Experimental animals were cultivated using smaller vials (25mm x 95mm), at larval densities of 50-60 larva per vial. These were put into both cages, with flies mixed when collecting virgins, as to avoid cage bias. Flies were collected between 2 and 4 hours after eclosion to adulthood, with experimental individuals randomly allocated to one of 40 diet treatments.

Artificial diets:

40 artificial diets, which varied in both protein (P) to carbohydrate (C) ratio and nutritional content, were created using the established protocol outlined in Simpson and Abisgold (Simpson & Abisgold, 1985). This was a two-stage process, the first generated a powdered form of each diet using the methods outlined in South et al. (2011). Five P:C rails were added, and one removed, compared to South et al. (2011) for a total of 10 rails to provide a more comprehensive coverage of the nutritional space (Fig. 2.1). The second stage used the powdered diet to make an agar-based medium, through combination with water, agar and Nipagin (At a ratio of 10:10:1:0.1 diet, water, agar and Nipagin). Agar was added to water, which was then boiled, and left to cool to <80^{o}C, after which Nipagin and diet was then added. Food colouring was used to provide a contrast to fly eggs. My artificial diets were designed to cover the nutritional space in greater detail, than the yeast and sucrose based diets used by Lee et al. (2008) and the holidic diets used by Fanson and Taylor (2012) and Jensen et al. (2015).

Diets in holidic medium form were provided in 'vial caps' (1.6 cm diameter, 1.6cm deep) that could be securely fitted to the vials in which experimental individuals were housed. Caps were changed before and after a mating, with mating's taking place every 5 days. The holidic medium was not only a food source, but also a moisture source and site of oviposition for experimental females.

Experimental Protocol:

To determine the effects of P and C on LS and reproduction on *D. melanogaster*, on the day of eclosion, 20 flies of each sex were individually assigned to one of 40 artificial diets at random (*N*=1600). Individual flies were fed, and reproduction assessed, every 5 days for their entire lifetime. Starting on day five, all experimental individuals were paired with a 5-day-old virgin mating partner for a 12-hour period, mating partners were collected in the same manner as experimental individuals and housed individually until mating events. This mating regime was continued for the duration of an experimental individual's lifetime and each time a new 5-day-old mating partner was used. All experimental flies had their food caps changed 24 hours prior to mating, to allow focal individuals to 'settle' on new food and allow greatest chance of copulation success. The food caps were also changed 6 hours after mating partner removal, at which time female reproductive effort was measured, by counting the number eggs oviposited on food cap; only un-hatched eggs were counted. Male reproductive effort, also measured every five days, was carried out using the methods outlined in Jensen et al. (2015). We counted the number offspring produced by a focal male when in competition with a 5-day-old virgin male with the Krüppel dominant eye mutation. This allowed for offspring paternity to be easily assigned and provided us with a biological relevant

measure of male fitness that we could compare to previous work. After the 12-hour mating period, the Krüppel male and female were removed, and the female established on 7ml of standard 'Jazz mix' diet for a 14-day period. On the day 14, vials were frozen and offspring phenotyped sexed and counted. LS was assessed through daily mortality checks. Diet treatment resulted in differential LS and as such reproductive effort was assessed across an individual's lifetime (Total female eggs laid or male offspring sired) and assessed daily (female egg production rate or male offspring production rate). Daily reproductive effort was calculated by dividing lifetime reproductive effort by LS. Upon finishing data collection, I collected LS and reproductive effort data on 1717 individuals, flies that died before their first mating or escaped during the experiment were not included in analysis (a total of 117 flies). Thus, my total sample size was 1600 individuals (800 males and 800 females).

Statistical Analysis:

A multivariate response-surface approach, as outlined in South et al. (2011) was used to estimate the linear and nonlinear (i.e. quadratic and correlational) effects of P and C intake on LS and reproduction within each sex. To visualise the multivariate nutritional landscapes for each trait, I used non-parametric thin-plate splines, which were constructed in R using the *Tps* function in the Fields package (Nychka et al., 2015) of R (R Core Team, version 3.1.2, Vienna, Austria, www.r-project.org). I also estimated the location of the nutritional optima and their 95% confidence regions (CRs) using the *OptRegionTps* function in the 'OptimaRegion' package (del Castillo et al*.,* 2016) in R. Full details of this approach can be found in Rapkin et al. (*In Press*).

A sequential model-building approach (Draper & John, 1988) was then used to determine whether the linear and nonlinear (quadratic and correlational) effects of nutrient intake differed across my response variables. Full details of this approach can be found in the supplementary material. Although the sequential model has utility in statistical analysis of the difference in magnitude of the linear and nonlinear gradients across response variables, it is unable to shed light on the direction of this difference in nutritional space (Rapkin et al., 2015; Bunning et al., 2016). It is, therefore, possible for response variables to show differences in the magnitude of linear and nonlinear gradients, but actually occupy a similar location in nutritional space. I, therefore, also calculate two additional measures to quantify any difference in the location of nutritional optima. Firstly, I calculated the angle ($\boldsymbol{\theta}$) and 95% confidence interval (CI) between nutritional vectors for the two response variables of interest using the procedure outlined in Bunning et al*.* (2015) (R code to calculate θ can be found in Appendix 1). Finally, I also estimated the divergence between the global nutritional maxima (calculated from my 95% CR of the nutritional landscape) using the Euclidean distance ($\boldsymbol{d}$)

and corresponding 95% CIs using the *CRcompare* function in the 'OptimaRegion' package in R. To provide measures of $\boldsymbol{\theta}$ and ***d*** that are comparable across studies, I express these parameters as a percentage of their maximum values ($\boldsymbol{\theta}$ = 180°, ***d*** = 82.11). See Rapkin et al. (*In Press*) for a full overview and justification of this analysis.

3.4. Results:

LS and both daily and lifetime reproduction are greatly influenced by variation in the intake of P and C (Figure 3.1 A & B, Table 3.1). I found that high protein diets are ultimately detrimental to LS in both sexes, with LS maximised on low P:C diets. Using my calculated global nutritional optima (Figure 3.2 A & B), I show that a P:C ratio of 1:15.93 for males, and 1:15.88 for females, was optimal for maximal LS across the sexes. I also found that LS decreased as diets became more P biased, for any given nutritional content, which one can see when following any isocaloric line (Figure 3.1 A & B). Also, my results indicate that LS generally increased with total nutritional content, which provides evidence in opposition to the notion of caloric restriction being responsible for LS extension, although this effect was reduced as diets became increasingly C biased. Formal analysis shows that whilst the optimal ratios are very similar, there are differences between the sexes in the effect of P and C on LS. This is due to the linear effects of P and C, where males have steeper positive gradients (Table 3.2), and the non-linear effects of P (Table 3.2). However, the nutritional optima fall on similar places on the nutritional landscape as shown by the small angle between the linear nutritional vectors ($\boldsymbol{\theta}$ = 5.91°, 95% CI: 0.00°, 16.64°) and the relatively small Euclidean distance between the optima ***d***= 21.14 (95% CI: 19.00, 21.93) compared to the maximum possible ***d***= 82.11.

