

Food for Thought: The Importance of Nutrition in Sexual Selection

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ABSTRACT

Sexual selection and sexual conflict are two fundamental evolutionary mechanisms that are responsible for the diversification of a range of morphological, behavioural and physiological traits in the sexes, across most animal taxa. Decades of empirical research has shown that the expression of many (if not all) of these traits is dependent on diet. Few studies have, however, provided a detailed view of how diet influences the operation of sexual selection and sexual conflict. The traditional view that nutritional resources are of a single form, namely energy or calories, has recently been challenged by the idea that it is the combination of various micro- and/or macronutrients that is key to trait expression and in maintaining reproductive fitness. While this established dogma is changing, more empirical studies are needed that focus on the how the intake of specific nutrients influence the expression of key traits that regulate the operation of sexual selection and sexual conflict.

In this thesis I examine the role of nutrition on the operation of sexual selection and sexual conflict. To achieve this I perform a number of experiments utilising the Geometric Framework (GF) of nutrition to tease apart specific effects of two macronutrients (protein and carbohydrates) on a number of important phenotypic traits in two field cricket species; the decorated cricket *Gryllodes sigillatus* and the Australian black field cricket *Teleogryllus commodus*. I also combine the GF with conventional quantitative genetic experiments to examine the potential for the genetics of dietary choice to constrain the evolution of key traits that regulate sexual selection and sexual conflict.

I start by examining the effect of protein (P) and carbohydrate (C) intake on a male sexual trait, the cuticular hydrocarbon (CHC) profile, known to be subject to strong pre-copulatory sexual selection in *G. sigillatus*. I find that diet influences the expression of male CHCs and how attractive a male is to a female. Specifically, I show that CHCs are maximized at a high intake of nutrients in a P:C ratio of 1:1.5, that female pre-copulatory choice exerts significant selection on this variation in male CHCs and that the nutritional optima for male mating success almost perfectly matched the optima for CHC expression. However, this change in CHC expression was not the only pathway for the effects of nutrient intake on male pre-copulatory attractiveness to females suggesting that other trait(s) are also important in mediating this relationship (Chapter 2). Next, I examine the effect of the intake

of these nutrients on the regulation of sexual conflict in *G. sigillatus*. Males in this species produce a large, gelatinous nuptial gift (the spermatophylax) that the female consumes during mating and that prevents her from prematurely removing the sperm-containing ampulla and terminating mating. The size and amino acid composition of the spermatophylax are known to prolong the attachment time of the ampulla and, therefore, prevent the female from exerting post-copulatory mate choice. I show that the size and amino acid composition that increases the gustatory appeal of the spermatophylax to females is maximised at a high intake of nutrients in a P:C ratio of 1:1.3 (Chapter 3). Furthermore, I show that the nutritional optima for these properties of the spermatophylax are almost perfectly aligned with the optima for ampulla attachment time. This suggests that the balanced intake of P and C is fundamental to the regulation of sexual conflict in this species.

A key assumption in life-history theory is that phenotypic traits important to fitness will be subject to trade-offs as they compete for a limited pool of resources. In most empirical studies, the nutrients in food are considered the resource that life-history traits compete for during development, yet the diets provided are typically poorly resolved so that the specific nutrients regulating any trade-off cannot be determined. While the GF provides a powerful way to examine how specific nutrients influence the trade-off between traits, this framework currently lacks a robust protocol to quantify the presence and magnitude of nutritionally based trade-offs. In Chapter 4, I start by developing a standardized protocol for quantifying the presence and magnitude of nutritionally based trade-offs when using the GF. This work shows that nutritionally based trade-offs occur when life-history traits are maximised in different regions of nutrient space and that this divergence can be quantified by the overlap in the 95% confidence region (CR) of the global maxima, the angle (θ) between the linear nutritional vectors and the Euclidean distance (d) between the global maxima for each trait. As these metrics are measured in a standardized way, they can be directly compared across different traits, the sexes and model organisms. Next, I test this protocol by examining the nutritional basis of the trade-off between reproductive effort and immune function in male and female *G. sigillatus*. I show that encapsulation ability and egg production in females increased with the intake of both nutrients, being maximised at a P:C ratio of 1.04:1 and 1:1.17, respectively. In contrast, encapsulation ability in males only increased with the intake of P being maximised at a P:C

ratio of 5.14:1, whereas calling effort increased with the intake of C but decreased with the intake of P and was maximized at a P:C ratio of 1:7.08. Consequently, the trade-off between reproduction and encapsulation ability is much larger in males than females and this is supported by the non-overlapping 95% CRs on the global maxima for these traits in males and the larger estimates of θ and d .

Sexual selection promotes the evolution of sex differences in life history strategies and this often requires different intakes of nutrients. Indeed, the sexes in many different species have evolved divergent nutritional optima for a range of important fitness-related traits. If dietary choice for the intake of these nutrients is genetically uncoupled in the sexes, males and females should evolve sex differences in nutrient intake and each sex should evolve to their sex-specific nutritional optima. However, if the sexes have different nutritional optima but dietary choice is positively genetically correlated between the sexes, this will constrain the evolution of sexual dimorphism in nutrient intake and prevent one or both sexes from reaching their nutritional optima: a process known as intralocus sexual conflict (ISC). In Chapter 5, I examine the potential for ISC over the optimal intake of nutrients for reproduction and lifespan in male and female black field crickets, *T. commodus*. I show that males and females have distinct dietary optima for lifespan and reproductive effort. Male lifespan and nightly calling effort were both maximised at a high intake of nutrients in a P:C ratio of 1:8, whereas female lifespan and daily egg production were maximised at a high intake of nutrients in a P:C ratio of 1:2 and 1:1, respectively. Using a half-sib quantitative genetic breeding design I also showed positive genetic correlations between the intake of P and C in the sexes. Together this provides the potential for ISC over the optimal intake of nutrients to influence the evolution of sexual dimorphism in reproductive effort and lifespan. However, by measuring the genetic constraint (which compares the predicted evolutionary response of these traits when there is genetic covariance between the sexes for nutrient intake, to the predicted response when the genetic covariance is set to zero (i.e. no genetic constraint)), I show that the positive genetic correlations over nutrient intake had little effect on the predicted response of nutrient regulation in the sexes. Furthermore the within sex, additive genetic variance-covariance matrix appeared to play more of a role in constraining the predicted response of nutrient regulation in the sexes.

When presented with a nutritionally imbalanced diet, animals often show a range of compensatory feeding behaviours to increase their intake of any nutrient(s) that are deficient. However, such behaviours may come at a cost, as the more common nutrients are over-consumed and may impact homeostatic functioning and health. Due to the different nutritional demands of reproduction in the sexes, males and females often show different compensatory feeding behaviours that target different nutrients and this has been shown to have differential health consequences in the sexes. Little is known, however, about the role that genes play in this process: are the sexes genetically predisposed to under or over-ingest nutrients when encountering a nutritionally imbalanced diet and is any genetic predisposition for dietary regulation linked to genes for health in the sexes? In my final chapter (Chapter 6), I examine the quantitative genetics of dietary choice in male and female *T. commodus* and the potential health consequences this may have in terms of lipid deposition. Using a split-brood breeding design, where different full-sibling offspring of each sex were provided with alternate pairs of imbalanced diets, I showed significant genotype-by-sex and genotype-by-diet pair interactions for the total diet consumed, the total preference for nutrients and lipid deposition. Furthermore, there was also substantial genetic covariation between these traits in each diet pair within the sexes. Collectively, this work shows that different genotypes respond to an imbalanced diet in different ways and that this is genetically linked to lipid deposition. Moreover, the significant genotype-by-sex interactions suggests that these genotypes respond differently in the sexes and is likely to explain why various diet related health issues (such as obesity) are more prominent in one sex over the other.

Collectively, my thesis demonstrates the importance of considering the multifaceted nature of nutrition when examining the role that diet plays in regulating sexual selection and sexual conflict. My work challenges the longstanding view that calories and/or energy content are the main drivers of costly sexual traits and sexually dimorphic life-history strategies and shows that a balanced intake of specific nutrients (namely P and C) plays a far more important role. My work also highlights that the genetics of dietary choice can also have important consequences for how important life-history traits, such as lifespan and reproductive effort, are able to evolve independently in the sexes and the implications this has for the regulation of nutrients and the potential risks to health when consuming an imbalanced diet.

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FIGURES

Figure 1.1. An example of a nutritional landscape. Animals can feed along the nutritional rails (dashed grey lines) and a trait of interest is regressed onto the intake data for nutrients A and B. Regions on the surface in red correspond to the optimal expression of the trait of interest, with blue regions showing low values for trait expression. The dashed black lines are iso-caloric lines which connect diets of equal caloric value across the nutritional rails.

Figure 1.2. The distribution of the 24 artificial diets used in this thesis that define the nutritional space. Solid black lines represent the six nutritional rails (P:C), with four diets per rail that differ in total nutritional content (P+C). Each black dot represents an individual diet with red dots representing the diets used to make the dietary choice feeding pairs. The diets on each rail that have the same total nutrition are connected by iso-caloric lines (dashed, black lines).

Figure 1.3. (A) A male decorated cricket, *Gryllodes sigillatus*, calling to attract a mate; (B) a mating pair with the male (bottom) transferring a spermatophore containing a gelatinous spermatophylax (blue arrow) and sperm containing ampulla (yellow arrow) to the female (top); (C) a female consuming the spermatophylax (blue arrow), with the ampulla (white arrow) remaining attached, transferring sperm. After consuming the spermatophylax the female will remove the ampulla and terminate sperm transfer. The male can be seen in the background guarding the female to prevent premature removal of the ampulla and the courtship of other nearby males. Photos are courtesy of David Funk and Scott Sakaluk.

Figure 1.4. (A) a mating pair of *Teleogryllus commodus* with a male (bottom) and female (top); (B) a male transferring a sperm containing ampulla (black arrow) to the female. Photos are courtesy of John Hunt.

Figure 2.1. Path diagram illustrating the alternate models used to predict male mating success (MS) from the standardized linear and quadratic effects of protein (P) and carbohydrate (C) intake and the three principal components describing the variation in CHC expression (PC1, PC2, PC3). In the *partial mediation model*, I model the effects of nutrient intake as directly influencing MS (red pathways) and indirectly influencing MS through their effects on CHC expression. In the *full mediation model*, I model the effects of nutrient intake as influencing MS exclusively through their effects on CHC expression (i.e. the red pathways are constrained to zero).

Figure 2.2. Nutritional landscapes illustrating the effects of protein and carbohydrate intake on (A) PC1 and (B) PC3 that describe the variation in CHC expression and (C) mating success in male *Gryllodes sigillatus*. High values of these response variables are given in red and low values in blue. The black dots represent the actual nutrient intake for each male cricket in this experiment and the white cross on each landscape represents the regulated intake point (\pm SE) estimated in Chapter 3 for male *G. sigillatus* when given dietary choice.

Figure 2.3. Thin-plate spline contour view visualization of the fitness surface on the two major axes of significant nonlinear sexual selection, m_2 and m_3 . The open symbols represent individual data points for each male in this experiment. Colours represent the relative mating success of males (\bar{w}), with red representing the highest relative fitness and blue representing the lowest relative fitness.

Figure 3.1. A typical chromatograph showing the amino acids contained in the spermatophylax of male *Gryllodes sigillatus*. The x-axis shows the retention time for each amino acid and the y-axis shows the abundance of each amino acid, measured as the area under the peak and expressed in actual quantities based on standard curves. The numbers above the amino acid peaks correspond to the peak numbers provided in the Table 3.1.

Figure 3.2. Nutritional landscapes illustrating the effects of protein and carbohydrate intake on (A) spermatophylax weight, (B) the gustatory appeal of the spermatophylax and (C) ampulla attachment time in *Gryllodes sigillatus*. High values of these traits are given in red and low values in blue. The black dots represent the actual nutrient intake data for each individual cricket and the red cross on each landscape represents the regulated intake point (\pm SE) that is presented in Figure 3.4 and derived from the choice experiment (Experiment 2).

Figure 3.3. The mean (\pm SE) consumption of diets in each diet pair. White bars represent the high-P diet and grey bars the high-C diet in each diet pair. The P:C ratio of the diet is provided above the bar and the total nutritional content of the diet (%) is provided within the bar in bold. In each diet pair, males consumed significantly more of the high-C diet than the high-P diet (Diet pair 1: $t_{39} = 9.58$, $P = 0.0001$; Diet pair 2: $t_{39} = 10.32$, $P = 0.0001$; Diet pair 3: $t_{39} = 2.42$, $P = 0.020$; Diet pair 4: $t_{39} = 9.70$, $P = 0.0001$).

Figure 3.4. The mean (\pm SE) intake of protein (P) and carbohydrates (C) for the 4 diet pairs (black symbols, labelled by number) and the regulated intake point (\pm SE, red symbol), calculated as the mean P and C intake across diet pairs. The black dashed lines represent the outer boundaries of the choice experiment design (P:C ratios of 5:1 and 1:8) and therefore

crickets are able to feed to any point in nutritional space within these rails. The red dashed line represents the P:C ratio that passes through the regulated intake point, estimated at a P:C ratio of 1:1.74. This is the P:C ratio that crickets actively defend when given dietary choice.

Figure 4.1. A hypothetical example demonstrating the typical protocol of the Geometric Framework (GF) of nutrition to study nutrient effects on lifespan. (A) The geometric array of diets presented in nutrient space. This particular geometric array consists of 9 different nutritional rails, each with a different fixed ratio of nutrient A to nutrient B (moving left to right: 1:8, 1:5, 1:3, 1:2, 1:1, 2:1, 3:1, 5:1 and 8:1). There are four different diets (black dots) located along each nutritional rail that have the same nutrient ratio but increase in total nutrition (i.e. caloric content) as they move away from the origin. Across different nutritional rails the four diets are arranged so that they are connected by iso-caloric lines (dashed lines). These iso-caloric lines connect diets with different A:B ratios but the same caloric content. This hypothetical array therefore consists of 36 unique diets that differ in both the A:B ratio and total nutrition. (B) The distribution of actual feeding data (small black dots) recorded from animals restricted to each of the 30 unique diets. The consumption of diet by each animal is precisely measured over a defined feeding period and because the nutritional composition of the diets is known, this consumption of diet can be easily converted to an intake of nutrient A and B. As each animal is restricted to a single diet, they can only feed along the length of the nutritional rail by eating more or less of the diet (thereby ingesting more or less nutrients and calories). (C) An example of a nutritional landscape for lifespan. For each animal where the intake of nutrient A and B has been measured, the researcher also measures lifespan. This enables lifespan to be superimposed on the nutrient intake data and the linear and nonlinear effects of nutrient intake on lifespan can be quantified statistically using response surface methodologies. The relationship between nutrient intake and lifespan can also be visualized using thin-plate splines to plot the nutritional landscape in contour view. In the hypothetical example provided, the nutritional landscape is provided in contour view where regions in red represent increased lifespan and regions in blue represent reduced lifespan. The peak in lifespan appears to be centred at 50mg of nutrient A and 125 mg of nutrient B, which represents an A:B ratio of 1:2.5. To test whether animals are “optimally” regulating their intake of nutrients to maximise lifespan, a researcher can present animals with alternate

pairs of diets differing in the ratio of A to B and total nutrition. A typical dietary choice design might pair diets 1 and 3 (diet pair 1), 1 and 4 (diet pair 2), 2 and 4 (diet pair 3) and 2 and 3 (diet pair 4) (red dots, panel A) and measure the consumption of both diets and the subsequent total intake of A and B over a predefined time period. The average intake of nutrient A and B across these diet pairs is referred to as the regulated intake point (RIP) and represents the point in nutrient space that individuals actively defend when given dietary choice. The RIP (white cross, panel C) can be mapped onto the nutritional landscape and its proximity to the peak used to determine whether dietary choice is optimal for lifespan.

Figure 4.2. A hypothetical example illustrating how to quantify differences in the strength of nutritionally based trade-offs between two life-history traits. In each panel, the nutritional landscape of two competing life-history traits is provided in contour view where the darker shading represents an increased expression of the trait and light shading a decreased expression of the trait. The dashed black lines in each panel are the A:B nutritional rail that passes through the nutritional optima for each trait. The pair of curved, solid black lines that connect the nutritional rails passing through the optima represents the angle (θ) between these rails and the red dashed line represents the Euclidean distance (d) between the global maxima for each life-history trait. Panels A to C represent the case where the nutritional optima for both life-history traits occur at the same (or very similar) caloric intake. Consequently, both θ and d provide an accurate measure of how divergent the nutritional optima are for the two life-history traits and therefore the strength of the nutritionally based trade-off between these traits. In moving from panel A to C, the nutritional optima for the two life-history traits move closer together (ending with overlap in panel C) and both θ and d get smaller indicating the strength of the nutritionally based trade-off between these traits is getting weaker. Panel D represents the case where the nutritional optima for the two life-history traits are located at two different caloric intakes. Consequently, θ and d provide different measures of the extent of the nutritionally based trade-off between life-history traits. In this instance d provides a better estimate of the divergence between optima and the strength of the life-history trade-off than θ .

Figure 4.3. Nutritional landscapes illustrating the linear and nonlinear effects of protein and carbohydrate intake on (A) calling effort and (B) encapsulation ability in males and (C) egg production and (D) encapsulation ability in female *G. sigillatus*. On each landscape, high values of these traits are given in red and low values in blue. The open black circles

represent the actual nutrient intake data for each cricket and the closed white circles represent the global maxima on each landscape.

Figure 4.4. The 95% confidence region (solid grey fill) for the global maxima (closed black circle) on each landscape for (A) calling effort and (B) encapsulation ability in males and (C) egg production and (D) encapsulation ability in female *G. sigillatus*. On each landscape, the regulated intake point (\pm SE) is provided as a black cross and the dashed black line represents the boundary of the data. The regulation of nutrient intake under dietary choice is considered optimal for a given trait if the regulated intake point overlaps the 95% confidence region for the global maxima.

Figure 4.5. The mean (\pm SE) absolute consumption of each diet in the four diet pairs by (A) female and (B) male *G. sigillatus*. Grey bars represent the consumption of the high carbohydrate diet in the pair, whereas white bars represent the consumption of the high protein diet in the pair. The actual P:C ratio of alternate diets in each pair are provided above each bar and the total nutrient content of each diet are provided within the bar. The asterisks above each diet pair represents a significant difference (tested using a paired *t*-test) in the consumption of diets at $P < 0.05$. For each diet pair, males and females consumed significantly more of the high carbohydrate diet than the high protein diet. The difference in protein (white bars) and carbohydrate (grey bars) consumption from that expected if (C) females and (D) males fed at random from the diets in a pair. The asterisks above each bar represent a significant deviation from a mean of zero (tested using an unpaired *t*-test) which is expected under random feeding. For each diet pair, males and females consumed significantly more carbohydrates than expected by random feeding and less protein.

Figure 4.6. The mean (\pm SE) protein and carbohydrate intake of male (open squares) and female (open circles) *G. sigillatus* on each of the four diet pairs (labelled by number). The regulated intake point, calculated as the mean intake of nutrients across diet pairs is also presented for males (solid black square) and females (solid black circle) at a P:C ratio of 1:2.00 and 1:1.84, respectively. The red dashed lines and red solid lines represent the outer boundaries of my choice experimental design for males and females, respectively. The dashed black lines represent the expected intake of nutrients at a P:C ratio of 1:8, 1:3 and 1:1 (left to right of figure), respectively.

Figure 5.1. The nutritional landscapes for female and male LS (A & B, respectively), female DRE and male DRE (C & D, respectively) and female and male LRE (E & F, respectively). In each landscape, the red regions represent higher values for the response variable, whereas blue regions represent lower values. The black cross represents the regulated intake point (and 95% CIs) estimated in Experiment 3. The small black circles represent the actual feeding data for individual crickets.

Figure 5.2. The nutritional landscapes female and male LS (A & B, respectively), female DRE and male DRE (C & D, respectively) and female and male LRE (E & F, respectively) with the two major eigenvectors (and 95% CIs) of G_f and G_m overlaid (grey ellipsoids). The black cross represents the regulated intake point (and 95% CIs) estimated in Experiment 3.

Figure 5.3. The mean (and 95% CIs) protein (P) and carbohydrate (C) intake of mated female and male (A & B, respectively; closed symbols) and virgin female and male (C & D, respectively; open symbols) *T. commodus* on the four different diet pairs in Experiment 3. Diet pairs are labelled by their number. The black dashed lines (at P:C ratios of 5:1 and 1:8) represent the outer nutritional rails for the individual diets contained in the diet pairs. Consequently, any dietary choice will occur within these outer margins.

Figure 5.4. The regulated intake point (RIP), calculated as the mean intake of protein (P) and carbohydrate (C) across diet pairs for mated and virgin males (blue symbols, solid and open symbols, respectively) and mated and virgin females (red symbols, solid and open symbols, respectively). The black dashed lines (at P:C ratios of 5:1 and 1:8) represent the outer nutritional rails for diets. The solid and dashed red lines represent the RIP for mated and virgin females, respectively. The solid and dashed blue lines represent the RIP for mated and virgin males, respectively.

Figure 6.1. The mean (\pm SE) (A) total amount of diet eaten, (B) total nutrient preference (P intake/C intake) and (C) lipid mass for male (blue bars) and female (red bars) *T. commodus* on each of the four experimental diet pairs.

Figure 6.2. The mean (\pm SE) intake of P and C by male (blue symbols) and female (red symbols) *T. commodus*. The open symbols represent the mean intake of nutrients in each of the four diet pairs (denoted by pair number), whereas the solid symbols represent the regulated intake point (RIP), calculated as the mean of the four diet pairs. The solid blue and red lines represent the nutritional rails (lines in nutrient space that represents a fixed intake of nutrients) that passes through the RIP for males (P:C ratio of 1:2.02) and females

(P:C ratio of 1:1.71). The black dashed lines (P:C ratios of 5:1 and 1:8) represent the outer nutritional rails of the nutritional landscape.

Figure 6.3. Thin-plate spline (contour view) visualizations of the effects of protein (P) and carbohydrate (C) intake on lipid mass in (A) female and (B) male *Teleogryllus commodus*. In each spline, the red regions represent higher values for the measured trait, whereas blue regions represent lower values.

Figure 6.4. Reaction norms illustrating the genotype-by-diet pair interaction (G:DP) for the total amount of diet eaten (TE) in male and female *T. commodus*. Females are presented above the diagonal (grey background) and males beneath the diagonal (white background). In each panel, lines represent the response of a given genotype across two diet pairs.

Figure 6.5. Reaction norms illustrating the genotype-by-diet pair interaction (G:DP) for the total nutrient preference (TP, defined as the intake of P divided by the intake of C) in male and female *T. commodus*. Females are presented above the diagonal (grey background) and males beneath the diagonal (white background). In each panel, lines represent the response of a given genotype across two diet pairs.

Figure 6.6. Reaction norms illustrating the genotype-by-diet pair interaction (G:DP) for lipid mass (LM) by male and female *T. commodus*. Females are presented above the diagonal (grey background) and males beneath the diagonal (white background). In each panel, lines represent the response of a given genotype across two diet pairs.

Figure 6.7. Reaction norms illustrating the genotype-by-sex interaction (G:S) for TE in the different diet pairs in *T. commodus*. In each panel, lines represent the response of a given genotype across two diet pairs.

Figure 6.8. Reaction norms illustrating the genotype-by-sex interaction (G:S) for TP in the different diet pairs in *T. commodus*. In each panel, lines represent the response of a given genotype across two diet pairs.

Figure 6.9. Reaction norms illustrating the genotype-by-sex interaction (G:S) for LM in the different diet pairs in *T. commodus*. In each panel, lines represent the response of a given genotype across two diet pairs.

TABLES

Table 1.1. Protein (P) and carbohydrate (C) composition of the 24 artificial diets used in this thesis. The total nutrient concentration in each diet is given as the sum of the percentage P and percentage C, with the remaining percentage consisting of indigestible crystalline cellulose plus micronutrients. Diets highlighted in bold are the four diets highlighted in red in Figure 1.2 which are used in choice feeding experiments.

Table 2.1. Principal Component (PC) analysis of CHC expression in male *Gryllobes sigillatus*. PCs with eigenvectors exceeding one are presented and used in subsequent analysis. Factor loadings >0.30 (in bold) are interpreted as biological significant (Tabachnick & Fidell 1989). CHCs are named where known and unnamed CHCs (asterisks) are described by basic chemical structure. CHCs are listed in order of increasing carbon chain length.

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Table 2.5. Canonical analysis to locate the major dimensions of the γ matrix given in Table 2.4. θ_i and λ_i are the linear and nonlinear selection acting on each vector, respectively. Significance tested via permutation test: *** $P < 0.0001$, ** $P < 0.001$.

Table 2.6. Structural equation model parameter estimates for protein (P) and carbohydrate (C) intake on the three principal components describing variation in CHCs (PC1, PC2, PC3) and male mating success (MS) taken from the partial mediation model. Significance values: * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

Table 2.7. The linear effects of protein (P) and carbohydrate (C) intake on male mating success mediated through the three principal components (PC1, PC2, PC3) that describe the

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Table 3.1. The amino acids that correspond to the peak numbers found on the example chromatograph in Figure 3.1.

Table 3.2. The effect of protein (P) and carbohydrate (C) intake on spermatophylax (SPHYLAX) weight, the gustatory appeal of the SPHYLAX and ampulla (AMP) attachment time in *Grylloides sigillatus*.

Table 3.3. Sequential model comparing the linear and nonlinear effects of protein (P) and carbohydrate (C) intake on spermatophylax (SPHYLAX) weight, the gustatory appeal of SPHYLAX and ampulla (AMP) attachment time in *Grylloides sigillatus*.

Table 4.1. The linear and nonlinear effects of protein (P) and carbohydrate (C) intake on early life reproductive effort (calling effort and egg production) and immune function (encapsulation, pro-phenyloxidase (PO) activity and activated phenyloxidase activity) in male and female *Grylloides sigillatus*.

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Table 5.1. Linear and nonlinear effects of protein (P) and carbohydrates (C) on lifespan (LS), daily reproductive effort (DRE) and lifetime reproductive effort (LRE) in the sexes. DRE and LRE was measured as calling effort and egg production in males and females, respectively.

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AUTHOR'S DECLARATION

The research contributing to this thesis was conducted by James Rapkin (JR) who was supported by a studentship from NERC. This thesis is presented as a series of discrete papers that were written by JR with comments and editing from John Hunt (JH), however, others contributed to each chapter as detailed below.

CHAPTER TWO

The data in this chapter was collected by JR. Scott K. Sakaluk (SKS) established the cricket cultures used in this chapter and provided advice on experimental design. Kim Jensen (KJ) and Clarissa M. House (CMH) provided advice on experimental design. JR, JH and John K. Sakaluk (JKS) conducted the statistical analyses. JR wrote the manuscript with comments and editing from JH, SS and JKS. A version of this chapter has been submitted to the *Journal of Evolutionary Biology*.

CHAPTER THREE

The data in this chapter was collected by JR and Sarah M. Lane. SKS established the cricket cultures used in this chapter and provided advice on experimental design. KJ and CMH provided advice on experimental design. JR and JH conducted the statistical analyses. JR wrote the manuscript with comments and editing from JH, SKS, KJ and CMH. A version of this chapter has been published in the *Journal of Evolutionary Biology* **29**: 395-406.

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CHAPTER FIVE

The data in this chapter was collected by JR, RCA, and Charles Grant. RCA and JH established the cricket cultures used in this chapter. JR and JH conducted the statistical analyses with advice and assistance from Alastair Wilson (AW) on genetic analyses using the animal model. JR wrote the manuscript with comments and editing from JH.

CHAPTER SIX

The data in this chapter was collected by JR. RCA and JH established the cricket cultures used in this chapter. JR and JH conducted the statistical analyses with advice and assistance from AW on genetic analyses using the animal model. JR wrote the manuscript with comments and editing from JH.

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CHAPTER 1:
GENERAL INTRODUCTION

1.1. Sexual Selection and Sexual Conflict

Sexual selection arises because one sex (typically females) invests more resources in reproduction (fewer, larger, highly nutritious, macrogametes: eggs) than the other (typically males) (numerous, small, highly motile, microgametes: sperm) (Trivers, 1972; Andersson, 1994). This means that males typically have more resources free for behaviours and displays used to compete for access to females (Trivers, 1972). The opportunity for and the intensity of sexual selection is, therefore, generally greater in males (Bateman, 1948; Trivers, 1972) which also results in male fitness typically being more variable than in females (Trivers, 1972).

There are two components of sexual selection: mate choice and competition for mates. Typically, because of the resources invested in reproduction, it is the females that are more choosy than males and indeed males that are more competitive than females, although that is not to say that there are not cases where males are choosy and females competitive (Andersson, 1994; Andersson & Simmons, 2006). Male-male mate competition was easily accepted as a mechanism of sexual selection when Darwin first posited his theory (Darwin, 1872) probably because male-male competition and the evolution of traits to enhance male competitive ability were obvious. Female choice proved controversial until around 50 years ago when the benefits of female choice were explored and evidence for female choice became increasingly well documented (Fisher, 1915; Lande, 1981; Andersson, 1994; Andersson & Simmons, 2006), however debate over female preference remains.

The benefits of female choice are divided into direct and indirect benefits. In models of direct benefits, female preference through choice is under natural selection with females expressing a preference in order to gain, for example, resources (e.g. nuptial gifts), greater investment by males in parental care, protection from other males and/or predators and fewer parasites transferred at mating (Price *et al.*, 1993; Chapman *et al.*, 2003). In models of indirect benefits female preference evolves because it becomes genetically associated with genes that produce sexy sons (Fisher's effects) (Fisher, 1915; Lande, 1981) and/or produce high viability offspring (good genes process) (Kirkpatrick, 1982; Kokko *et al.*, 2002; Chapman *et al.*, 2003).

One of the advances of sexual selection theory in recent years was the theory by Parker (1970) that sexual selection does not stop at the pre-copulatory stage but can

continue post-copulation. Current theory suggests that females can gain direct phenotypic and indirect genetic benefits from multiple matings. By mating with multiple males, there will be competition among sperm of different males to fertilise available ova (Andersson & Simmons, 2006). This is commonly seen as the post-copulatory equivalent of male-male competition with cryptic female choice and the ability of a female to bias paternity, seen as the post-copulatory equivalent of female choice (Chapman *et al.*, 2003; Andersson & Simmons, 2006).

Males and females clearly have distinct reproductive roles and divergent reproductive interests, with each sex seeking to optimise their own reproductive success (Chapman, 2006). These divergent interests can lead to conflict over the outcome of the interaction between males and females. This type of conflict is termed 'interlocus' sexual conflict and can lead to the evolution of a number of traits and behaviours in one sex to benefit their own reproductive interests at the expense of the other sex. In males for example, interlocus conflict can lead to strategies such as sexual coercion (Clutton-Brock & Parker, 1995), mate guarding (Sakaluk, 1991; Watson *et al.*, 1998), harassment of females (Sakaluk, 1991; Chapman, 1995), genital morphology (House *et al.*, 2013) and the manufacture of accessory gland products (Chapman *et al.*, 2000). Such sex-specific adaptations can induce a counter adaptation in the other sex, for example in females, resistance to harassment (Watson *et al.*, 1998), counter adaptations to accessory gland products (Chapman, 1995; Arnqvist & Rowe, 2005) and ending copulations early (Sakaluk *et al.*, 2006; Gershman & Sakaluk, 2010). Such sexually antagonistic adaptations have led to a number of 'arms-races' between the sexes (Arnqvist & Rowe, 2005; van Doorn, 2009) and are responsible for a number of the elaborate sexual traits seen in the natural world.