Male daily offspring production was maximised in a near identical region of the nutritional landscape as LS, at a P:C ratio of 1:15.92 (Figures 3.1 A & C and 2 C) and, therefore, the nutritional landscapes look qualitatively similar (Figure 3.1 A & C). Conversely, female daily egg production was maximised at a P:C ratio of 1:1.21 (Figure 3.2 D) meaning that the daily rate of reproduction differed between the sexes (Tables 3.1 & 3.2 and Figure 3.1 C & D). This was due to the linear effects of P and non-linear effects of P and C (Table 3.2). These effects lead to optima that are located in different regions of the nutritional space, as evidenced by the large angle between the linear nutritional vectors ($\boldsymbol{\theta}$ = 90.02°, 95% CI: 72.81°, 107.45°) and relatively large Euclidean distance (***d***= 50.53, 95% CI: 49.79, 51.60). I find similar effects of P and C on lifetime reproductive effort in both sexes. Male lifetime offspring production peaks in the same region as daily reproductive effort, 1:15.92 (Figure 3.2 E), and female lifetime egg production maximised at a more C biased P:C ratio of 1:1.85 (Figure 3.2 F). Differences between the sexes were again due to the linear effects of P and non-linear effects of P

and C (Table 3.2), resulting in optima that fell in different regions of the nutritional space. This is another relatively large angle between the linear nutritional vectors for lifetime reproductive effort between the sexes (θ = 59.79°, 95% CI: 46.01°, 73.85°), all be it reduced from that of daily rate of reproduction. This is also the case for the Euclidean distance (***d***= 33.98, 95% CI: 33.09, 35.15), the reduction is likely due to female lifetime egg production being maximized at a higher P:C ratio, relative to daily egg production.

The differences in nutritional optima for LS and reproduction, between the sexes, results in differing degrees of trade-offs within the sexes. For males, the landscapes and P:C ratios suggest that there would be minimal trade-offs between LS and measures of daily and lifetime reproductive effort, with all three responses maximised on high nutrition, high P:C diets. However, the sequential model indicates that there are small differences in the nutritional optima for LS and daily reproductive effort, as a result of the linear and nonlinear effects of P and C (Table 3.2). I find that P has a greater negative effect on LS than daily reproductive effort and C has a greater positive effect on LS than daily reproductive effort, with similar responses seen in the quadratic effects of P and C for both responses. Whilst the sequential model highlights differences in the effects of P and C between these traits in males, the angle between the linear nutritional vectors and the Euclidean distance show that they fall on very similar regions of nutritional landscape (θ **=** 5.84°, 95% CI: 0.00°, 16.94° and ***d*** = 6.74, 95% CI: 2.81, 10.66). I found no evidence of trade-offs between LS and lifetime reproductive effort in males (Table 3.2) and again these were located on similar regions of the nutritional landscape (θ **=** 5.89°, 95% CI: 0.00°, 14.77° and ***d*** = 13.24, 95% CI: 10.37, 15.02). Finally, I discovered some evidence of a trade-off between daily and lifetime reproductive effort, in males, caused by the linear and nonlinear effects of P and C (Table 3.2), yet like the other male traits these fall in a similar region of the nutritional space, again evidenced by the angle and distance between the linear nutritional vectors (θ = 5.68, 95% CI: 0.00, 16.43 and ***d*** =15.05 CI: 12.58, 16.69).

Females show a much greater degree of trade-offs between lifespan and both measures of reproductive effort, as implied by the nutritional landscapes. This highlights that what is optimal for one trait may not be optimal for the other. I found significant metabolic trade-offs between LS and daily reproductive effort due to the linear and nonlinear effects of P but not C (Table 3.2), and nutritional optima that fell in very different regions of the nutritional landscape (θ= 81.97, 95% CI: 65.82°, 98.65° and ***d***= 44.08 95% CI: 43.31, 44.73). This is also the case for the metabolic trade-off between LS and lifetime reproductive effort, which was due to the linear and nonlinear effects of P (Table 3.2). The angle between the linear nutritional vectors is again large (θ= 49.81, 95% CI: 32.60°,

66.22°) but, like the Euclidean distance (***d***=32.97, 95% CI: 31.95, 33.84), is reduced relative to the difference in nutritional optima between LS and daily reproductive effort. Further, lifetime reproductive effort is maximized on higher P:C ratios compared to daily reproductive effort, and upon closer inspection of the sequential model it is clear that the linear and nonlinear effects of P (Table 3.2) were causing the apparent metabolic trade-off between these traits. The traits' optima do fall on slightly dissimilar regions of the nutritional space (Figure 3.1 D and F), which results in an angle between the linear nutritional vectors of $\boldsymbol{\theta}$= 32.10 (95% CI: 16.67°, 47.68°), and a Euclidean distance of ***d***= 21.32 (95% CI: 20.38, 22.74), that is smaller than that between LS and lifetime reproductive effort. As such, I found evidence of trade-offs between LS and reproduction in females, but less so for males, which is mediated by specific nutrient intake. This is particularly the case between female LS and daily reproductive effort where the optima lie on very different areas of the nutritional space. In summary, my results show that P and C intake have linear and nonlinear effects on LS, DRE and LRE in male and female *D. melanogaster.* I find no sex differences in P and C intake on LS but there were divergent nutritional effects on DRE and LRE between the sexes, as a consequence the trade-off between LS and DRE/LRE was greater in females than males.

3.5. Discussion:

There is a consensus throughout the literature that DR extends LS, with this finding consistent for a wide array of species (Nakagawa et al., 2012; Fontana & Partridge, 2015; Brooks & Garratt, 2016). The effect is also typically stronger in females than males, which is explained through females having larger energy expenditure in terms of reproduction (Barnes & Partridge, 2003; Bonduriansky et al., 2008). Whilst caloric restriction was thought to be the best explanation for the observed LS extension, there is now a growing body of work which suggests the effects of specific nutrients could be more important (Nakagawa et al., 2012; Brooks & Garratt, 2016). Here, I present work that opposes the caloric restriction hypothesis by showing that it is the effect of specific nutrients, namely protein and carbohydrates, rather than calories, that is responsible for the extension of LS in *D. melanogaster*. Evidently, my work supports the growing number of studies which show that the effects of specific nutrients, not caloric restriction, are responsible for extending LS, and mediating the trade-off between LS and reproduction (Lee et al., 2008; Bonduriansky et al., 2008; Fanson et al., 2009; Fanson & Taylor, 2012; Simpson & Raubenheimer, 2007; Simpson & Raubenheimer, 2009; Nakagawa et al., 2012; Bruce et al., 2013; Solon-Biet et al., 2014; Jensen et al., 2015; Brooks & Garratt, 2016).