Far less attention has been paid to 'intralocus' sexual conflict (ISC) whereby the sexes are displaced from their phenotypic optima due to sex-specific sexual selection on shared traits (Bonduriansky & Chenoweth, 2009). Shared traits are assumed to be controlled by a common genetic machinery in both sexes (Rice, 1984), reflected by a strong, positive intersexual genetic correlation which measures the extent of the similarity between the additive effects of alleles when expressed in different sexes (Bonduriansky & Chenoweth, 2009). However, the strongly divergent reproductive strategies of the sexes generates sex-specific selection on many shared traits, with selection on one sex causing a displacement of the other sex from its phenotypic optimum and thereby reducing its fitness. An example of

such sexually antagonistic selection can be seen in Soay sheep where larger horn size increases success in male-male competition but horns prove costly for females to produce (Robinson *et al.*, 2006), with increased horn size being negatively associated with breeding success and longevity. Overall how ISC impacts the evolution of male and female traits and whether resolution to the conflict is possible remain important but unanswered questions.

1.2. The Link between Sexual Selection and Diet

Sexual selection has long been acknowledged as the primary driving force behind the evolution of exaggerated male sexual traits (Andersson, 1994; Andersson & Simmons, 2006). An important model in sexual selection theory is the handicap model (Zahavi, 1975; 1977; Johnstone *et al.*, 2009) which posits that male sexual traits should function as reliable indicators of male quality for female mate choice (Trivers, 1972; Johnstone, 1995; Lailvaux & Irschick, 2006) and male-male competition (Zahavi, 1975; 1977; Andersson, 1994; Andersson & Simmons, 2006). Reliability of these “honest signals” is maintained by the fact that exaggerated sexual traits are costly to produce meaning that only high quality males can afford the costs of producing and/or maintaining these traits and are impossible to mimic by low quality males (Zahavi, 1975; 1977; Johnstone *et al.*, 2009). A central prediction of the handicap hypothesis is that, if these exaggerated traits are costly to produce and/or maintain then they should be sensitive to variation in the acquisition of resources and also subject to trade-offs due to variation in resource allocation (Zahavi, 1975; 1977; Stearns, 1992; Rowe & Houle, 1996). Exaggerated traits should, therefore, co-vary positively with condition which can be conceptually defined as the available “pool of resources” an animal can allocate to fitness enhancing traits (Rowe & Houle, 1996; Hunt *et al.*, 2004; Tomkins *et al.*, 2004). The resource responsible for the variation seen in condition-dependent traits is typically defined as energy, acquired from food (Cotton *et al.*, 2004a; 2006; Hunt *et al.*, 2004). A number of examples exist across a broad range of animal taxa over the importance of food on the condition-dependent expression of a broad variety of traits, for example; behaviour (activity (Mikolajewski *et al.*, 2004) and migration (Brodersen *et al.*, 2008)), growth (Forsman & Lindell, 1996; Johns *et al.*, 2014), immunity (Hill, 2011; Triggs & Knell, 2012; Córdoba-Aguilar *et al.*, 2016), lifespan (Chen & Maklakov, 2012; 2014), morphology (genitals (Soto *et al.*, 2007; Cayetano & Bonduriansky, 2015) and ornaments (Kotiaho, 2002;

Cotton *et al.*, 2004a; Hill, 2011; Morehouse, 2014)), physiology (stress response (Herring *et al.*, 2011); oxidative damage (Schantz *et al.*, 1999; Garratt & Brooks, 2012)) and reproduction (courtship (Gray & Eckhardt, 2001; Kotiaho, 2002; Hunt *et al.*, 2004); ejaculate production (Perry & Rowe, 2010; Kaldun & Otti, 2016); fitness (Hill, 2011; Janicke & Chapuis, 2016) and sexual attractiveness (Holzer *et al.*, 2003; Cotton *et al.*, 2004a)).

Traditional studies of the nutritional ecology of life-history and sexually selected traits have focused on a quantitative resource constraints paradigm which assumes that animals forage to maximise the intake of a single nutritional resource which is then allocated between the competing fitness components (Cotton *et al.*, 2004b; Cotter *et al.*, 2011). A number of studies have, therefore, manipulated the caloric content or quantity of available food when studying the effect of varying resources on condition dependent life-history traits. For example, calling frequency in male crickets (*Gryllus campestris*) has been shown to decline under a restricted diet (Scheuber *et al.*, 2003) while conversely, lifespan has been shown to increase under a restricted diet (Masoro, 2002; Piper & Partridge, 2007). Poor diet quality has also been shown to affect the ability of Trinidadian guppies and house finches to convert dietary carotenoids into colourful ornaments (Grether, 2000; McGraw *et al.*, 2002).

The theory that the nutritional composition of available food is more important to the evolution and maintenance of life-history traits (qualitative resource constrains) than the caloric content has been gaining acceptance (Morehouse *et al.*, 2010; Cotter *et al.*, 2011). A number of studies have shown the importance of specific nutrients on the expression of various sexually selected traits in various animal species (South *et al.*, 2011; Sentinella *et al.*, 2013; Bunning *et al.*, 2015; House *et al.*, 2015) as well as the importance of a specific balance of nutrients in mediating trade-offs between various life-history traits such as lifespan and reproduction (Lee *et al.*, 2008a; Maklakov *et al.*, 2008; Cotter *et al.*, 2011; Reddiex *et al.*, 2013; Jensen *et al.*, 2015). Furthermore, sex differences have also been found between nutrition dependent trade-offs between life-history traits, due to the different nutritional requirements resulting from the divergent reproductive interests of the sexes (Lee *et al.*, 2008a; Maklakov *et al.*, 2008; Cotter *et al.*, 2011; Reddiex *et al.*, 2013; Jensen *et al.*, 2015). It is therefore, important to gain a complete understanding of the consequences of dietary intake on life-history traits and the resultant trade-offs by not just looking at the caloric content of food but also the combined effect of specific nutrients

(Morehouse *et al.*, 2010; Cotter *et al.*, 2011). A powerful protocol to account for the multifaceted effects of nutrients is the geometric framework of nutrition, which, has been used successfully in a number of studies (Simpson & Raubenheimer, 1993; 1995; 2012).

1.3. The Geometric Framework of Nutrition

Animals simultaneously require a wide range of nutrients (including macro- and micronutrients) to support numerous biological functions for example, growth, reproduction and somatic maintenance (Simpson & Raubenheimer, 1993; 1995; 2012). However, most studies that have manipulated diet, whether it be to investigate the condition-dependence of different sexually selected traits (Cotton *et al.*, 2004a; Hunt *et al.*, 2004; Tomkins *et al.*, 2004) or to understand trade-offs between life-history traits (Magwere *et al.*, 2004; Partridge *et al.*, 2005; Piper & Partridge, 2007; King *et al.*, 2011; Attisano *et al.*, 2012), have used relatively simple dietary approaches. Traditionally, this has been achieved either by manipulating the diet quality of a small number of laboratory diets or restricting the caloric intake of an animal (Simpson & Raubenheimer, 2012), under the assumption that foraging is considered a process of acquiring energy rather than a complex balance of obtaining specific nutrients for investment in all life-history traits (Simpson & Raubenheimer, 2012). While these simple approaches have generated valuable insights into the importance of diet during resource acquisition and allocation they have however, contributed very little to our understanding of the specific nutrient requirements and combination of nutrients required by animals to invest optimally in various traits (Simpson & Raubenheimer, 2012). To address these questions, one would need a nutritionally explicit model that is able to represent the animal, the environment and the nutritional basis for the interactions between the animal and its environment (Raubenheimer & Simpson, 2009). Furthermore, such a model must be able to take into account that any interaction between the animal and its environment might involve multiple food components (Simpson & Raubenheimer, 2012). Finally, such a nutritionally explicit model must be grounded in an evolutionary biological framework where the consequences of, for example, reproductive effort and death, to an animal over its nutritional choices can be represented (Simpson & Raubenheimer, 2012).

The geometric framework (GF) of nutrition is such a model designed with these core requirements in mind (Simpson & Raubenheimer, 1993; 1995; 2012). Using the GF, the behaviour and consequences of an animal's nutritional environment and feeding choices can be investigated in a multidimensional nutritional framework which aims to differentiate between nutrient intake and utilisation (Archer *et al.*, 2009; Simpson & Raubenheimer, 2012). The basic methodology of the GF is the production of a range of diets that vary in the concentration and ratios of nutrients that they consist of and then accurately measuring their consumption in a series of feeding trials (Simpson & Raubenheimer, 1995; Archer *et al.*, 2009). Quantifying the intake for each unique nutrient combination (referred to throughout this thesis as a "no-choice feeding trial") allows for the construction of a fine-scale nutritional landscape (Simpson & Raubenheimer, 1995; Archer *et al.*, 2009) to identify the nutritional optima of a trait. A nutrient landscape is a geometric space with two or more axes (dependent on the number of nutrients being examined) representing a food component that is suspected to play a role in a particular trait which can then be mapped onto the landscape (Archer *et al.*, 2009; Simpson & Raubenheimer, 2012). This enables the use of powerful response surface methodologies (Lande & Arnold, 1983) to examine both the independent effect and the interaction between the different dietary components and the trait being examined (Archer *et al.*, 2009). An example of such a nutritional landscape can be seen in Figure 1.1 where the ratio of macronutrients vary along a series of fixed 'nutritional rails' with points along this rail representing diets that vary in the total caloric content from low to high (Simpson & Raubenheimer, 1995; South *et al.*, 2011). These points are connected by iso-caloric lines across the rails to other points that have the same total nutrition (Figure 1.1).

An important part of the interactions between an animal and its environment is the amount and balance of nutrients that are required by an animal (nutritional requirements) to invest maximally in a trait(s) (Simpson & Raubenheimer, 2012). The GF allows for feeding trials where individuals are given a choice between two diets (referred to throughout this thesis as a "choice feeding trial") to examine the behavioural rules that govern dietary intake and for predicting how an animal should respond to its dietary environment (Archer *et al.*, 2009; Simpson & Raubenheimer, 2012). It is, therefore, possible to identify the "intake target" or regulated intake point (RIP) towards which an animal feeds and actively defends (Simpson & Raubenheimer, 2012). This intake target may be "static", i.e. a point

integrated over time, or “dynamic” whereby it is possible to track its trajectory during an animal’s development (Archer *et al.*, 2009; Simpson & Raubenheimer, 2012). The GF has been successfully utilised to examine the effects of caloric and specific nutrient intake on a variety of life-history traits, including condition dependent sexual traits (House *et al.*, 2015), reproductive effort (Maklakov *et al.*, 2008; South *et al.*, 2011), fecundity (Bunning *et al.*, 2015), lifespan and ageing (Lee *et al.*, 2008a; Reddix *et al.*, 2013; Jensen *et al.*, 2015) and immune function (Cotter *et al.*, 2011) in a variety of animal taxa ranging from invertebrates to humans (Simpson & Raubenheimer, 2012). I will, therefore, use the GF approach extensively in this thesis.

1.4. Nutritional Optima and Trade-offs

Trade-offs are central to life-history theory (Stearns, 1989; 1992) and are key to predicting the optimal life-history of an organism in a given environment (Stephens & Krebs, 1986; Stearns, 1992; 2000; Roff, 1993; 2002; Roff & Fairbairn, 2007). As stated previously, trade-offs exist because different life-history traits compete for a finite pool of resources, meaning that the allocation of resources to one trait causes a reduction in available resources to allocate to other traits (Stearns, 1992; Roff, 2002). An important model in predicting the extent of trade-offs is the acquisition-allocation model or “Y-model” of Van Noordwijk & de Jong (Van Noordwijk & de Jong, 1986), however direct empirical tests of the Y-model have proven challenging due to difficulties in quantifying resource acquisition (Stearns, 1989; Roff, 2002; Roff & Fairbairn, 2007). Due to the use of a number of different measures as proxies for resource acquisition for example, body size (Biere, 1995) and lipid stores (Chippindale *et al.*, 1998), our knowledge of the nutritional underpinnings of trade-offs is limited and leaves trade-offs an important but unsolved problem in life-history research. A more powerful approach to measure trade-offs in life-history studies is to experimentally manipulate resource acquisition ability through dietary manipulation of the type and quantity of resources available to an animal. Indeed, empirical studies across a range of taxa have shown that this type of dietary manipulation can have profound effects on life-history trade-offs (e.g. invertebrates (Hunt *et al.*, 2004); fish (Kolluru & Grether, 2005); amphibians (Lardner & Loman, 2003); reptiles (Brown & Shine, 2002); birds (Karell *et al.*, 2007); and mammals (Hill & Kaplan, 1999)). A limitation common to most of these

studies is the use of only a few diets of poorly defined nutritional composition (e.g. “good” versus “bad” diets) with consumption of these diets rarely measured (e.g. Holzer *et al.*, 2003; Hunt *et al.*, 2004). This approach makes it difficult (if not impossible) to statistically partition the effects of specific nutrients from calories or to examine any effect that the interaction between nutrients may have on the trade-offs between life-history traits. As explained previously, to accurately quantify resource allocation trade-offs requires the use of a multidimensional approach such as the GF to measure the intake and allocation of nutrients to life-history traits (Stearns, 1992; Simpson & Raubenheimer, 2012; Flatt & Heyland, 2011).

1.4.1. Trait-Specific Nutritional Optima

Optimal foraging theory predicts that animals regulate their energy intake to maximise their fitness (Stephens & Krebs, 1986). Traditionally, foraging models assumed that fitness increased with increasing energy intake (Stephens & Krebs, 1986), however the use of the GF in nutritional studies has highlighted how foragers must in fact regulate both their energy intake and the nutrients this energy comes from (Simpson & Raubenheimer, 1993; 2012). This is because fitness determining traits are often optimised at different specific amounts and combinations of nutrients which suggests that the competitive allocation of available resources leads to trade-offs between fitness enhancing traits (Zera & Harshman, 2001; Schwenke *et al.*, 2016). In female *D.melanogaster* for example, fecundity is maximised in females that eat energy rich food containing protein (P) and carbohydrate (C) in a 1:2 ratio, while lifespan is maximised on food containing P:C in a 1:16 ratio (Lee *et al.*, 2008a; Jensen *et al.*, 2015). Further examples of life-history trade-offs can be found between other important fitness determining life-history traits such as, reproduction and immune function (Schwenke *et al.*, 2016), reproduction and lifespan (Lee *et al.*, 2008a; Jensen *et al.*, 2015), between different immune responses (Cotter *et al.*, 2011) and between reproductive and dispersal trait development (Clark *et al.*, 2015).

The increasing use of the GF for nutrition has provided many important insights into how animals balance their nutrient intake in complex nutritional environments and in determining the nutritional optima of specific traits (Simpson & Raubenheimer, 2012). However, many conclusions are reached by a visual inspection of the nutritional landscapes produced by the geometric approach, but such conclusions can be subjective and can only

'suggest' a trade-off. For example, the location of the nutritional optima for a given trait and the extent to which the regulated intake point aligns with this optima are typically derived by visualizing the nutritional landscape but such observations can be difficult to ascertain with any precision. This issue is perhaps even more striking when considering nutritional trade-offs between different life-history traits. A nutritional trade-off will exist when the optimal expression of two traits occur in different regions of the nutritional landscape and are thus optimized at different intakes of specific combinations of nutrients. Characterizing any nutritionally based trade-offs would require formally locating the nutritional optima for each life-history trait and quantifying the extent of any nutritional differences. However, such a robust conceptual framework for quantifying nutritionally based life-history trade-offs does not currently exist.

1.4.2. Sex-Specific Nutritional Optima

Examples of trait-specific nutritional optima have been shown in the previous section. However, given the role of sexual selection in driving the evolution of a number of sexually selected traits and the strongly divergent reproductive strategies of males and females, the sexes should differ in their trait-specific nutritional optima. Shared traits should be under divergent selection for dietary preference and resource utilization to maximize sex-specific fitness. This has been demonstrated in studies using the GF for example, reproductive effort in the field cricket *Teleogryllus commodus*. Male reproductive effort through calling (a metabolically intensive sexual advertisement which is under strong selection through female choice (Bentsen *et al.*, 2006)) was maximised on diets high in carbohydrate (Maklakov *et al.*, 2008). In contrast, female reproductive effort through egg laying rate was maximised on diets high in protein (Maklakov *et al.*, 2008). Furthermore, when examining dietary preference by looking for the regulated intake point, Maklakov *et al.* (2008) found that both sexes actively regulated their nutrient intake with females consuming more protein relative to carbohydrate than males, indicating a preference in the direction of dietary consumption to maximise reproductive fitness. However, both sexes were shown to have similar dietary preferences suggesting that one or both of the sexes is constrained from reaching their sex-specific optima (Maklakov *et al.*, 2008). Work by Jensen *et al.* (2015) found a similar result in *D.melanogaster* whereby, sex-specific fitness would be best maximised by independent regulation, by the sexes, of protein and carbohydrate.

Indeed, Jensen *et al.* (2015) went further by measuring the number of offspring produced by males using competitive matings trials rather than relying on a proxy of mating success (i.e. calling effort) and again found that male reproductive fitness increased with increasing C intake (P:C 1:8) (Jensen *et al.*, 2015). However, as was found by Maklakov *et al.* (2008) there was little divergence between the regulated intake point of males and females suggesting that the sexes were constrained from reaching their sex-specific optima for reproduction and lifespan (Jensen *et al.*, 2015).

The presence of sex differences in optimal nutritional intake provides the potential for sexual conflict over dietary choice, specifically intralocus sexual conflict (ISC), because many of the genes responsible for optimal trait expression are shared by the sexes (Bonduriansky & Chenoweth, 2009). As suggested previously, ISC may constrain selection and prevent one or either sex from reaching their optima, in this case, their nutritional optima to maximise fitness. While a number of studies have provided general evidence for ISC and nutritional studies have specifically found evidence for sex-specific nutritional optima, only one study has investigated both aspects. However, this study only found limited evidence for ISC over nutritional optima in *D.melanogaster* (Reddiex *et al.*, 2013), although there were limitations to the study. For example, nutrient intake was only measured over four days which limits the opportunity for differences in male and female regulation to become apparent and may explain this limited finding. Our current understanding of the underlying genetics behind nutritional choice and the impact of any conflict over sex-specific nutritional optima on life-history traits is limited and requires further study.

1.5. The Link between Dietary Preference, Sexual Selection and Sexual Conflict on Reproduction and Ageing

1.5.1. Introduction to Ageing

Ageing (or senescence) is an irreversible decline of an individual's physiological performance with age, which progressively increases the risk of mortality over time (Finch, 1994). Ageing is an almost universal process (Archer & Hosken, 2016) and can be seen not only in humans but also in other mammal species, insects (Jones *et al.*, 2014) and also

bacteria (Ackermann *et al.*, 2003) making the understanding of its evolution and mechanisms, important biological questions (Hughes & Reynolds, 2005).

Evolutionary theories of ageing are based upon the observation that because very few individuals survive environmental hazards such as predation and disease to reach an old age, the strength of natural selection grows weaker over an individual's lifetime (Haldane, 1942; Hamilton, 1966). Such a decline in natural selection over time would allow for the accumulation of late acting deleterious effects (Mutation Accumulation Theory (Medawar, 1952)) and would favour alleles with positive effects early in life, even if these alleles have a negative effect later in life (Antagonistic Pleiotropy (Williams, 1957)) and promote the age related declines in somatic maintenance (Disposable Soma Theory (Kirkwood, 1977)) that are associated with ageing.

1.5.2. Sexual Selection and Ageing

Evolutionary theories of ageing rely on the assumption that the strength of natural selection on senescence is constant until the age of the first reproductive event, at which point it begins to decline (Haldane, 1942). While this is true and not without experimental support (for example: comparative studies in mammals (Austad, 1997) and insects (Keller & Genoud, 1997) and experimental evolution studies (Stearns, 2000)) the importance of sexual selection in determining the schedule of reproductive effort which, therefore, affects the strength of natural selection and thus the rate of ageing has, until recently, been largely ignored in ageing research (Archer & Hunt, 2015). As explained previously, the vastly divergent reproductive strategies of males and females and consequently the differing intensity of sexual selection on males and females, will have consequences for how males and females invest in reproductive effort over their lifetime (Bonduriansky *et al.*, 2008).

Female reproductive success relies upon having time to accrue the resources necessary to produce and rear offspring, with natural selection expected to promote a low-risk, low wear and tear strategy of female reproductive effort to yield moderate rates of return over extended time periods (Bonduriansky *et al.*, 2008). In contrast, males are predicted to adopt a "live fast, die young" strategy of reproductive effort (Hunt *et al.*, 2004; Bonduriansky *et al.*, 2008), whereby males invest intensively in early life reproductive effort to yield high fitness returns over a short and immediate time frame but at a sacrifice to lifespan (Kokko, 1997; 1998; Kokko *et al.*, 2002). A number of studies support this prediction

showing that male mortality is higher than female mortality in a variety of taxa (Promislow, 1992) and that differences in lifespan and ageing across the sexes vary with the intensity of sexual selection in both comparative studies (Promislow, 1992; Clutton-Brock & Isvaran, 2007) and experimental evolution studies (Maklakov *et al.*, 2009). By influencing the scheduling of reproductive success, sexual selection can affect the evolution of ageing and senescence (Bonduriansky *et al.*, 2008). However, it is not always the case that sexual selection will favour a shorter lifespan in males. Indeed there are cases where selection favours a longer lifespan in males when female choice for high quality males is also selecting for traits with a positive pleiotropic effect on ageing (Promislow, 2003; Graves, 2007; Bonduriansky *et al.*, 2008).

In addition to sexual selection driving reproductive scheduling and affecting lifespan and ageing, the potential exists for sexual conflict to also influence the evolution of ageing and senescence (Promislow, 2003; Bonduriansky *et al.*, 2008). A number of studies have explored how interlocus sexual conflict and antagonistic co-evolution can drive an evolutionary 'arms race' between the sexes, for example males influencing the lifespan of their mate, either through direct physical damage during mating (Crudgington & Siva-Jothy, 2000; Stutt & Siva-Jothy, 2001) or by manipulating a female's reproductive schedule to increase short-term reproductive output at a cost of longevity (Chapman, 1995). However, much less work has focused on the role of ISC (Bonduriansky & Chenoweth, 2009) and the possibility that sex differences in the optimal timing and cost of reproductive effort could be driving ISC over ageing and senescence (Zajitschek, 2007; Bonduriansky *et al.*, 2008; Zajitschek *et al.*, 2012).

1.5.3. Diet and Ageing

The importance of diet on condition-dependent investment in lifespan has been shown previously (Chen & Maklakov, 2012; 2014), surprisingly however, studies using dietary restriction (DR, a reduction in food intake without malnutrition) have reported an increase in longevity across a range of species (Masoro, 2002; Archer *et al.*, 2009; Nakagawa *et al.*, 2012). This increase in lifespan is often attributed to caloric restriction (CR) (Masoro, 2002; Partridge & Brand, 2005) where the restriction of certain nutrients increases longevity, for example, a lower proportion of available protein or sugar in food increased the longevity of *D. melanogaster* (Piper & Partridge, 2007). Additionally, DR studies have

reported a trade-off between lifespan and reproduction where an increase in longevity is often linked with a corresponding decrease in reproductive output (Bonduriansky *et al.*, 2008). Life-history theory predicts such a trade-off is due to the cost of reproduction and therefore, a decrease in reproductive output is expected if longevity and somatic maintenance is prioritised (Roff, 2002; Partridge *et al.*, 2005). Furthermore, DR studies have also reported sex differences in the response to DR and CR due to the different nutritional requirements and variation in the nature of trade-offs between reproduction and longevity (Magwera *et al.*, 2004). The effect is typically more pronounced in females (Nakagawa *et al.*, 2012) where the energetic demands of reproduction are generally considered to be higher than in males and so the trade-off between lifespan and reproduction is more pronounced (Bonduriansky *et al.*, 2008). However, these simple caloric manipulations offer only a limited insight into the effect of food and diet on sexually selected and general life-history traits and do not take into account the multifaceted nature of nutrients on trait expression (Archer *et al.*, 2009; Morehouse *et al.*, 2010; Cotter *et al.*, 2011). Indeed a number of recent studies using the GF have shown that it is the intake of specific nutrients and not calories *per se* that mediates the trade-off between reproduction and lifespan and are, therefore, responsible for the increase in longevity seen in DR studies (Lee *et al.*, 2008a; Maklakov *et al.*, 2008; Fanson *et al.*, 2009; Fanson & Taylor, 2012; Solon-Biet *et al.*, 2014; Jensen *et al.*, 2015).

As discussed previously, reproductive effort and lifespan are responsive to the dietary environment and trade-offs between these two traits exist due to the competing demands for resources (Bonduriansky *et al.*, 2008) with experimental evidence for this trade-off shown in DR studies (Masoro, 2002; Piper & Partridge, 2007). More recently, the development and use of the GF has further shown that reproductive effort and lifespan are subject to not just trait-specific but also sex-specific nutritional optima which reflects the resource requirements of the strongly divergent reproductive interests of males and females (Lee *et al.*, 2008b; Maklakov *et al.*, 2008; Jensen *et al.*, 2015). Therefore, the consumption of a single blend of nutrients is not enough to maximise every required trait and instead animals require the ingestion of a variety of different nutrient combinations (Simpson *et al.*, 2004) expressed as a preference for specific combinations of nutrients.

1.5.4. Conflict over Dietary Preference

Dietary preferences play an important role in determining how an animal can regulate its nutritional intake from multiple food sources or to compensate for any limited availability of specific nutrients (Edgecomb *et al.*, 1994; Raubenheimer & Jones, 2006). This regulation of nutritional intake, subject to the availability of nutrients, should allow an individual to invest resources to maximise fitness (Simpson & Raubenheimer, 2009; Simpson *et al.*, 2010). Such regulation of intake has been demonstrated for female egg laying rate and offspring production for males in *D.melanogaster* (Lee *et al.*, 2008a; Jensen *et al.*, 2015), male sexual attractiveness in cockroaches (*Nauphoeta cinerea*, South *et al.*, 2011) and in male and female reproductive effort in crickets (*T.commodus*, Maklakov *et al.*, 2008) where the regulated intake of nutrients reflected a blend of nutrients that maximised the measured fitness component. This regulation of available food would, therefore, impact the strength of sexual selection and its consequences on lifespan and ageing.

Despite a growing understanding of the effects of the nutritional composition of foods on various life-history and sexually selected traits using the GF (Lee *et al.*, 2008a; Maklakov *et al.*, 2008; Fanson *et al.*, 2009; Fanson & Taylor, 2011; South *et al.*, 2011; Reddiex *et al.*, 2013; Archer *et al.*, 2014) our understanding of the underlying genetic architecture for nutritional preference is still limited. Any genetic variation that underlies nutritional preference would be important for populations in adapting to generational changes in dietary availability (Warbrick-Smith *et al.*, 2009; Reddiex *et al.*, 2013) and could determine the extent to which sexually dimorphic nutritional preferences can evolve to match sex-specific dietary optima (Maklakov *et al.*, 2008). Any evolutionary constraints over sexually dimorphic nutritional preferences could help in driving ISC for dietary preference and sexually dimorphic traits. A number of studies have found the potential for ISC to act on dietary preference and thus drive conflict over other sexually dimorphic traits (Maklakov *et al.*, 2008; Jensen *et al.*, 2015), however only one study to date has specifically investigated ISC over dietary preference and found only limited evidence for ISC over nutrient optimisation (Reddiex *et al.*, 2013). This does not necessary rule out the presence of ISC over nutrient optimisation, as there were a number of weakness with the study by Reddiex (2013) for example, a very short feeding period (four days) which may explain why there were only minor differences in nutritional landscapes between the sexes which is contrary to the differences reported between the sexes in other studies (Maklakov *et al.*, 2008;

Jensen *et al.*, 2015). It is clear, therefore, that further study over the potential for and impact of ISC on nutritional preference is required.

1.6. Sexual Selection and Nutritional Imbalance

The growing number of studies using the GF has helped to uncover the importance of nutrient regulation in determining which trait- and sex-specific nutritional optima an individual chooses to invest in and the strength of the trade-offs between these investments. Furthermore, studies are starting to investigate how these nutritional preferences can help to drive differences between traits, conflict between the sexes and the consequences of these preferences on important life-history traits, for example lifespan and ageing.

As discussed previously, optimal foraging theory has already predicted that animals regulate their nutrient and energy intake (Stephens & Krebs, 1986) to maximise fitness. Individuals can forage in a number of ways, for example, through compensatory feeding when animals are restricted to a diet that is deficient in important nutrients and individuals must balance the costs of over- and under-ingesting certain nutrients (Simpson & Raubenheimer, 2012). Alternatively, animals can forage by eating from different nutritionally imbalanced foods to consume an optimal nutrient blend, therefore, allowing an individual to adjust their intake to consistently eat the same ratio of nutrients to reach their optimum intake point (Simpson & Raubenheimer, 2012).

While a number of studies have shown evidence that animals can regulate their intake of nutrients (Lee *et al.*, 2008a; Maklakov *et al.*, 2008; Jensen *et al.*, 2012; Harrison *et al.*, 2014; Bunning *et al.*, 2015; Jensen *et al.*, 2015) recent insights from nutritional ecology have found separate appetite systems for protein, carbohydrate and fat (Simpson & Raubenheimer, 2005; Gosby *et al.*, 2014; Raubenheimer *et al.*, 2015). When restricted to diets of fixed macronutrient intake, animals have been shown to strongly regulate their intake of protein, much more so than the intake of carbohydrate and fat (Sorensen *et al.*, 2008; Gosby *et al.*, 2014; Raubenheimer *et al.*, 2015). This dominant appetite for protein has been termed the protein leverage hypothesis (PLH) (Simpson & Raubenheimer, 2005) and posits that when the proportion of dietary protein falls, the powerful protein appetite stimulates an increased energy intake in an attempt to gain limiting protein. If the diet shifts

towards an increased proportion of foods that are higher in carbohydrate and/or fat (thereby diluting the available protein), energy intake will increase (Gosby *et al.*, 2014).

This increase in energy intake when the proportion of available protein is low, has led to PLH being theorized to play a role in obesity and its associated health problems, for example, diabetes and cardiovascular disease (Martens *et al.*, 2013; Raubenheimer *et al.*, 2015). A number of studies using the geometric framework of nutrition have provided evidence in support of the PLH and its effects on fat deposition in a variety of animal species (e.g. caterpillars (Warbrick-Smith *et al.*, 2006); *Drosophila* (Skorupa, 2008); and mice (Sorensen *et al.*, 2008; Huang *et al.*, 2013)). The strong role of sexual selection and sexual conflict in determining trait- and sex-specific nutritional preferences and trade-offs between trait investments has been outlined throughout this introduction, however, there is a lack of understanding about how sexual selection might interact with the PLH. Could the strength of sexual selection in controlling the nutritional choices that maximise fitness in the sexes in fact drive nutritional regulation, in concert with the PLH, beyond the optima and have a negative effect on an individual's long term health and thus lifespan? Furthermore, given the currently limited understanding of the underlying genetic architecture for nutritional preference there is also a lack of understanding over the genetics behind the PLH and the regulated intake point and whether a potential interaction exists between the nutritional environment and the genetics driving nutritional preference. Therefore, further study is required to effectively tease apart the links between diet, sexual selection, genetics and the nutritional environment.

1.7. Outline and Objectives

1.7.1. Outline

The primary objective of this thesis is to examine the role of nutrition on sexual selection and sexual conflict in males and females and the impact this has on various life-history traits. I present this thesis as a series of discrete papers, each with its own literature review, methodology, results and discussion. While each chapter is directly related to the overall theme of nutrition, sexual selection and sexual conflict, the findings are related to a

number of specific topics within the field of sexual selection. I outline my general approach below in order to link together the individual papers that are presented in my thesis.