My results show that intake of P and C greatly influenced the LS of both male and female *D. melanogaster*. A P:C ratio of 1:15.93 for males and 1:15.88 for females was shown to be best for LS extension across both sexes (Figure 3.1 A & B). I also found that LS decreased as you move down any given caloric rail, or rather diets with low nutritional content had the shortest LS for any given P:C ratio, excluding the most carbohydrate rich rails. Therefore, my findings are aligned with previous studies which found that it was the ratio of P relative to C that caused an extension of LS rather than calories *per se* (Lee et al., 2008; Fanson et al., 2009; Fanson & Taylor, 2012; Bruce et al., 2013; Jensen et al., 2015). My landscapes also appear qualitatively similar to work carried out on female *D. melanogaster* by Lee et al., (2008) and on male and female *D. melanogaster* by Jensen et al., (2015), which both found that LS was maximised on high intake of nutrients at a P:C ratio of 1:16, although these diets were delivered through a liquid capillary rather than a medium-based diet. Moreover, they are largely similar to nutritional surfaces from work carried out on other species including female Queensland fruit flies – *Bactrocera tryoni* (Fanson et al., 2009; Fanson & Taylor, 2012) and field crickets – *T. commodus* (Maklakov et al., 2008). Moreover, they are largely similar to nutritional surfaces from work carried out on other species including female Queensland fruit flies – *Bactrocera tryoni* (Fanson et al., 2009; Fanson & Taylor, 2012) and field crickets – *T. commodus* (Maklakov et al., 2008). However, the optimal P:C ratio for LS varies between species, with *T. commodus* LS being maximised at a P:C of around 1:8 (Maklakov et al., 2008) and *B. tryoni* LS being maximised at a P:C of 1:21 using yeast-based diets and 1:32 using holidic diets (Fanson et al., 2009; Fanson & Taylor, 2012).

Unlike LS, there were large differences in the effect of P and C intake on reproduction between male and female *D. melanogaster* (Fig. 3.1). My results show that male daily reproductive effort was maximised at a similar P:C ratio as LS, P:C=1:15.92 (Fig. 3.1 C), whereas female daily reproductive effort was maximised at a more P biased P:C ratio of 1:1.21 (Fig. 3.1 D). Females also need a more P biased diet, relative to LS, in order to maximise their lifetime reproductive effort, a P:C ratio of 1:1.85 (Fig. 3.1 F). My results show that male lifetime reproductive effort was also maximised at P:C ratio indistinguishable from LS and daily reproductive effort, P:C=1:15.92 (Fig. 3.1 E). The divergence between male and female nutritional demands for reproduction appears to be consistent across different studies (Maklakov et al., 2008; Jensen et al., 2015), and is attributed to the divergent reproductive strategies between the sexes. Males typically invest less into reproduction, as they have small cheap to produce microgametes, compared to females who have larger, more expensive macrogametes (Trivers, 1972). However, males often have a larger variance in reproductive success,

and thus are subject to higher intensities of sexual selection. Males compete with each other for access to mates using sexual displays and behaviours, the most elaborate of which has access to females (Bonduriansky et al., 2008). These traits, displays and behaviours are energetically demanding and require large amounts of C to fuel them (Maklakov et al., 2008). C provides the necessary easily digestible energy that males need to out compete one another in order to maximise the number offspring they can sire (Maklakov et al., 2008; Jensen et al., 2015). Conversely, there is typically less variance in female reproductive success, as females do not usually compete for matings, with their reproductive success being determined by the number of eggs they produce. Oogenesis plays a key role in female egg production, and oogenesis is a nutrient limited process (Wheeler, 1996). Further, P intake is known to stimulate oogenesis and regulate vitellogenesis, thus a high intake of P is required to fully realise female egg production, and female reproduction should be maximised at more P rich diets relative to males (Wheeler, 1996; Maklakov et al., 2008). My findings regarding female reproductive effort are consistent with other studies including work on this species (P:C= 1:2 (Lee et al., 2008; Jensen et al., 2015)), other species of fly (*B. tryoni* P:C= 1:2.3 (Fanson et al., 2009) P:C 1:1 (Fanson & Taylor, 2012)) and field crickets (*T. commodus* P:C= 1:1 (Maklakov et al., 2008)). My results for both measures of male reproductive effort were consistent with Jensen et al., (2015) with both daily and lifetime reproductive effort being maximised at P:C = 1:16 in their study. The similarity between the results of my study and Jensen et al. (2015), for both LS and reproduction, would suggest that any differences in the actual measures of these traits between studies would be due to the type of diet.

The trade-off between LS and reproduction, caused by these traits competing for limited resources, has been one of the most studied in life-history theory in part due to its direct tie to fitness (Williams, 1966; Barnes & Partridge, 2003; Partridge et al., 2005; Lee et al., 2008; Nakagawa et al., 2012; Jensen et al., 2015). My research is the latest in a recent trend which implicates the intake of specific nutrients, most commonly P and C, in mediating the trade-off between LS and reproduction (Lee et al., 2008; Maklakov et al., 2008; Fanson et al., 2009; Fanson & Taylor, 2012; Solon-Biet et al., 2014; Jensen et al., 2015). My findings are also consistent with the long-standing idea that trade-offs are greater for females, relative to males, due to divergent reproductive strategies between the sexes (Bonduriansky et al., 2008). I find little evidence of trade-offs for males between LS and either reproductive measure, as the nutritional optima were located at nearly identical regions of nutritional space (Fig. 3.1 A,C & E). The limited evidence for trade-offs between male LS and daily reproductive effort is due to the magnitude of the effect each macronutrient has on LS (Table 3.2), yet when I formally compared the landscapes I found little variance in the location of the nutritional

optima. However, I found that for female *D. melanogaster* the LS and reproductive measures were maximised in very different areas of the nutritional landscape (Fig. 3.1 B, D & F). Female LS is maximised on C biased diets, with the optima being at P:C= 1:15.88, whereas daily reproductive effort was maximised at P:C= 1:1.21 with deviations from this point to either more C or P rich diets resulting in a reduced rate of egg production. Interestingly, female lifetime reproductive effort was maximised at P:C= 1:1.85 with a move towards a more C biased diet resulting in a smaller reduction in egg production, compared to a move towards more P biased diets. This could in part be due to the greatly increased LS of the flies on the more C biased diets allowing for more reproductive events. Nonetheless, the pattern I see overall in females, is that what is optimal for LS is sub-optimal for reproduction, and vice versa, a finding consistent with the Y-model of trade-offs (Noordwojk & de Jong, 1986). The premise of this model states that increasing reproductive output diverts resources away from somatic maintenance and subsequently has a negative effect on LS.

Although, an important point to note is that the differences between the nutritional landscapes of females could be due to the level P intake and the effects of this. The Lethal Protein hypothesis, suggests that the direct costs of P over ingestion could mediate the trade-off between LS and reproduction (Simpson & Raubenheimer, 2009; Fanson et al., 2012; Jensen et al., 2015). Clearly, protein is deleterious to LS in both sexes yet females needed a higher intake of P to maximise their egg production, resulting in a trade-off between these traits. Studies have shown that excess ingestion of P can cause an increase in mitochondrial production of reactive oxygen species (ROS) (Gredilla et al., 2004; Sanz et al., 2004; Ayala et al., 2007). ROS cause oxidative damage to nuclear and mitochondrial DNA, which has a negative effect on somatic maintenance and thus LS (Sanz et al., 2004). My study, is added to the list of previous work supporting the Lethal P hypothesis, and calls for further investigation to directly test for and measure the effects of P (Fanson & Taylor, 2012; Jensen et al., 2015).