1.7.2. Approach

If sexually selected traits are costly to produce, they should be responsive to the nutritional environment (Parker, 1979; Andersson, 1994; Cotton *et al.*, 2004a; Andersson & Simmons, 2006; Cotton *et al.*, 2006). A number of studies have shown that the expression of sexual traits is dependent on the amount and quality of diet available (Andersson, 1994; Cotton *et al.*, 2004b; Hunt *et al.*, 2004; Lailvaux & Irschick, 2006) by varying the diet quantity and/or caloric content (Cotton *et al.*, 2004b; Magwere *et al.*, 2004). The limitation of only manipulating the total nutrients available or just one specific nutrient, is that it does not account for what effect any interaction between nutrients may have on sexually selected life-history traits (Morehouse *et al.*, 2010). Furthermore, such simple manipulations do not allow for differences between the amount of food ingested and the amount/type of nutritional resource directly incorporated into sexual traits (Tomkins *et al.*, 2004). The growing use of the GF (Simpson & Raubenheimer, 1993) in sexual selection and life-history studies has overcome these limitations and a number of studies have started to look at the effect of specific nutrient intake on fitness-enhancing traits, however, there is still a gap in our understanding of how nutrition might mediate or strengthen sexual selection and conflict.

The GF is a powerful tool in understanding how the acquisition of specific nutrients can influence the expression of life-history traits and fitness measures (Simpson & Raubenheimer, 1993; 2012) and to that end is used extensively throughout this thesis. Following an established GF protocol (Simpson & Abisgold, 1985) my work focuses on two major macronutrients; protein (P) and carbohydrate (C). Proteins are an important class of complex nitrogenous substances found in the cells of both animals and plants and serve multiple roles including enzymatic, structural, hormonal and genetic elements of various life processes (Saxena, 2006). Proteins are polymers of molecular units called amino acids of which there are approximately 20 commonly found in proteins. A number of these amino acids (approximately 10) are capable of being synthesized within an animal (non-essential amino acids), however, the remaining 10 amino acids cannot be synthesized in any sufficient quantity to maintain normal function and must be obtained from the diet (essential amino

acids) (Saxena, 2006). A deficiency in any one of these essential amino acids can result in the failure of important metabolic processes leading to an increase risk of infection and ultimately death. It is, therefore, unsurprising that a number of studies have shown various animal taxa maintaining a relatively constant P intake while allowing the intake of other macronutrients to vary (Simpson & Raubenheimer, 2005; Sorensen *et al.*, 2008). This tight regulation of P intake, therefore, maintains the intake of required amino acids to prevent the failure of various metabolic processes, however, the potential exists for a strict regulation of P intake to prevent the detrimental effect on lifespan and reproduction of the over ingestion of P, which has been termed the lethal P hypothesis (Simpson & Raubenheimer, 2009; Fanson & Taylor, 2012). While C intake may fluctuate more than P intake, carbohydrates are still biologically significant nutrients providing a number of structural and metabolic roles but primarily are a major source of energy for all life processes (Saxena, 2006). Similarly to proteins, carbohydrates can exist in many forms with simple sugars being sources of readily metabolized energy stores and more complex sugar chains acting as dense high-energy storage molecules to provision for long term/future energy requirements (Saxena, 2006). In total, I construct my nutritional landscape using 24 dry granular diets, that varied in both their ratio of protein to carbohydrate (P:C) and in the absolute amount of protein and carbohydrate (P+C) present. The 24 diets are split equally between six P:C ratios (5:1, 3:1, 1:1, 1:3, 1:5 and 1:8), with four diets on each P:C ratio and each of these four diets varying in total P+C present (12%, 36%, 60% and 84%). The placement of the 24 diets on the nutritional landscape can be seen in Figure 1.2 and their composition can be seen in Table 1.1, while the protocol for diet manufacture can be found in Appendix 1.

In conjunction with the GF, I have used two cricket species in this thesis: the decorated house cricket *Grylodes sigillatus* (previously *Grylodes supplicans*) (Walker, 1869) in Chapters 2, 3 and 4 and the Australian black field cricket, *Teleogryllus commodus* (Walker, 1869) in Chapters 5 and 6. Each species has specific benefits that make each one an ideal study species to address the topics put forward in this thesis. *G.sigillatus* are ideal candidates for looking at the effects of nutrition on sexual conflict, sexually selected traits and behaviours and interactions between these and other life-history traits. As is the case in other cricket species (Sakaluk & Cade, 1980; Burpee & Sakaluk, 1993; Ivy & Sakaluk, 2005), female *G.sigillatus* will mate multiple times during their lives and with a number of different

males. Over 20 days a female will mate on average 22 times, while a male will mate on average 10.9 times during their life (Sakaluk, 1987). There is, therefore, intense sexual selection on males for competition for mates and sexual conflict between the sexes with females selected to acquire multiple matings and males selected to reduce female mating frequency. This has led to the evolution of a number of adaptations in males and counter-adaptations in females to maximise sex-specific reproductive success (Sakaluk, 1987) which can be seen most clearly as a series of mating behaviours that are a prerequisite for a successful copulation. Males do compete for access to females through direct physical aggression (Sakaluk, 1987) however, they primarily rely on an acoustic advertisement call to attract females (Figure 1.3A) (Cade, 1979; Sakaluk, 1987), who respond by moving towards the calling males (Cade, 1979; Sakaluk, 1987) and engaging in pre-copulatory mate choice amongst the competing males (Ivy & Sakaluk, 2007) by judging male vigour and other condition dependent signals (Weddle *et al.*, 2012; 2013). Once a female has chosen a male, she mounts him and allows the male to transfer a large, sperm containing spermatophore, attaching it beneath the base of her ovipositor (Figure 1.3B) and remaining outside of the female's body following mating (Sakaluk, 1984; 1987). The spermatophore consists of a large spermatophylax, which does not contain any sperm and is eaten by the female and a sperm containing ampulla (Sakaluk, 1984). Immediately after mating ends, the female dismounts the male, removes and begins to consume the spermatophylax but leaves the ampulla attached (Figure 1.3C), allowing the ampulla to transfer sperm into the female's sperm storage organ. Once consumed the female will then remove and consume the remainder of the ampulla, therefore, terminating sperm transfer (Sakaluk, 1984). The length of time a female spends consuming a male's spermatophylax is, therefore, important to a male's reproductive success. The longer a female spends consuming a spermatophylax, the longer the sperm containing ampulla remains attached to the female and, therefore, the more sperm are transferred (Sakaluk, 1984). Females can exert post-copulatory mate choice and regulate how much sperm is transferred by a particular male, by leaving the ampulla attached for longer and thus accept more sperm from attractive males than unattractive males (Ivy & Sakaluk, 2007). In response males will actively guard females post mating to prevent a female from prematurely terminating sperm transfer and to exclude rival males (Bateman & MacFadyen, 1999), although females will attempt to evade their guards to gain access to new males (Bateman & MacFadyen, 1999). In addition to these easily observable

and quantifiable behaviours it is easy to assess the reproductive effort and fitness across the sexes with female fecundity measured by counting the number of eggs produced (Hunt *et al.*, 2006; Zajitschek, 2007; 2012; Archer *et al.*, 2012), and male calling effort recorded and measured on specialist equipment (Hunt *et al.*, 2004; 2006; Zajitschek, 2007; 2012).

The field cricket *T.commodus* is also an ideal species for studying the effect of nutrition on sexual selection since it shares many of the mating behaviours (apart from producing a spermatophylax) seen in *G.sigillatus* (Figure 1.4). Likewise, it is easy to measure the reproductive effort and fitness of both male and female *T.commodus* using the same established methods used with *G.sigillatus*. However, the real suitability of *T.commodus* as a study species is with the quantitative analysis of the underlying genetics behind nutrient regulation and investment of nutrients in life-history traits. *T.commodus* has been used successfully as a study species in a number of quantitative genetic studies (Bentsen *et al.*, 2006; Hunt *et al.*, 2007; Pitchers *et al.*, 2013), and there already exists an understanding over the regulatory choices of this species and the nutritional requirements for optimal investment in life-history traits (Maklakov *et al.*, 2008; Zajitschek *et al.*, 2012).

1.7.3. Chapter Justification and Objectives

Sexual selection is a major driving force in the evolution of exaggerated male sexual traits (Andersson, 1994; Andersson & Simmons, 2006) with these traits serving as reliable indicators of male quality (Zahavi, 1975; 1977) in female mate choice (Johnstone, 1995; Lailvaux & Irschick, 2006) and male-male competition (Zahavi, 1975; 1977; Andersson, 1994; Andersson & Simmons, 2006; Johnstone *et al.*, 2009). Reliability is maintained by these male sexual traits being costly to produce (Johnstone *et al.*, 2009) and thus they should also be sensitive to variation in the acquisition of resources and also be subject to trade-offs resulting from variation in resource allocation (Rowe & Houle, 1996; Hunt *et al.*, 2004). A number of empirical studies have tested this core prediction and many examples now exist that demonstrate the condition-dependence of male sexual traits (Cotton *et al.*, 2004a; Hunt *et al.*, 2004; Tomkins *et al.*, 2004). Typically, studies that manipulate condition tend to vary the quantity and/or caloric content of diet with little understanding of the specific nutrient content or effects of compensatory feeding (Cotton *et al.*, 2004a; Hunt *et al.*, 2004). The use of the GF would overcome these limitations with recent studies showing that the balanced intake of nutrients is more important for sexual traits than caloric intake *per se*

(Maklakov *et al.*, 2008; Sentinella *et al.*, 2013; Bunning *et al.*, 2015; House *et al.*, 2015). Most empirical studies on the condition-dependence of male sexual traits in insects have focused on conspicuous sexual traits, for example, morphological structures (Johns *et al.*, 2014); ornaments (Cotton *et al.*, 2004a); displays (Kotiaho, 2002); and acoustic signals (Hunt *et al.*, 2004). The condition-dependence of chemical signals is relatively poorly studied which is surprising given that sex pheromones and cuticular hydrocarbons (CHCs) play an important role in the recognition of species, the sexes and kin, in insects (Wyatt, 2003; Blomquist & Bagnères, 2010). There is growing evidence that sex pheromone and CHC expression is affected by diet but to date only two studies have used the GF to examine the condition-dependence of chemical signals in *Drosophila* (Fedina *et al.*, 2012) and cockroaches (South *et al.*, 2011). This work demonstrates that nutrients can affect the condition-dependence of chemical signals but also have important knock-on consequences for male mating success and subsequently for sexual selection. In *G.sigillatus*, CHCs play an important role in regulating sexual selection with females previously shown to be exerting a complex pattern of linear and nonlinear sexual selection on male CHCs during pre-copulatory mate choice (Steiger *et al.*, 2015). Furthermore, CHC expression has been shown to be influenced by diet but this is a sex-specific effect with significant genotype-by-diet interactions on CHC expression seen in males suggesting that CHCs may be under sex-specific selection to maintain signal reliability (Weddle *et al.*, 2012). Unfortunately the high and low quality diets used by Weddle *et al.* (2012) make it impossible to determine whether the observed dietary effects on male CHCs are due to specific nutrients or calories *per se*. Furthermore, it is currently unknown if diet also influences male mating success and if so, what role condition-dependent CHC expression might play in mediating this effect. To resolve this lack of understanding, in Chapter 2 I use the GF to examine the effects of P and C intake on CHC expression in male *G.sigillatus* and utilize multivariate selection analysis to determine how any condition-dependent variation in male CHC expression influences male mating success and subsequently the strength and form of sexual selection targeting male CHCs. Finally, I use a structural equation modelling approach to determine if any effect of nutrients on mating success is mediated exclusively through condition-dependent CHC expression or through other sexually selected traits.

Sexual conflict arises whenever the reproductive interest of males and females do not perfectly coincide (Parker, 1979) which can lead to the evolution of adaptations that

enhance the fitness of one sex at the expense of the other (Parker, 1979; Arnqvist & Rowe, 2005). These adaptations can include mating behaviours (Rowe *et al.*, 1994; Córdoba-Aguilar, 2009) and morphological structures (Crudgington & Siva-Jothy, 2000; Stutt & Siva-Jothy, 2001) but more importantly are not limited in their effects to just pre- or during copulations, with males being able to continue to manipulate females long after copulation has finished (Arnqvist & Rowe, 2005). A classic example is the seminal fluid proteins found in the ejaculate of male *D.melanogaster* which are known to have a wide range of physiological effects on females (Wolfner, 2002). However despite being found in a number of taxa, relatively little is known about the chemical manipulation of females through the male ejaculate, outside of *D.melanogaster* (Arnqvist & Rowe, 2005). Nuptial gifts refer to any material beyond obligatory gametes that is provided by a donor to a recipient during courtship or copulation to improve the fitness of the donor (Lewis *et al.*, 2014). They are taxonomically widespread with males of various field cricket and katydid species synthesizing their own gifts which, constitute a major form of reproductive investment and their production has been shown to be costly to the male (Vahed, 1998; Sakaluk *et al.*, 2004; Leman *et al.*, 2009). In many species males provision gifts with nutrients or defensive compounds that are otherwise absent from a female's diet (Lewis & South, 2012) and, therefore, provide a fitness advantage to her offspring through consumption of the gift (Gwynne, 1997; 2008). However, in other species, no such benefit has been detected with nuptial gifts predominantly serving to protect the male ejaculate and cause the female to relinquish some of her control over sperm transfer and paternity (Vahed, 1998; 2007). In *G.sigillatus*, the production of a nuptial gift, in the form of a gelatinous spermatophylax, is an important part of courtship behaviour in this species. A female will consume the spermatophylax and after which will immediately remove and consume the ampulla, thereby terminating sperm transfer. Females have been shown to vary considerably in the length of time that they will feed on a spermatophylax with size being an important factor as it takes a female longer to fully consume a larger spermatophylax (Sakaluk, 1984; 1985). While production of a nuptial gift will come at a direct cost to immune function in males, consumption of this gift by the female clearly enhances a male's fitness by prolonging sperm transfer, while there appears to be little direct benefit to the female in its consumption (Will & Sakaluk, 1994). Recently, research has begun to show that the chemical composition of the spermatophylax produced by male *G.sigillatus* has a role to play in mediating sexual

conflict in this species (Warwick *et al.*, 2009; Gershman *et al.*, 2012; 2013). Multivariate selection analysis has shown that the specific combination of amino acids (the primary component after water) could prolong or shorten the time a female spent feeding on the spermatophylax (Warwick *et al.*, 2009; Gershman *et al.*, 2012). Very little is known about how amino acids are regulated in the spermatophylax, although evidence from studies using *D.melanogaster* suggest that diet is a prime candidate (McGraw *et al.*, 2007; Fricke *et al.*, 2008). Unfortunately these studies lack an understanding of the relationship between specific nutrients and the production of manipulative chemicals. In Chapter 3, I use the GF to specifically look for any relationship between the specific nutrients P and C and the amino acid composition of the spermatophylax produced by male *G.sigillatus* and whether any dietary effect could mediate sexual conflict by influencing the time spent by a female in consuming a gift before terminating sperm transfer. Furthermore, I conduct a choice feeding experiment to determine if males can regulate their intake of P and C to optimize the size and chemical composition of the spermatophylax.

Trade-offs between life-history traits are an important part of life-history theory and are central for predicting the optimal life-history of an organism in a given environment (Reznick, 1985; Stearns, 1992; 2000; Roff, 1993; Rolff, 2002; Roff & Fairbairn, 2007; Reznick *et al.*, 2000). Life-history trade-offs exist because different life-history traits compete for a finite pool of resources, with the allocation of resources to one trait necessarily causing a reduction in available resources to allocate to other traits (Stearns, 1992; Rolff, 2002). . Typically, life-history studies examine trade-offs by experimentally altering resource availability through dietary manipulations (Reznick, 1985; Reznick *et al.*, 2000; Roff & Fairbairn, 2007). Indeed, a number of empirical studies across a range of animal taxa have shown that manipulating the quantity and/or quality of the available diet can have a profound effect on the trade-offs between different life-history traits (Hill & Kaplan, 1999; Brown & Shine, 2002; Lardner & Loman, 2003; Hunt *et al.*, 2004). Two major determinants of fitness are variance in reproduction and immune function (Reznick, 1985; Stearns, 1992; Rolff, 2002). Both processes are energetically demanding and have been shown to trade-off in a range of animal taxa (Ahtiainen *et al.*, 2004; French *et al.*, 2007; McCallum & Trauth, 2007; Mills *et al.*, 2010; Schwenke *et al.*, 2016) as well as differing optimal reproductive strategies driving the evolution of sex differences in the trade-off between these traits (Rolff, 2002; Zuk & Stoehr, 2002; Zuk, 2009). A major limitation with previous life-history

trade-off studies that use diet to manipulate resource acquisition is that typically only a few diets of poorly defined nutritional composition are used and diet consumption is rarely measured (Holzer *et al.*, 2003; Hunt *et al.*, 2004). The use of the GF can overcome these limitations and partition the effects of the intake of specific nutrients and calories on the expression of different life-history traits and indeed empirical studies using the GF have shown nutritional trade-offs between lifespan and reproduction (Lee *et al.*, 2008a; Maklakov *et al.*, 2008; Jensen *et al.*, 2015). While demonstrating that life-history traits have different nutritional optima can be seen as evidence for nutrient based trade-offs, a robust framework that formally documents the existence and strength of any nutritionally based trade-offs is currently lacking. Such a framework would have to incorporate a standardized way of comparing not only the effects of nutrient intake on a number of different life-history traits but also comparing life-history traits between the sexes and across different species. Furthermore, any comparative framework would have to accurately quantify the magnitude of any nutritional based differences between life-history traits. In Chapter 4, I present a conceptual framework to show that nutritional trade-offs occur when life-history traits are maximised in different regions of nutrient space and quantify this divergence by calculating the 95% confidence region of the global nutritional maxima, the angle (θ) between linear nutritional vectors and the Euclidean distance (d) between the global nutritional maxima for each trait. I then empirically test this framework by examining the effects of P and C intake on the trade-off between reproduction and immune function in both male and female *G. sigillatus*.

Chapter 4 highlighted trade-offs between trait-specific nutritional optima for reproductive effort and immune function as well as differences in the strength of these trade-offs between the sexes. Another important set of life-history trade-offs has been shown between reproductive effort and lifespan, with CR shown to increase lifespan across a range of species (Nakagawa *et al.*, 2012). This trade-off is typically more pronounced in females and is explained through the divergent energetic costs of reproduction of the sexes (Bonduriansky *et al.*, 2008) with increased lifespan in females through CR explained through a corresponding decrease in fecundity (Chapman & Partridge, 1996) whilst males having comparably lower energetic demands for reproduction, show less of a trade-off between reproduction and lifespan (Bonduriansky *et al.*, 2008). Recent GF studies have shown that it is the balanced intake of specific nutrients rather than caloric intake *per se* that mediates

the trade-off between reproduction and lifespan, with the sexes having very different nutritional optima for these traits (Lee *et al.*, 2008a; Maklakov *et al.*, 2008; Reddix *et al.*, 2013; Jensen *et al.*, 2015). Given the shared genetic architecture between the sexes for the expression of life-history traits as well as the controlled regulation of dietary intake, the divergent sex-specific nutritional optima for the maximal expression of these life-history traits provides the potential for ISC, whereby the one or both of the sexes are displaced from their nutritional fitness optima (Lande, 1980; Bonduriansky & Chenoweth, 2009). ISC is theoretically at its strongest whenever selection acting on shared phenotypic traits is directly opposing in the sexes and the genetic correlation for the shared trait(s) is strong and positive between the sexes (i.e. the inter-sexual genetic correlation) (Bonduriansky & Chenoweth, 2009). To empirically demonstrate ISC over optimal nutrient intake, one must first show sex differences in the effects of nutrients on shared phenotypic traits, i.e. reproductive effort and lifespan (Bonduriansky & Chenoweth, 2009; Lewis *et al.*, 2011). One must then calculate the genetic (co)variances within and between the sexes for these life-history traits and show the presence of a positive genetic covariance between the sexes for the intake of nutrients (Bonduriansky & Chenoweth, 2009; Lewis *et al.*, 2011). A number of GF studies have shown sex differences in the required nutrient intake for the optimal expression of reproduction and lifespan (Lee *et al.*, 2008a; Maklakov *et al.*, 2008; Jensen *et al.*, 2015), however, only one study has gone further and estimated the genetic (co)variances acting within and between the sexes to specifically test for a positive genetic covariance between the sexes (Reddix *et al.*, 2013). Despite finding limited ISC, there are limitations to the study by Reddix *et al.* (2013), for example, only having a four day feeding period which would not have allowed enough time to see significant differences between nutrient intake in the sexes. Due to these limitations, the findings of Reddix *et al.* (2013) might have under-estimated the strength of ISC acting on nutritional choice. To address this gap in knowledge, in Chapter 5 I use the field cricket *T.commodus* to demonstrate the differences between the sexes for optimal nutrient intake for maximal expression of reproductive effort and lifespan. I then use a half-sib breeding design to conduct a quantitative genetic study of nutritional choice in males and females and show positive genetic covariances between the sexes for P and C intake. With this evidence of ISC over nutritional choice I perform further cutting edge statistical tests to predict with confidence the evolutionary responses of reproductive effort and lifespan in males and females to this

opposing selection and the strength of genetic constraints acting on these shared life-history traits.

The previous chapters of this thesis and the findings of other GF studies (Lee *et al.*, 2008a; Maklakov *et al.*, 2008; Reddiex *et al.*, 2013; Solon-Biet *et al.*, 2014; Bunning *et al.*, 2015; 2016; House *et al.*, 2015; Jensen *et al.*, 2015) have all shown sex- and trait-specific nutritional optima for numerous life-history traits. Optimal foraging theory predicts that animals will evolve foraging mechanisms that maximise their fitness (Stephens & Krebs, 1986) with a number of regulatory mechanisms found to exist. For example, compensatory feeding if restricted to a single diet that is deficient in an important nutrient or by feeding from a number of different nutritionally imbalanced foods to maintain a constant intake of a specific nutrient ratio (Simpson & Raubenheimer, 2012). Despite these regulatory mechanisms, animals are often constrained from reaching their nutritional optima for a specific life-history trait, either due to trade-offs with other traits (Holzer *et al.*, 2003; Hunt *et al.*, 2004; Lee *et al.*, 2008a; Maklakov *et al.*, 2008; Cotter *et al.*, 2011; Jensen *et al.*, 2015) or due to the shared genetic basis between the sexes for nutritional preference preventing either sex from expressing an independent nutritional preference (Chapter 5; Maklakov *et al.*, 2008; Reddiex *et al.*, 2013; Jensen *et al.*, 2015). Such genetic constraints could cause the over-ingestion of nutrients when in nutritionally imbalanced environments. It is this over-ingestion of nutrients, specifically energy-rich C and fat, that is blamed for the unprecedented increase in worldwide rates of obesity (Martens *et al.*, 2013) in numerous animal species ranging from vertebrates to invertebrates (Simpson & Raubenheimer, 2012). Despite evidence for a genetic control of obesity (Mathes *et al.*, 2011) and of nutrient regulation in both sexes (Chapter 5; Reddiex *et al.*, 2013), our understanding of the interactions between these genes and the dietary environment is lacking, especially for the consequences of gene by dietary environment interactions on lipid deposition and the potential of sexual selection to mediate any effects. In Chapter 6, I address these questions by performing further analyses on the genetic estimates of dietary preference in male and female *T.commodus* presented in Chapter 5 and include the lipid mass of the individuals in the analysis. I find clear gene by sex by dietary environment interactions but deeper analysis reveals complex relationships between weight gain, nutrient preference and dietary environment which hints at the difficulty of predicting the effects of genetic interactions on obesity.

In Chapter 7, I conclude by summarising the findings of these studies and discuss them in the broad context of sexual selection and conflict before theorising on future areas of research to build upon the work of this thesis.

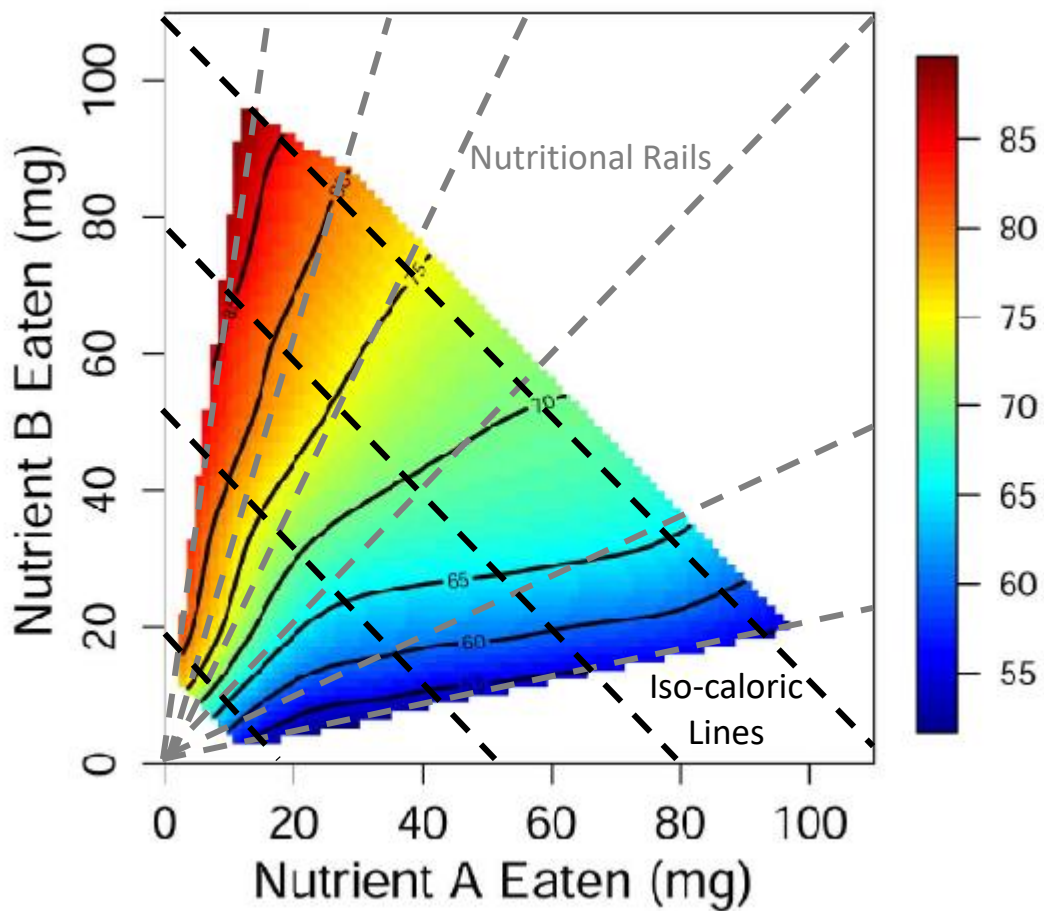


Figure 1.1. An example of a nutritional landscape. Animals can feed along the nutritional rails (dashed grey lines) and a trait of interest is regressed onto the intake data for nutrients A and B. Regions on the surface in red correspond to the optimal expression of the trait of interest, with blue regions showing low values for trait expression. The dashed black lines are iso-caloric lines which connect diets of equal caloric value across the nutritional rails.

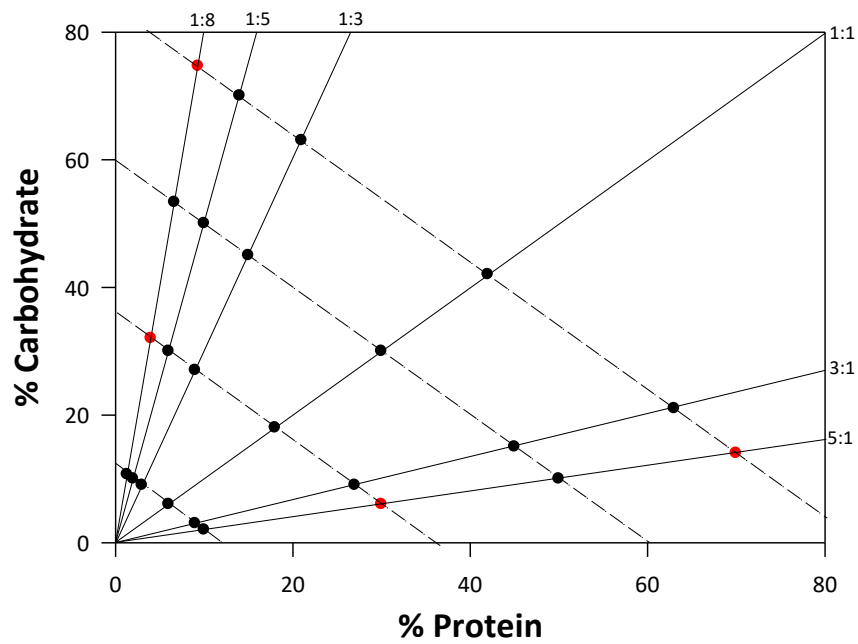


Figure 1.2. The distribution of the 24 artificial diets used in this thesis that define the nutritional space. Solid black lines represent the six nutritional rails (P:C), with four diets per rail that differ in total nutritional content (P+C). Each black dot represents an individual diet with red dots representing the diets used to make the dietary choice feeding pairs. The diets on each rail that have the same total nutrition are connected by iso-caloric lines (dashed, black lines).

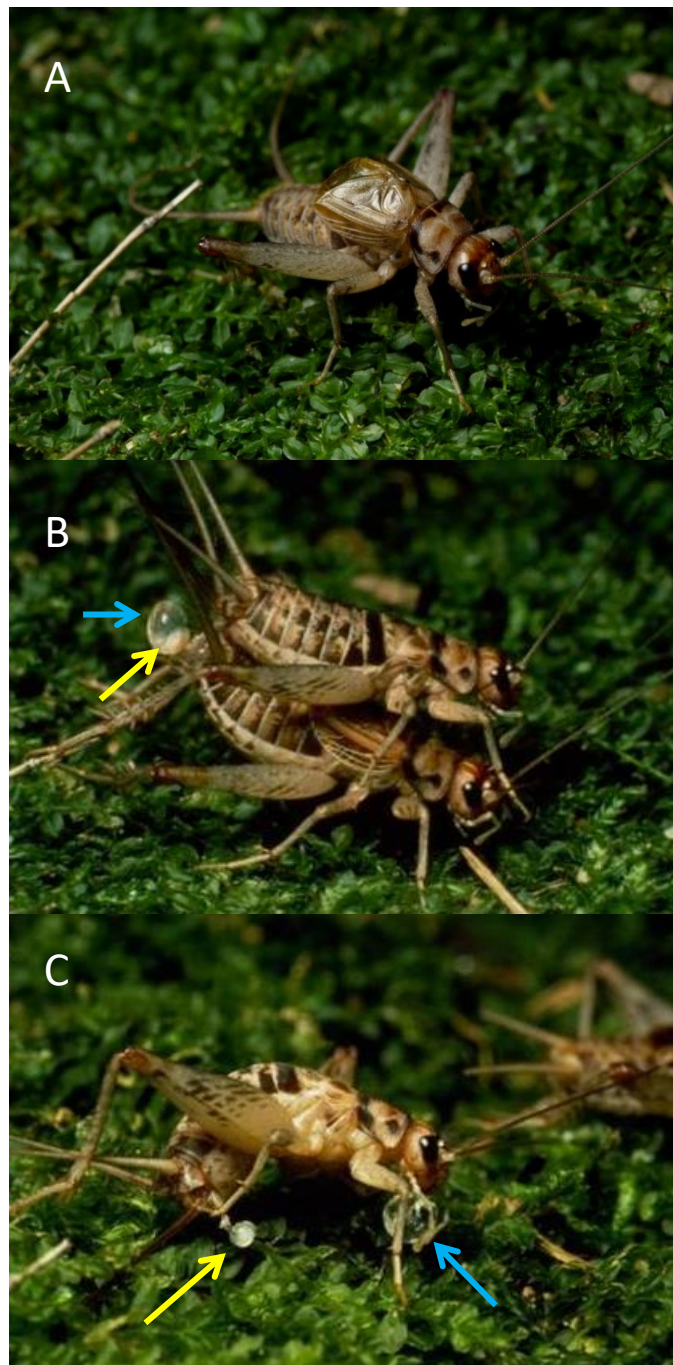


Figure 1.3. (A) A male decorated cricket, *Gryllodes sigillatus*, calling to attract a mate; (B) a mating pair with the male (bottom) transferring a spermatophore containing a gelatinous spermatophylax (blue arrow) and sperm containing ampulla (yellow arrow) to the female (top); (C) a female consuming the spermatophylax (blue arrow), with the ampulla (white arrow) remaining attached, transferring sperm. After consuming the spermatophylax the female will remove the ampulla and terminate sperm transfer. The male can be seen in the background guarding the female to prevent premature removal of the ampulla and the courtship of other nearby males. Photos are courtesy of David Funk and Scott Sakaluk.