Although there has been a recent trend towards holidic mediums when studying nutrition in flies (Piper et al., 2014; Bonduriansky et al., 2016; Hawley et al., 2016), there is yet to be a study to formally compare whether there is a benefit to this type of diet compared to the more frequently used CAFÉ assays. Therefore, perhaps the most interesting aspect of my study comes from its comparison with earlier work carried out by Jensen et al., (2015). My work and that of Jensen et al. (2015), were carried out in a near identical manner, the main exception being the type of diet. In this study, I used holidic medium diets, an agar based substrate which flies could spend their entire lifecycle on and a more accurate representation of natural feeding conditions, compared to the

liquid capillary CAFÉ assay used by Jensen et al. When informally comparing my results, it would appear that LS was greater on the holidic medium diets compared to the CAFÉ assay diets, with this being the case for a range of rails varying in P:C ratio. For example, if I consider the 'worst' diet for LS in both studies (the highest P:C ratio and lowest nutrition) I find that the average LS for females on holidic medium diets was 11.1 ± 1.10 days, compared to 6.06 ± 0.25 days on the CAFÉ assay diets (Jensen et al., 2015). The same is true for males on the 'worst' diet where my males lived on average 8.1 ± 0.53 days, compared to 6.19 ± 0.21 days. However, perhaps the most pronounced difference is found when comparing C rich diets where flies lived longest. Female *D. melanogaster* fed using a holidic medium had an average LS of 36.8 ± 2.65 when consuming the best diet for LS on the 1:16 rail, compared to an average LS of 21.76 ± 1.37 days for flies on the CAFÉ assay diets. This represents an increase of 69.12% for female LS. Again, a similar trend is found when comparing male LS, where flies on the holidic medium diets had an average LS of 33.85 ± 2.47 days and those on the CAFÉ assay had an average LS of 26.13 ± 1.17, an increase of 29.54% for male LS. This suggests that flies fed a holidic medium diet are longer lived than those fed using capillary feeders.

The comparison between the types of diet and their effect on reproduction is not as definitive. Female egg production rate would appear to be maximised on holidic medium diets, with females on the best diet for this trait producing an average of 5.5 ± 0.66 eggs a day, in comparison to Jensen et al.'s (2015) best CAFÉ assay diet on which females produced 1.24 ± 0.23 eggs per day. However, when considering male offspring production rate, I find that males on the best holidic medium diets sired an average of 2.23 ± 0.35 offspring per day this is fewer than males on CAFE assay diets which sired 8.23 ± 0.66 offspring per day on average. In both experiments males were mated in competition with a marker male and female, the female was then put into in a separate vial containing Jazz mix. There are a number of possibilities that could account for males on holidic medium diets producing fewer offspring, including variance in the quality of Krüppel females between studies. However, another possibility is that the increase in male LS came at the cost of their reproductive effort. Whilst it is commonly believed that LS extending effects are greater in females due to greater costs of reproduction (Nakagawa et al., 2012), recent work has shown that sperm production is more costly than one would expect (Bunning et al., 2015). Thus, it is possible that males on the most C rich diets are living longer but as a consequence of a lower reproductive output and reduced sperm production. Alternatively, an aged male *D. melanogaster* may not be able to compete with a younger competitor male for access to mates (competitor males were 5 days old in my study), yet live long enough to reduce my measure of rate of reproduction.

Thus perhaps the key finding of my work, is that despite the obvious differences in diet-type between my diets and those of Jensen et al. (2015), and the clear difference in their effect on LS and reproduction, the way P and C influence LS and reproduction is very similar. Further, the effect of P and C can be clearly seen even when intake is not measured. Whilst some have previously suggested that capillary feeders substantially reduce LS and egg laying, compared to flies fed on a standard agar-gelled medium, the most common argument for their continued use is that they can accurately measure intake and this is necessary to understand the effects of P and C on a given trait (Lee et al., 2008; Wong et al., 2009; Deshpande et al., 2014; Tennessen et al., 2014). However, here I have shown that this may not be the case and that in fact, a medium-based diet shows similar effects of P and C but also allows for greater trait expression, i.e. longer lived flies with greater reproductive effort. This, of course, can only be said of comparisons between holidic diets, where other dietary factors have been minimalized. Clearly, research is needed to determine if diet-type has an effect on trait expression, and what causes any effect seen. Perhaps it could simply be due to the difficulties a fly faces when feeding using a capillary where a fly has to feed upside down and at an angle it is not accustom to – *D. melanogaster* typically feed on microorganisms, on the surface of fruit (Werger, 1977; Kimura et al., 1977; Wong et al., 2009). Nonetheless, these results add weight to the argument that capillary feeders, whilst accurate, are a sub-optimal method of diet for studying nutrition in flies (Piper et al., 2011; Jensen et al., 2015; Wong et al., 2009; Deshpande et al., 2014; Tennessen et al., 2014). In addition, capillary feeders may have affected the expression of other traits measured in studies using the GF. In studies where nutrition has a clear effect on a given trait this may not present a problem, however in studies where the effect was small or perhaps close to significant, the type of diet could influence these results. As such, I feel this warrants further investigation into the type of diets, not only in *D. melanogaster*, but also other commonly used model organisms in nutritional research such as crickets, cockroaches and nematodes.

In conclusion, my work adds to the growing body of evidence that challenges the notion that caloric restriction is the mediator in the trade-off between LS and reproduction, by showing that it is the intake of P and C that actually mediates this trade-off in *D. melanogaster.* I show that there are sex-specific effects of P and C intake on reproduction but not LS and as a result there are differing levels of trade-offs between males and females. My landscapes were similar to other studies which have used the GF with CAFÉ assay capillary feeders to investigate LS and reproduction in this species, yet I saw an increase in average LS for both sexes and an increase in female daily egg production but not male daily offspring production. Thus, this work could provide a platform for research into, if a diet type effect is significant and if so why it occurs. What is apparent though is that the effects of P and

C on LS and reproduction remain remarkably consistent regardless of diet-type (liquid- or medium-based diet), and whether or not intake has been measured. Studies like this work to unravel the complex effects of nutrition on health and longevity and as such I, would encourage other researchers to continue to strive for as accurate results as possible.