Figure 1.4. (A) A mating pair of *Teleogryllus commodus* with a male (bottom) and female (top); (B) a male transferring a sperm containing ampulla (black arrow) to the female. Photos are courtesy of John Hunt.

Table 1.1. Protein (P) and carbohydrate (C) composition of the 24 artificial diets used in this thesis. The total nutrient concentration in each diet is given as the sum of the percentage P and percentage C, with the remaining percentage consisting of indigestible crystalline cellulose plus micronutrients. Diets highlighted in bold are the four diets highlighted in red in Figure 1.2 which are used in choice feeding experiments.

| Diet Number | Percentage Composition | | | P:C |
|-------------|------------------------|------------------|-----------|------------|
| | Protein (P) | Carbohydrate (C) | P+C | |
| 1 | 10 | 2 | 12 | 5:1 |
| 2 | 30 | 6 | 36 | 5:1 |
| 3 | 50 | 10 | 60 | 5:1 |
| 4 | 70 | 14 | 84 | 5:1 |
| 5 | 9 | 3 | 12 | 3:1 |
| 6 | 27 | 9 | 36 | 3:1 |
| 7 | 45 | 15 | 60 | 3:1 |
| 8 | 63 | 21 | 84 | 3:1 |
| 9 | 6 | 6 | 12 | 1:1 |
| 10 | 18 | 18 | 36 | 1:1 |
| 11 | 30 | 30 | 60 | 1:1 |
| 12 | 42 | 42 | 84 | 1:1 |
| 13 | 3 | 9 | 12 | 1:3 |
| 14 | 9 | 27 | 36 | 1:3 |
| 15 | 15 | 45 | 60 | 1:3 |
| 16 | 21 | 63 | 84 | 1:3 |
| 17 | 2 | 10 | 12 | 1:5 |
| 18 | 6 | 30 | 36 | 1:5 |
| 19 | 10 | 50 | 60 | 1:5 |
| 20 | 14 | 70 | 84 | 1:5 |
| 21 | 1.33 | 10.66 | 12 | 1:8 |
| 22 | 4 | 32 | 36 | 1:8 |
| 23 | 6.66 | 53.33 | 60 | 1:8 |
| 24 | 9.33 | 74.66 | 84 | 1:8 |

CHAPTER 2:
**THE COMPLEX INTERPLAY BETWEEN MACRONUTRIENT INTAKE,
CUTICULAR HYDROCARBON EXPRESSION AND MATING SUCCESS IN
MALE DECORATED CRICKETS *GRYLLODES SIGILLATUS***

2.1. ABSTRACT

The condition-dependence of male sexual traits plays a central role in sexual selection theory. Relatively little, however, is known about the condition-dependence of chemical signals used in mate choice and their subsequent effects on male mating success. Furthermore, few studies have isolated the specific nutrient(s) responsible for condition-dependent variation in male sexual traits. Here I used the geometric framework to determine the effect of protein (P) and carbohydrate (C) intake on male cuticular hydrocarbon (CHC) expression and mating success in male decorated crickets (*Gryllodes sigillatus*). I show that both traits are maximised at a moderate to high intake of nutrients in a P:C ratio of 1:1.5. I also show that female pre-copulatory mate choice exerts a complex pattern of linear and quadratic sexual selection on this condition-dependent variation in male CHC expression. Structural equation modelling revealed that although the effect of nutrient intake on mating success is mediated through condition-dependent CHC expression, it is not exclusively so, suggesting that other trait(s) must also play an important role. Collectively, these results suggest that the complex interplay between nutrient intake, CHC expression and mating success play an important role in the operation of sexual selection in *G. sigillatus*.

Key Words: Chemical Signals, Macronutrients, Mating Success, Selection Analysis, Sexual Selection, *Gryllodes sigillatus*

2.2. INTRODUCTION

Sexual selection is widely acknowledged as a major driving force in the evolution of exaggerated male sexual traits (Andersson, 1994; Andersson & Simmons, 2006). A dominant model in the sexual selection literature, known as the handicap model (Zahavi, 1975; 1977; Johnstone *et al.*, 2009), posits that male sexual traits should serve as reliable indicators of male quality in female mate choice (Johnstone, 1995; Lailvaux & Irschick, 2006) and male-male competition (Zahavi, 1975; 1977; Andersson, 1994; Andersson & Simmons, 2006; Johnstone *et al.*, 2009). According to this model, reliability is maintained by the fact that exaggerated sexual traits are costly to produce: because only males of high quality can afford these costs, exaggerated sexual traits should remain honest because they are impossible to mimic by low quality males (Zahavi, 1975; 1977; Johnstone *et al.*, 2009). If male sexual traits are costly to produce and/or maintain, they should be sensitive to variation in the acquisition of resources and also be subject to trade-offs resulting from variation in resource allocation (Zahavi, 1975; 1977; Rowe & Houle, 1996; Hunt *et al.*, 2004). Indeed a central prediction underlying handicap models of sexual selection is that male sexual trait expression should co-vary positively with condition, which can be conceptually defined as the amount of resources an organism has available for allocation to fitness-enhancing traits (Rowe & Houle, 1996; Hunt *et al.*, 2004; Tomkins *et al.*, 2004).

Almost two decades has been spent empirically testing this core prediction and many examples now exist in a range of species showing that male sexual traits show condition-dependent expression (Cotton *et al.*, 2004; Hunt *et al.*, 2004; Tomkins *et al.*, 2004). The most common approach used in empirical studies to experimentally manipulate condition is to vary diet quantity and/or the caloric content of the diet (Cotton *et al.*, 2004; Tomkins *et al.*, 2004). This approach, however, has two main limitations when studying the condition-dependence of male sexual traits. First, typically only a small number of diets are used (i.e. two or three) and these diets are often poorly defined with regard to specific nutrient content (e.g. Holzer *et al.*, 2003; Cotton *et al.*, 2004; Hunt *et al.*, 2004). This makes it difficult (if not impossible) to partition the effects of calories and specific nutrients on male sexual trait expression. Second, most studies do not precisely measure food consumption and therefore ignore any effects of compensatory feeding. Compensatory feeding, the ability of an individual to increase its food consumption to compensate for

reduced food quality (Simpson & Raubenheimer, 2012), appears widespread in animals (Behmer, 2009) and means that it is possible for individuals on poor quality diets to consume as many calories or nutrients as on a good quality diet. Compensatory feeding therefore has the potential to obscure any differences in condition-dependence across dietary treatments.

These limitations can be resolved using chemically defined (holidic) diets of known nutrient composition within the Geometric Framework (GF) for nutrition (Simpson & Raubenheimer, 2012). The GF is a multidimensional nutritional approach, within which the effects of the intake of multiple nutrients (n) can be separated in n -dimensional nutritional space by restricting individuals to a geometric array of diets that differ in known nutrient composition and concentration (i.e. calories)(Simpson & Raubenheimer, 2012). When combined with precise measurements of diet consumption (allowing nutrient intake to be calculated), the GF provides a powerful way to partition the effects of specific nutrient and caloric intake on condition-dependent sexual trait expression (Morehouse *et al.*, 2010). Indeed, empirical studies on a number of insect species have shown that a balanced intake of nutrients is more important to sexual trait expression than the intake of calories *per se* (Maklakov *et al.*, 2008; Sentinella *et al.*, 2013; Harrison *et al.*, 2014; Bunning *et al.*, 2015; Cordes *et al.*, 2015; House *et al.*, 2015).

Most empirical studies on the condition-dependence of male sexual traits in insects have focussed on conspicuous male sexual traits, including morphological structures that serve as weapons (e.g. Johns *et al.*, 2014) and ornaments (e.g. Cotton *et al.*, 2004), courtship displays (e.g. Kotiaho, 2002), acoustic signals (e.g. Hunt *et al.*, 2004) and colouration (e.g. Punzalan *et al.*, 2008). In contrast, the condition-dependence of chemical signals is relatively poorly studied, which is surprising given that sex pheromones and cuticular hydrocarbons (CHCs) play an important role in the recognition of species, the sexes and kin in insects (Wyatt, 2003; Blomquist & Bagnères, 2010). Male CHCs are also known to be the target of female choice in various cricket (Thomas & Simmons, 2009; 2011; Weddle *et al.*, 2012; Steiger *et al.*, 2013; 2015) and *Drosophila* (Ferveur, 2005; Ingleby *et al.*, 2013; 2014) species. There is also growing evidence that sex pheromone (e.g. Conner *et al.*, 1981; Clark *et al.*, 1997; McGuigan, 2006; Ming & Lewis, 2010) and CHC (e.g. Liang & Silverman, 2000; Hine *et al.*, 2004; Gosden & Chenoweth, 2011; Weddle *et al.*, 2012) expression is sensitive to diet, but the actual nutrient(s) responsible for these effects is poorly understood.

To date, only two studies have used the GF to examine the condition-dependence of chemical signals (South *et al.*, 2011; Fedina *et al.*, 2012). Fedina *et al.* (2012) provided female *D. melanogaster* with four diets differing in the percentage of sugar (S) to yeast (Y) in a factorial design and examined CHC expression at various ages. Diet composition was shown to have consistent and significant effects on female CHCs across ages, with dietary S and Y driving changes in CHCs in opposite directions. For example, there was nearly a two-fold increase in the total CHCs produced by females with age when consuming a high Y diet, whereas females consuming low Y diets maintained similar levels of CHCs with age. In contrast, the amount of dietary S consumed did not influence total CHC levels or their change with age. This study is limited, however, by the fact that the use of medium-based diets precluded the measurement of dietary intake, and yeast was used as the only source of protein. While yeast is high in protein, it also contains carbohydrate, lipids, salts and a number of vitamins making it impossible to identify which key nutrient(s) are responsible for the above effects of yeast consumption. In contrast, South *et al.* (2011) used a much larger number of holidic diets and precisely measured the intake of protein (P) and carbohydrate (C) to determine the effects of these macronutrients on the expression of the three male sex pheromones (3-hydroxy-2-butanone, 2-methylthiazolidine and 4-ethyl-2-methoxyphenol) in the cockroach *Nauphoeta cinerea*, and the subsequent effects on dominance and attractiveness. All three sex pheromones and male attractiveness increased with the intake of C (being maximised at a P:C ratio of 1:8) but were largely unaffected by the intake of P, whereas male dominance was not affected by the intake of either nutrient. Furthermore, when given a choice between alternate diets, males preferentially consumed high C diets to maximize their attractiveness to females. This work therefore not only illustrates how nutrients can have very different effects on the condition-dependent expression of chemical signals, but also that this can have important consequences for male mating success and the subsequent operation of sexual selection.

In the decorated cricket (*Gryllodes sigillatus*), CHCs play an important role in regulating sexual selection. Females have been shown to exert a complex pattern of linear and nonlinear (mainly stabilizing) sexual selection on male CHCs during pre-copulatory mate choice and this preference appears independent of the similarity in CHC profile of the male and choosing female (Steiger *et al.*, 2015). As CHC expression has a strong genetic basis in *G. sigillatus* (Weddle *et al.*, 2012; 2013), this finding suggests that females do not use CHCs

during mate choice to avoid inbreeding. This is supported by the fact that females do not prefer unrelated males over full-sib or half-sib brothers in pre-copulatory mate choice trials (Weddle *et al.*, 2013). CHCs also play an important role in regulating polyandry in *G. sigillatus* (Ivy *et al.*, 2005; Weddle *et al.*, 2013). Female *G. sigillatus* mate frequently through their lifetime (Sakaluk, 2002) and have been shown to prefer mating with novel males over previous mates (Ivy *et al.*, 2005; Weddle *et al.*, 2013). Females are able to discriminate against previous mating partners by physically imbuing males with their own CHCs during mating, rendering the CHC profile of the male to more closely resemble his female partner (Ivy *et al.*, 2005; Weddle *et al.*, 2013). Experimental perfuming of males with female CHCs has shown a female aversion towards males bearing their chemical signature (Weddle *et al.*, 2013), and recent work has shown that females use a simple form of “online processing”, whereby a female assesses her own CHC profile and compares it to the CHC profile of her potential mate before deciding whether or not to mate, to regulate this process (Capodeanu-Nagler *et al.*, 2014). CHC expression in *G. sigillatus* is known to be influenced by diet but this effect is sex-specific (Weddle *et al.*, 2012). Using a series of inbred lines, Weddle *et al.* (2012) varied the quality of the diet fed to juvenile and adult crickets and found that while the effects of diet and genotype-by-diet interactions on CHC expression were pronounced in males, dietary effects were small and genotype-by-diet interactions were absent in females. This differential response in males and females suggests that CHCs may be under sex-specific selection to maintain signal reliability: the lack of dietary effects and genotype-by-diet interactions for CHCs would facilitate chemosensory self-referencing by females (Weddle *et al.*, 2012). Unfortunately, the high and low quality diets used in this study varied in both nutrient composition and overall energy content, so it is impossible to determine whether the observed dietary effects on male CHCs is due to the intake of specific nutrients or calories *per se*. Furthermore, it is currently not known if diet also influences male mating success and if so, the role that condition-dependent CHC expression plays in mediating this effect.

In this study, I examine the effects of P and C intake on CHC expression in male *G. sigillatus*, as well as the subsequent effects of nutrient intake and CHC expression on mating success. I start by using a GF approach and restricting adult males to one of 24 unique artificial, holidic diets in a geometric array to document the linear and nonlinear effects of nutrient intake on CHC expression and male mating success. Using data from my other work

on *G. sigillatus* (Chapter 3), I also map the regulated intake point for P and C (defined as the point in nutrient space that individuals actively defend when given dietary choice (Simpson & Raubenheimer, 1993)) onto the nutritional landscapes for CHC expression and mating success to determine if males optimally regulate their intake of nutrients to maximise these traits. Next, I use multivariate selection analysis to determine how this condition-dependent variation in male CHC expression influences male mating success and therefore the strength and form of sexual selection targeting male CHCs. Finally, I use a structural equation modelling (SEM) approach to determine whether the effects of nutrient intake on mating success are mediated exclusively by condition-dependent CHC expression or whether other sexual traits(s) are also involved in mediating this relationship. If nutrient intake has similar effects on male CHC expression and mating success, I predict that the landscapes for these traits will be closely aligned in nutrient space. Furthermore, if the condition-dependent expression of CHCs is a key determinant of male mating success, I predict that female mate choice will exert significant sexual selection on male CHC expression and that the SEM model will show that the effect of nutrients on mating success is mediated exclusively through CHC expression.

2.3. MATERIALS AND METHODS

2.3.1. Experimental animals

The *G. sigillatus* used in this study were descended from 500 adults crickets collected in Las Cruces, New Mexico in 2001 which were used to initiate a laboratory culture maintained at a population size of approximately 5000 crickets and allowed to breed panmictically (Ivy & Sakaluk, 2005). Cricket cultures were housed in ten 15L plastic containers in an environmental chamber (Percival I-66VL) maintained at $32 \pm 1^\circ\text{C}$ on a 14h:10h light/dark cycle and provided with cat food (Go-Cat Senior[®], Purina, St Louis, MO, USA), rat food pellets (SDS Diets, Essex, UK) and water *ad libitum* in 60ml glass test tubes plugged with cotton wool and an abundance of cardboard egg cartons to provide shelter. Each generation, nymphs were collected at hatching and randomly allocated across culture containers to enforce gene flow in the cultures.