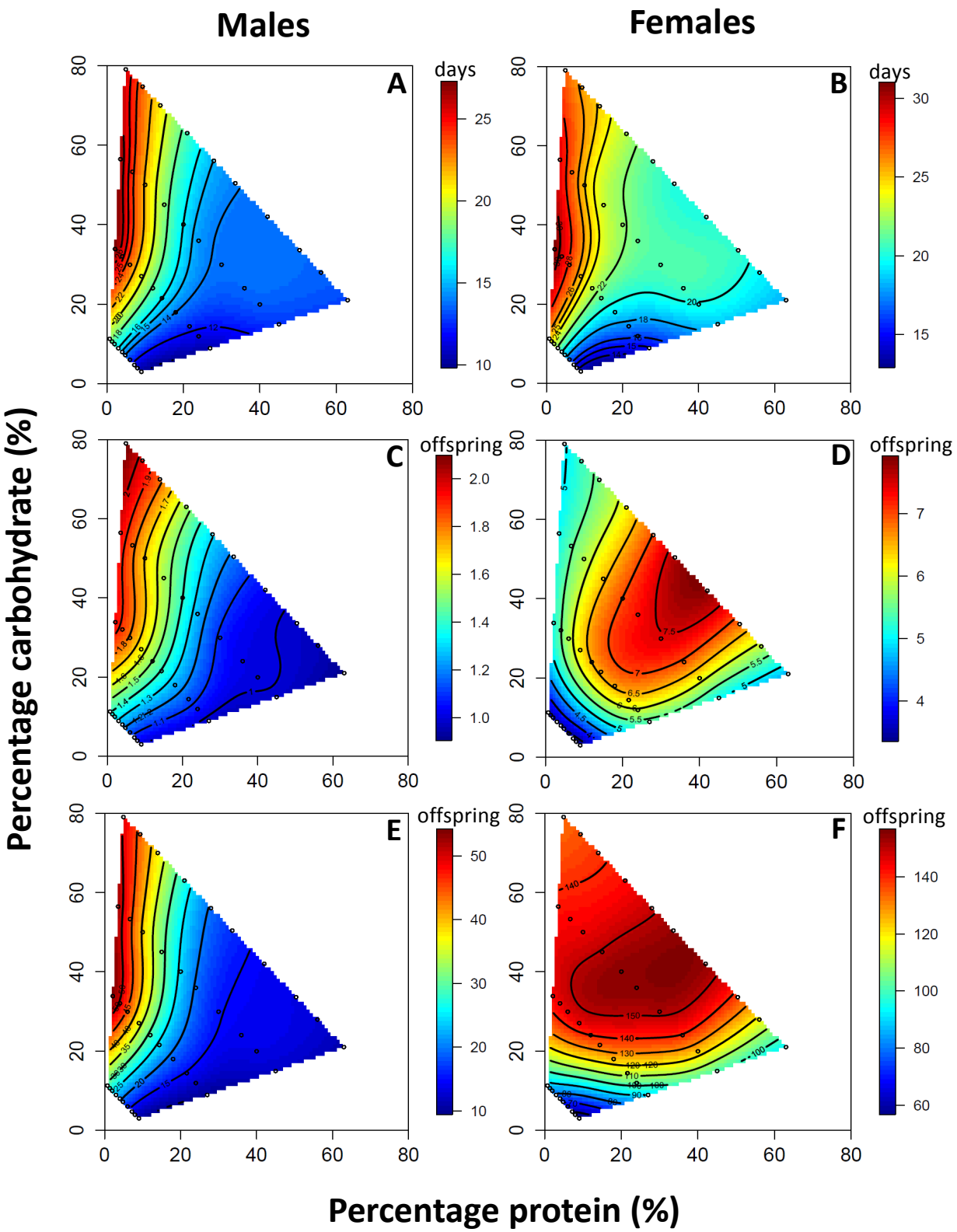

FIGURE 3.1. Nonparametric thin-plate spline contour visualizations of the responses surfaces describing the effects of protein and carbohydrate intake on (A) male lifespan (LS), (B) female LS, (C) male offspring production rate, (D) female egg production rate, (E) lifetime offspring production in males, and (F) lifetime egg production in females in *Drosophila melanogaster*.

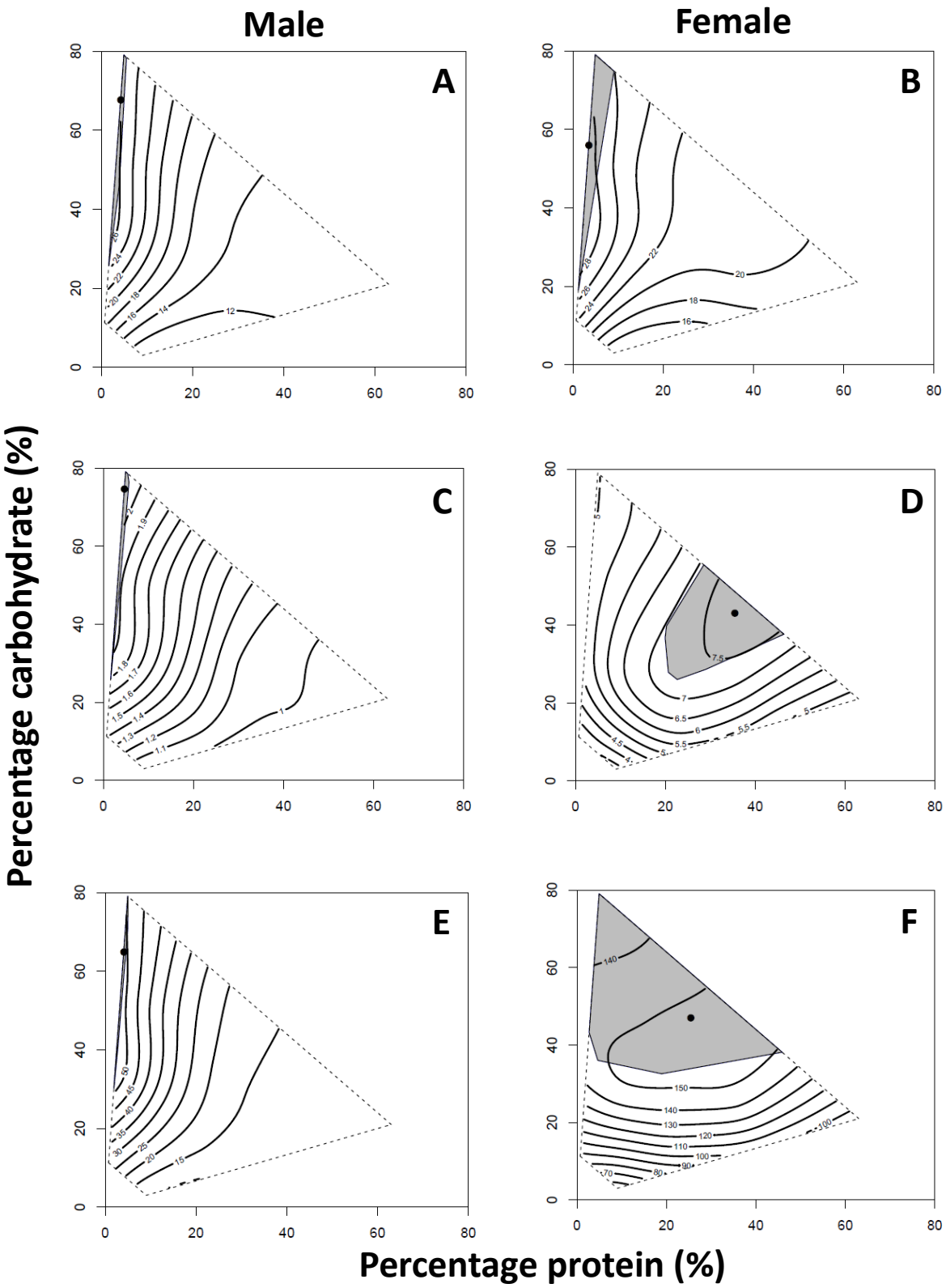

FIGURE 3.2. Confidence regions, with nutritional optima given as X,Y coordinates: (A) male LS = 4.25,67.70, (B) female LS = 3.53, (C) male DRE = 4.68,74.58, (D) female DRE = 35.43, 43.06, (E) male LRE = 4.08,64.96, 56.07, (F) female LRE = 25.43,47.10

TABLE 3.1. The linear, quadratic and correlational effects of protein (P) and carbohydrates (C) on lifespan (LS), daily reproductive effort (DRE) and lifetime reproductive effort (LRE) for male and female *Drosophila melanogaster*

	Linear effects			**Nonlinear effects**		
Response variables	P	C		**P x P**	**C x C**	**P x C**
(A) Males						
LS						
Coefficient ± SE	-0.31 ± 0.03	0.38 ± 0.03		0.27 ± 0.03	-0.31 ± 0.03	-0.32 ± 0.05
t_{799}	9.94	12.14		9.99	10.20	7.00
P	0.0001	0.0001		0.0001	0.0001	0.0001
DRE						
Coefficient ± SE	-0.19 ± 0.03	0.21 ± 0.03		0.08 ± 0.03	-0.10 ± 0.04	-0.17 ± 0.06
t_{799}	5.57	6.02		2.31	2.80	2.99
P	0.0001	0.0001		0.021	0.005	0.003
LRE						
Coefficient ± SE	-0.30 ± 0.03	0.30 ± 0.03		0.20 ± 0.03	-0.25 ± 0.03	-0.30 ± 0.05
t_{799}	9.34	9.38		6.68	7.62	5.94
P	0.0001	0.0001		0.0001	0.0001	0.0001
(B) Females						
LS						
Coefficient ± SE	-0.17 ± 0.03	0.25 ± 0.03		0.18 ± 0.03	-0.26 ± 0.04	-0.12 ± 0.05
t_{799}	5.13	7.39		5.55	7.45	2.15
P	0.0001	0.0001		0.0001	0.0001	0.032
DRE						
Coefficient ± SE	0.26 ± 0.03	0.24 ± 0.03		-0.26 ± 0.03	-0.29 ± 0.03	0.01 ± 0.05
t_{799}	8.00	7.40		8.65	8.76	0.18
P	0.0001	0.0001		0.0001	0.0001	0.86
LRE						
Coefficient ± SE	0.09 ± 0.03	0.33 ± 0.03		-0.09 ± 0.03	-0.35 ± 0.03	-0.07 ± 0.05
t_{799}	2.63	9.81		2.80	10.20	1.32
P	0.009	0.0001		0.005	0.0001	0.19

TABLE 3.2. Sequential model building analysis that contrasts the linear and nonlinear effects of protein (P) and carbohydrate (C) on lifespan (LS), daily reproductive effort (DRE) and lifetime reproductive effort (LRE), both between the sexes, and between traits within the sexes.

	SS_R	SS_C	DF_1	DF_2	F	P	ϑ	95% CI
Males vs. Females								
LS								
Linear	1356.44	1343.05	2	1594	7.95	0.0004[A]	5.91°	0.00°, 16.64°
Quadratic	1169.55	1164.35	2	1590	3.55	0.03[B]		
Correlational	1137.26	1131.30	1	1588	8.37	0.004		
DRE								
Linear	1514.20	1430.81	2	1594	46.45	0.0001[C]	90.02°	72.81°, 107.45°
Quadratic	1366.31	1295.16	2	1590	43.67	0.0001[D]		
Correlational	1291.52	1287.03	1	1588	5.54	0.019		
LRE								
Linear	1425.72	1365.03	2	1594	35.44	0.0001[E]	59.79°	46.01°, 73.85°
Quadratic	1254.97	1207.66	2	1590	31.14	0.0001[F]		
Correlational	1188.58	1181.13	1	1588	10.02	0.002		
Male								
LS vs. DRE								
Linear	1371.22	1354.25	2	1594	9.99	0.0004[G]	5.84°	0.00°, 16.94°
Quadratic	1256.99	1230.12	2	1590	17.37	0.0001[H]		
Correlational	1196.06	1192.78	1	1588	4.36	0.037		
LS vs. LRE								
Linear	1276.97	1274.67	2	1594	1.43	0.24	5.89°	0.00°, 14.77°
Quadratic	1097.12	1094.11	2	1590	2.19	0.11		
Correlational	1039.76	1039.68	1	1588	0.12	0.74		
DRE vs. LRE								
Linear	1406.37	1398.04	2	1594	4.75	0.009[I]	5.68°	0.00°, 16.43°
Quadratic	1331.72	1319.71	2	1590	7.23	0.0007[J]		
Correlational	1288.77	1286.41	1	1588	2.91	0.09		
Female								
LS vs. DRE								
Linear	1495.95	1419.61	2	1594	42.85	0.0001[K]	81.97°	65.82°, 98.65°
Quadratic	1314.35	1229.38	2	1590	57.61	0.0001[L]		
Correlational	1227.76	1225.54	1	1588	2.87	0.09		
LS vs. LRE								
Linear	1463.56	1433.41	2	1594	16.76	0.0001[M]	49.81°	32.60°, 66.22°
Quadratic	1312.80	1277.90	2	1590	21.71	0.0001[N]		
Correlational	1273.06	1272.75	1	1588	0.40	0.53		
DRE vs. LRE								
Linear	1412.56	1397.80	2	1594	8.42	0.0002[O]	32.10°	16.67°, 47.68 °
Quadratic	1196.47	1183.11	2	1590	8.98	0.0001[P]		
Correlational	1182.60	1181.74	1	1588	1.15	0.28		

Univariate test: [A] P: $F_{1,1594} = 8.73$, $P = 0.003$, C: $F_{1,1594} = 7.74$, $P = 0.005$; [B] P x P: $F_{1,1590} = 7.08$, $P = 0.008$, C x C: $F_{1,1590} = 0.20$, $P = 0.66$; [C] P: $F_{1,1594} = 91.53$, $P = 0.0001$, C: $F_{1,1594} = 0.67$, $P = 0.41$; [D] P x P: $F_{1,1590} = 62.34$, $P = 0.0001$, C x C: $F_{1,1590} = 17.21$, $P = 0.0001$; [E] P: $F_{1,1594} = 70.16$, $P = 0.0001$, C: $F_{1,1594} = 0.29$, $P = 0.59$; [F] P x P: $F_{1,1590} = 50.91$, $P = 0.0001$, C x C: $F_{1,1590} = 6.80$, $P = 0.009$; [G] P: $F_{1,1594} = 6.68$, $P = 0.01$, C: $F_{1,1594} = 13.98$, $P = 0.0001$; [H] P x P: $F_{1,1590} = 23.02$, $P = 0.0001$, C x C: $F_{1,1590} = 15.23$, $P = 0.0001$; [I] P: $F_{1,1594} = 5.57$, $P = 0.018$, C: $F_{1,1594} = 4.26$, $P = 0.039$; [J] P x P: $F_{1,1590} = 8.77$, $P = 0.003$, C x C: $F_{1,1590} = 7.21$, $P = 0.007$; [K] P: $F_{1,1594} = 85.67$, $P = 0.0001$, C: $F_{1,1594} = 0.02$, $P = 0.90$; [L] P x P: $F_{1,1590} = 106.38$, $P = 0.0001$, C x C: $F_{1,1590} = 0.61$, $P = 0.43$; [M] P: $F_{1,1594} = 30.19$, $P = 0.0001$, C: $F_{1,1594} = 2.63$, $P = 0.11$; [N] P x P: $F_{1,1590} = 37.14$, $P = 0.0001$, C x C: $F_{1,1590} = 3.44$, $P = 0.06$; [O] P: $F_{1,1594} = 14.16$, $P = 0.0001$, C: $F_{1,1594} = 3.13$, $P = 0.077$; [P] P x P: $F_{1,1590} = 17.47$, $P = 0.0001$, C x C: $F_{1,1590} = 1.28$, $P = 0.26$.

CHAPTER 4: GENERAL DISCUSSION

There is an ever growing body of work that has investigated the relationship between diet and life-history traits, and any trade-offs that may exist between these traits (McCay et al., 1939; Noordwojk & de Jong, 1986; Chapman & Partridge, 1996; Stearns, 1989; Zera & Harshman, 2001; Roff, 2002; Nakagawa et al., 2012). Historically, empirical research has offered us a limited insight into the role of specific nutrients and the type of diet on life-history traits. This is due, in part, to the simplistic dietary manipulation used in these studies, where diet was categorised as 'good vs bad' or 'restricted vs ad libitum'. This leaves an element of uncertainty in the conclusions that can be drawn from this work about the effects of diet on life-history traits (McCay et al., 1935; Hunt et al., 2004; Piper et al., 2005; Piper et al., 2011). For example, is it the total intake of nutrients (or calories) or the balanced intake of specific nutrients that is responsible for any observed patterns?

However more recently, studies have begun to use more complex methods in order to gain deeper insights into the complex interaction between diet and life-history traits (Maklakov et al., 2008; Simpson & Raubenheimer, 2007; Simpson & Raubenheimer, 2012). This includes moving away from diet being thought of as a one-dimensional entity, as research has started to implicate the intake of specific nutrients as an important driver in life-history trait expression and in mediating trade-offs between traits (Lee et al., 2008; Maklakov et al., 2008; Fanson et al., 2009; South et al., 2011; Fanson & Taylor, 2012; Solon-Biet et al., 2014; Bunning et al., 2015; Jensen et al., 2015). Indeed, my research shows that the intake of two macronutrients, protein (P) and carbohydrates (C), play a pivotal role in the expression of important life-history traits, which are closely linked to an individual's fitness. In fact this thesis adds to a growing body of work that has investigated dietary effects on a whole host of different life-history traits (Lee et al., 2008; Fanson et al., 2009; Fanson & Taylor, 2012; Jensen et al., 2015; Archer et al., 2015; Bunning et al., 2015; Rapkin et al., 2017). Moreover, my work begins to question whether diet-type impacts lifespan and reproduction, in addition to the effects that dietary composition has on these traits. In Chapter 3, I used a novel medium-based diet to investigate the effects of P and C on LS and reproduction in *D. melanogaster* and compared my results to those of a similar study that used liquid-based diets (Jensen et al., 2015). This allowed for possible diet-type effects to be explored, as it has been suggested that liquid based diets negatively impact on *D. melanogaster* trait expression (Piper et al., 2011; Jensen et al., 2015; Wong et al., 2009; Deshpande et al., 2014; Tennessen et al., 2014), and therefore any conclusions drawn from studies that use liquid-based diets could possibly be confounded. However, I found that whilst medium-based diets lead to greater trait expression the effects of P and C remained consistent across studies.

When considering life-history traits, one cannot overlook trade-offs – a negative covariance between phenotypic traits – as they are considered a core tenant of life-history theory (Roff, 2002; Roff & Fairbairn, 2007). The incidence and magnitude of trade-offs is determined by the amount of resources an individual can acquire and the allocation of the resources to different, competing traits, i.e. when the number of resources acquired is low, there is strong likelihood of large trade-offs between traits (Noordwojk & de Jong, 1986; Stearns, 1989; Stearns, 1992; Roff, 2002). As stated above, when studying nutrition and life-history traits simple diet manipulations have had widespread use, the same is true of nutrition and life-history trade-offs (Stearns, 1989; Roff, 2002; Roff & Fairbairn, 2007). Therefore, I find a pattern in the literature where the nutrient composition of the diets is poorly defined and the caloric intake of an individual is difficult to measure accurately (Simpson & Raubenheimer, 2012). This results in a near impossible task of relating the acquisition and allocation of key nutrients to life-history traits, and thus impedes our understanding of how and whether these traits are subject to a trade-off.

A tool which has been used to provide greater clarity in our understanding of the effect key nutrients have on life-history traits, and trade-offs between them, is the Geometric Framework (GF). The GF is an innovative method for accurately quantifying the intake of specific nutrients and their effect on the expression of life-history traits and how they could be influenced by nutritionally based trade-offs (Simpson et al., 2004; Simpson & Raubenheimer, 2007; Simpson & Raubenheimer, 2012). This method has been used to show nutrients play an important role in regulating both life-history trait expression and trade-offs between these traits (Lee et al., 2008; Maklakov et al., 2008; Fanson & Taylor, 2012; Jensen et al., 2015). In addition, those studies which have compared the level of trade-offs between the sexes, for lifespan and reproduction, have shown that the trade-offs are typically more pronounced in females compared to males (Maklakov et al., 2008; Jensen et al., 2015). This is thought to be due to divergent reproductive strategies between the sexes, which leads to differences in the levels of sexual selection (Trivers, 1972). This then drives the evolution of sex differences in life-history strategies, that leads to sex-specific nutritional demands (Andersson, 1994; Andersson & Simmons, 2006; Hosken & House, 2011; Simpson & Raubenheimer, 2012). In fact my work in Chapter 3, adds to a number of studies which has shown sex-specific nutritional optima for reproductive effort (Maklakov et al., 2008; Jensen et al., 2015; Rapkin et al., 2017). Further work has shown sex-specific nutritional optima for other key life-history traits including lifespan (Maklakov et al., 2008) and immune function (Rapkin et al., 2017).

My thesis brings together some of the core evolutionary field (nutritional ecology, sexual selection and life-history theory) in an effort to highlight the multidimensional complexity of nutrition and the potential evolutionary consequences nutrition may have. Yet, there are still many possible lines of research one could undertake in order to expand on this work, the most apparent of which is the expansion of the GF used in this thesis. My research, and other studies like it, have utilised only two of the three macronutrients, P and C, which means any effects that the third major macronutrient, lipid (L), may have are unknown. Undoubtedly an individual's dietary intake in both the wild and many laboratory settings would not be limited to only P and C, and would also include L to some degree. Thus, the next logical expansion of the GF would be to include L in the nutritional space, especially in conjunction with P as this is relevant to predatory animals (Simpson & Raubenheimer, 2012). Indeed, P and L combinations have already started being used in a geometric studies to great effect, to investigate the optimal intake of these nutrients for the predatory ground beetle (Jensen et al., 2012) and mink (Mayntz et al., 2009). The inclusion of L in the GF would allow for a more accurate representation of an individual's dietary preference, and subsequently provide novel insights into the effect of an additional macronutrient on life-history traits. Currently, dietary manipulations are limited to the comparison of two macronutrients at a time. The introduction of L into fly diets represents a unique issue, as the source of L tends to disperse poorly into any given dietary solution (Piper et al., 2014). Therefore, a pure source of L, which avoids this issue, would need to be found in order for L to successfully incorporated into fly diets. More broadly, future development of the GF should centre around the design and production of diets that are comprised of all three macronutrients, which also includes corresponding analyses to allow for detailed examination of any interactions between these macronutrients.

The use of standard selection analyses (Lande & Arnold, 1983) should allow for easy expansion of the current analyses to include all three macronutrients. Visualisation of the effect of all three macronutrients has on a trait poses somewhat of a problem, as currently a 'right-angled mixture triangle' is the preferred method, suggested by Raubenheimer (2011), but this does have a number of drawbacks. The biggest of these drawbacks is that it is difficult to visualise the effects that nutrient concentration has on phenotype expression. This is because mixture triangles use nutrient proportions, rather than intake values, to visualise data, which means that mixture triangles must use cross sections to compare the effects of nutrient concentration. Thus, mixture triangles lack the detail that current two nutrient analysis can provide and can only offer an overall idea of the effect the three nutrients have on traits. However, mixture triangles currently represent the best means of comparison between field and lab studies in nutritional ecology (Raubenheimer, 2011). Presently it

is difficult to compare the results of controlled laboratory experiments to an animal feeding naturally in the wild and this is often cited as one of the biggest criticisms of the GF. As highlighted throughout this thesis, the GF allows for accurate and precise measures of dietary intake for a given individual in a laboratory setting, but to expect the same measures from field studies would be unreasonable. Field studies typically rely on measures of the proportion of nutrients utilised calculated through the differences between nutrient concentration of the food eaten and the faeces (Crossman et al., 2005; Raubenheimer et al., 2009; Raubenheimer, 2011; Coogan et al., 2014). As field studies use nutrient proportions, mixture triangles represent the best method of visualising these results and in turn provide the easiest means of comparisons to laboratory studies. Whilst I see some use of mixture triangles in the literature (Lehmann et al., 2013; Coogan et al., 2014), an increase in prevalence would aid in future developments to mixture triangle use in an effort to increase our understanding of nutrient utilisation in both field and laboratory settings.

Another way that the GF can be expanded is to include the manipulation of micronutrients. In fact, recent research has shown that the essential amino acid methionine has an effect on lifespan in *D. melanogaster* and some rodent species (Miller et al., 2005; Zid et al., 2009) and also increase aged female egg production in *Drosophila* (Grandison et al., 2010). In contrast, work by Archer et al. (2015) used powerful response surface methodologies and found that DL-Alpha-Tocopherol (Vitamin E) did not increase lifespan or reproduction in the black field cricket *T. commodus*. Another promising avenue of study would be investigating the effects of macro- and micronutrients on the underlying mechanisms that regulate life-history traits and trade-offs, with a number of mechanisms already shown to play a role in the trade-off between lifespan and reproduction (Flatt & Heyland, 2011). However, research that uses the GF to investigate dietary effects on underlying mechanisms is limited. To date only one study has used this approach and it found limited dietary effect on oxidative damage (Archer et al., 2015). Finally, as emphasized in Chapter 3 of this thesis the type of diet that researchers feed an organism is important. This can also include whether the diet is holidic or not, as illustrated through two different studies which looked at lifespan in the Queensland fruit fly and found that the optimal P:C ratio for lifespan varied depending on whether the diet was yeast based (P:C = 1:21 (Fanson et al., 2009)) or holidic (P:C=1:32 (Fanson & Taylor, 2012)). It would appear that the type of food researchers feed their model organisms also warrants further investigation in order to provide the best possible understanding of nutrients and their effect on traits and any underlying mechanisms.

To summarise, the work in this thesis begins to show the complexities of nutrition and emphasises the need for a multidimensional approach when examining the impact diet has on key life-history traits and in mediating trade-offs between these traits. The research community has made great progress but in reality, has only begun to scratch the surface of this field and there is a large amount of possibilities for future development, especially around realising the potential of GF to investigate macro- and micronutrients their effect on regulatory systems, life-history traits and trade-offs. The work I have carried out aids in our understanding of this complexity and I hope it facilitates further research into this area.

APPENDIX 1. EXEMPLAR R CODE FOR CALCULATING THE ANGLE (θ) BETWEEN NUTRITIONAL VECTORS AND CALCULATING 95% CONFIDENCE INTERVALS

```
library(MCMCglmm)
# read in selection data
data<-read.table("angledata.txt",h=T)
attach(data)
str(data)
prior<-list(R=list(V=1,nu=0.02))
# str(selection.data) should give a column for relative fitness
 (rel.fitness), and 6 columns of phenotypic measures, 3 female
# traits (f1, f2 and f3) and 3 male traits (m1, m2, and m3)
# Bayesian linear regression to estimate beta for each female trait,
 produces
# posterior distribution based on 15200 estimates of each parameter:
selection.model.1<-MCMCglmm(LSF~P+C-1,data=data,
 prior=prior,nitt=400000,burnin=20000,thin=25)

# and again for male traits:
selection.model.2<-MCMCglmm(LSM~P+C-
 1,data=data,prior=prior,nitt=400000,burnin=20000,thin=25)

#summary of models (check against response surface analysis)
summary(selection.model.1)
summary(selection.model.2)

angles<-numeric(15200)
# creates an empty vector the same length as the posterior distribution, in
 which angle estimates for each row of the posterior
# distribution will be stored as follows:
for(i in 1:15200){
 b.1<- selection.model.1$Sol[i,1:2]
 b.2<- selection.model.2$Sol[i,1:2]
 # creates a vector of beta estimates for each trait for each sex for each
 row of the posterior distribution (and the loop runs through all rows)
 angles[i]<- acos((t(b.1) %*% b.2) / ((sqrt(t(b.1) %*% b.1)) * (sqrt(t(b.2)
 %*% b.2)))) * (180/pi) }
 # calculates the angles between female beta and male beta for each row of
 the posterior distribution
summary(angles)
HPDinterval(as.mcmc(angles),0.95)
# etc. to examine angle estimates which are now stored in the vector called
'angles'
```

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