Experimental crickets were collected from laboratory cultures as newly hatched nymphs and housed individually in a plastic container (5cm x 5cm x 5cm). Each container

was provided with a piece of cardboard egg carton for shelter and water in a 2.5ml test tube plugged with cotton wool. Nymphs were fed ground cat food pellets for the first two weeks post hatching and thereafter solid pellets until eclosion to adulthood. Containers were cleaned and food and water replaced weekly. Experimental animals were checked daily for eclosion to adulthood. At eclosion, males were transferred to a larger individual plastic container (20cm x 10cm x 10cm) and then randomly assigned to a diet.

2.3.2. Artificial diets and measuring dietary intake

I made 24 artificial, dry diets that varied in P:C ratio, as well as overall nutritional content, based on the established protocol outlined in Simpson and Abisgold (1985). The composition of these diets can be seen in Table 1.1 and their distribution in nutritional space can be visualized in Figure 1.2.

Each experimental male was randomly assigned to a diet and provided with a single feeding platform of diet of measured dry weight on their day of eclosion to adulthood. As expected there was no difference in the pronotum width ($F_{23,744} = 0.70$, $P = 0.85$) or body weight ($F_{23,744} = 0.95$, $P = 0.53$) of males across these 24 diets. This diet was changed every 2 days for a total of 10 days (5 feeding sessions) until males were sexually mature and mating behaviour and CHC profile was assessed (see below). Food and water were provided in feeding platforms constructed by gluing a vial lid (1.6 cm diameter, 1.6 cm deep) upside down onto a petri dish (5.5 cm diameter). This design allowed any diet spilled during feeding to be collected in the petri dish. Diet was kept in a drying oven (Binder® model FD 115, Germany) at 30°C for 48 hours to remove any moisture prior to weighing. Feeding platforms containing diet were weighed before and after each feeding period, using an electronic balance (Ohaus Explorer Professional model EP214C, USA). Prior to the final weighing, any faeces were removed from the feeding platform using a pair of fine forceps. Diet consumption was calculated as the difference in dry weight of diet before and after feeding. This was converted to a P and C intake by multiplying by the percentage of these nutrients in the diet following the procedure outlined in South *et al.* (2011).

2.3.3. Experimental design and measuring male mating success

To determine the effects of P and C intake on the CHC profile of males, as well as their subsequent mating success, 32 males were established at random on each of the 24

diets (total $n=768$ males) on their day of eclosion to adulthood and fed for 10 days to quantify their intake of P and C. At day 10, each male was placed in a clear plastic arena (20 x 10 x 10cm), illuminated by red light and maintained in a constant temperature room maintained at $28 \pm 1^\circ\text{C}$, and allowed to acclimate for 60 seconds before a virgin, 10 day old female was introduced. The male was given 20 minutes to initiate courtship after the female had been introduced into the arena. If the male failed to court during this period, he was excluded from the study, as I could not be certain the females had assessed his quality. A mating was recorded as successful if the male transferred a spermatophore to the female in this 20 minute period. Successful males were assigned a score of 1 and those that were unsuccessful were assigned a score of 0. At the end of behavioural observations, each male was immediately placed in an individual micro-centrifuge tube and frozen at -80°C for CHC analysis.

2.3.4. Extraction and chemical analysis of CHCs

CHCs were extracted by whole-body immersion in a 5ml glass vial containing 3mL of HPLC-grade hexane (Fisher Scientific, UK) and 10 ppm dodecane as an internal standard for 5 minutes. The cricket was removed from the vial using metal forceps which were cleaned in methanol between each sample. 50 μL of the extract was then transferred to a glass auto-sample vial (Chromacol, UK).

CHC extracts were run on an Agilent Technologies 7890 Gas Chromatography with Flame Ionization Detector (GC-FID). A 2 μL volume of each extract was injected using an Agilent G4513A autosampler connected to an Agilent 7692 sample tray chilled to 5°C onto a DB-1 column (30m x 0.25mm internal diameter x 0.25 μm film thickness). Hydrogen was used as the carrier gas. Both inlet lines were set at 325°C and the injection was in pulsed splitless mode. Separation of the extract was optimized using a column profile which operated at 50°C for 30 seconds and then increased at $20^\circ\text{C min}^{-1}$ to 320°C where it then increased at $7.5^\circ\text{C min}^{-1}$ to 350°C where it was held for 5 minutes with a flow rate of 20 mL min^{-1} . The total run time for each extract was therefore 23 minutes. The area under each CHC peak was quantified using CHEMSTATION software (v. B.04.02.SP1, Agilent Technologies, UK).

Prior to statistical analysis, the area under each CHC peak was divided by the area of the internal standard (dodecane) to control for drift in the sensitivity of the GC-FID over

time. This proportion was then \log_{10} transformed to ensure normality of each CHC peak in our dataset (Weddle *et al.*, 2012; 2013). Two CHC peaks identified in previous studies on *G. sigillatus* (5,9-C₃₇diene and 3,9-C₃₇diene, Weddle *et al.*, 2012; 2013; Steiger *et al.*, 2015) were not present in all samples and were therefore excluded from further analysis.

2.3.5. Statistical analysis

Due to the large number of CHC peaks being examined (see Table 2.1), I extracted principal components (PCs) based on the correlation matrix and retained PCs with eigenvalues exceeding 1 for subsequent analyses (Tabachnick & Fidell, 2001). In total, 3 PCs were retained for further analysis based on this criterion. Component loadings exceeding $|0.30|$ were interpreted as biologically important (Tabachnick & Fidell, 2001).

I used a multivariate response surface approach (South *et al.*, 2011) to determine the linear and nonlinear effects of P and C intake on the PCs describing the variation in male CHC expression and mating success. Prior to analysis, I standardized nutrient intake and the response variables to a mean of zero and a standard deviation of one using a Z-transformation so that the nutritional gradients were in comparable units and to ensure that any differences between nutritional gradients were not driven by scale alone (see below). The measure of male mating success (0 or 1) did not conform to a normal distribution and while this does not influence the estimation of gradients from a response surface (Lande & Arnold. 1983), it can bias tests of their statistical significance (Mitchell-Olds & Shaw. 1987). I therefore, used a resampling procedure to assess the significance of the nutritional gradients (Mitchell-Olds & Shaw. 1987). I randomly shuffled mating success across males in the data set to create a null distribution for each nutritional gradient where there is no relationship between the intake of nutrients and mating success. I then used a Monte Carlo simulation to determine the number of times (out of 10,000 iterations) that each nutritional pseudo-gradient (β_{rand}) was greater than or equal to the original nutritional gradient (β_{real}) and this was used to calculate a two-tailed probability value following the protocol outlined in Manly (1997). I used nonparametric thin-plate splines (Green & Silverman. 1994) to visualize the nutritional landscapes for each of the response variables. Thin-plate splines were constructed using the *Tps* function in the 'FIELDS' package of R (version 2.15.1, www.r-project.org) and were visualized as contour maps using the value of the smoothing parameter (λ) that minimized the generalized cross-validation score (Green

& Silverman. 1994). Although analyses were conducted on standardized data, I visualized the nutritional landscapes on raw data for ease of interpretation.

I used a sequential model building approach (Draper & John. 1988) to determine whether the linear and nonlinear effects of P and C intake differed across the response variables (South *et al.* 2011). Full details of this approach are provided elsewhere (South *et al.*, 2011; Bunning *et al.*, 2015; and in Appendix 2). In brief, I started by fitting a linear model to the data, including a dummy variable (response type = PC1, PC2, PC3 or mating success) as a fixed effect, P and C intake as covariates and the actual measures associated with the dummy variable as the response variable. From this reduced model I extracted the residual sums of squares (SS_r). I then ran a second linear model that included all the interactions between the dummy variable and the covariates and again extracted the residual sums of squares for this complete model (SS_c). A partial *F*-test was then used to statistically compare SS_r and SS_c , whereby a significant reduction in SS_c compared to SS_r indicates that the complete model significantly increases the amount of variance explained and therefore demonstrates that the nutritional gradients differ significantly across the dummy variable. This model was repeated by sequentially adding the quadratic terms for nutrient intake (P x P and C x C) and then the correlational term (P x C). In cases where an overall significant difference was detected, univariate interaction terms from the complete model were used to determine which nutrient(s) contributed to this effect. Importantly, this approach only statistically compares the magnitude of linear and nonlinear nutritional gradients between the response variables and does not provide any information of the direction of this difference in nutritional space. Therefore, it is possible for two response variables to differ in the magnitude of their nutritional gradients, but be optimized in similar regions on the nutritional landscape. Consequently, I also calculated the angle (θ) between the linear vectors for the two response variables being compared using trigonometry and the 95% confidence interval for θ using a Bayesian approach implemented in the “MCMCglmm” package of R (version 2.15.1, www.r-project.org). When $\theta = 0^\circ$ the vectors are perfectly aligned and the optima are located in the same region of nutrient space, whereas $\theta = 180^\circ$ represents the maximum possible divergence between these vectors. Full details of these calculations and associated R code are presented in Appendix 3. The above statistical comparisons were only conducted between response variables that showed a statistically significant effect of nutrient intake.

I used standard multivariate selection analysis (Lande & Arnold, 1983) to evaluate the strength and form of linear and nonlinear sexual selection acting on male CHCs through female mate choice. Following convention (Lande & Arnold, 1983), I transformed the absolute measure of male mating success (i.e. 1 = successful, 0 = unsuccessful) to relative mating success by dividing by the mean absolute mating success of the population. I used this measure of relative mating success as a proxy for relative fitness in the population (e.g. Steiger *et al.*, 2015). To estimate the standardized linear selection gradients (β), a first-order linear multiple regression model was fitted using the three PCs describing the variation in male CHC expression as the predictor variables and relative fitness as the response variable (Lande & Arnold, 1983). I then used a second-order quadratic multiple regression model that included all linear, quadratic and cross-product terms to estimate the matrix of standardized nonlinear selection gradients (γ) that describes the curvature of the fitness surface (Lande & Arnold, 1983). As multiple regression analysis is known to underestimate the quadratic regression coefficients by a factor of 0.5, I doubled the standardized quadratic selection gradients derived from this model (Stinchcombe *et al.*, 2008). Since relative fitness does not conform to a normal distribution, I used the Monte Carlo procedure outlined above to test the significance of the standardized selection gradients.

The strength of nonlinear selection gradients are known to be underestimated by interpreting the size and significance of individual γ gradients (Blows & Brooks, 2003). I therefore used canonical analysis of the γ matrix to locate the major eigenvectors of the fitness surface (Phillips & Arnold, 1989). I used the double regression method (Bisgaard & Ankenman, 1996) to estimate the strength of linear selection (θ_j) operating along each eigenvector (\mathbf{m}_j). This approach, however, is known to inflate type I error when estimating the strength of nonlinear selection (λ_j) operating along \mathbf{m}_j . I therefore used the permutation procedure outlined in Reynolds *et al.*, (2010) to determine the strength and significance of nonlinear selection operating along λ_j . I used thin-plate splines (Green & Silverman, 1994) to visualize the major eigenvectors of the fitness surface following the procedure outlined above for the visualization of nutritional landscapes.

I used SEM to partition the direct effects of nutrient intake on mating success from the indirect effects of nutrient intake on mating success that are mediated through CHC expression. I evaluated two competing models. In the first, I modelled mating success as influenced directly by the standardized linear and quadratic effects of nutrient intake and

indirectly through their influence on CHCs (Figure 2.1). I refer to this model as the *partial mediation model*, as it estimates the residual influence of nutrient intake on mating success after controlling for the influence of CHC expression on mating success. In contrast, the second model constrained the residual influences of the standardized linear and quadratic effects of nutrient intake on mating success to zero (Figure 2.1). I refer to this model as the *full mediation model*, as the entire influence of nutrient intake on mating success is modelled to operate exclusively through their impact on CHC expression. As the interaction between nutrients in the response surface analysis (i.e. P x C) and between the PCs in the multivariate selection analysis (i.e. standardized correlational selection terms) were not statistically significant (see Results), and would require more demanding forms of model estimation given their nonlinearity, I omitted these terms from the SEM models. I analysed the SEM models using the LAVAAN package in R (Rosseel, 2012). As mating success was measured as a categorical variable, I used the diagonal weighted least-squares estimator when fitting all models. I then evaluated each of the models using two descriptive fit indexes—a relative index and an absolute index (Hu & Bentler, 1999)—and carrying out nested model comparisons using competing models' χ^2 fit statistics. The relative index used was the comparative fit index (CFI), which compares the fit of the estimated model to the fit of a null model in which all observed variables are uncorrelated; a CFI value greater than 0.90 indicates an acceptable fit (Hu & Bentler, 1999; Little, 2013). The absolute index used was the root mean square error of approximation (RMSEA), which indicates the amount of misfit in the model per degree of freedom; a RMSEA value less than 0.08 indicates an acceptable fit, and a 90% confidence interval can be estimated to perform a test of “close fit” of the data (i.e. $RMSEA \leq 0.05$).

2.4. RESULTS

PC analysis of the 16 individual CHC peaks yielded three PCs with eigenvalues exceeding 1, which collectively explain 72.72% of the total variation in male CHC expression (Table 2.1). PC1 accounts for 45.25% of the total variation in CHC expression and is positively loaded to each CHC peak (Table 2.1). Consequently, this vector represents the absolute amount of CHCs possessed by males. PC2 explains a further 18.97% of the total variation in male CHC expression and is positively loaded to shorter-chained CHCs (less than

C₃₇) and negatively loaded to longer-chained CHCs (greater than C₃₇)(Table 2.1). This vector therefore describes the trade-off between long- and short-chained CHCs. PC3 explains the remaining 8.50% of the total variation in male CHC expression that is positively loaded to the two unidentified alkatrienes (C₃₉H₇₄) and negatively loaded to 9,31-C₄₁diene (Table 2.1). This vector therefore describes the trade-off between these specific CHCs.

2.4.1. The effects of nutrition on CHCs and male mating success

The intake of P and C had clear linear and nonlinear effects on the variation in CHC expression described by PC1 and PC3 (Table 2.2, Figure 2.2A & B). PC1 and PC3 increased linearly with the intake of P and C and these traits were equally responsive to the intake of both nutrients (Table 2.2). The significant negative quadratic terms indicate a peak in PC1 and PC3 with the intake of both nutrients (Table 2.2) and inspection of the nutritional landscapes show that these peaks occur at high intakes of P and C, centred around a P:C ratio of approximately 1:1.5 for PC1 (Figure 2.2A) and 1:1 for PC3 (Figure 2.2B). There were no significant correlational effects of nutrients on PC1 or PC3 (Table 2.2). Formal statistical comparison using a sequential model building approach showed that the linear, quadratic and correlational effects of P and C intake on PC1 and PC3 did not differ significantly (Table 2.3). Furthermore, the angle between the linear nutritional gradients for PC1 and PC3 was small (9.74°) indicating that the peaks for these traits occupy a similar region in nutrient space (Table 2.3, Figure 2.2A & B). In contrast to PC1 and PC3, there was no linear or nonlinear effect of P and C intake on PC2 (Table 2.2).

Male mating success also increased linearly with the intake of P and C and this trait was equally responsive to the intake of both nutrients (Table 2.2). There was also significant negative quadratic terms indicating a peak in mating success with the intake of both nutrients (Table 2.2) and inspection of the nutritional landscape shows that this peak occurs at a high intake of P and C at a P:C ratio of approximately 1:1.5 (Figure 2.2C). The correlational effect of P and C intake on mating success was not significant (Table 2.2). Formal comparison showed that the linear effects of nutrient intake on mating success differed significantly from the linear effects on PC1 and PC3 (Table 2.3). In the case of PC1, this difference was due to the fact that PC1 was more responsive to the intake of C than mating success, whereas for PC3 was due to the fact that PC3 was more response to the intake of both nutrients than mating success (Table 2.3). Despite these differences, the

angles between the linear nutritional gradients for PC1 and mating success (16.07°) and between PC3 and mating success (16.11°) were small indicating that all these traits occupy similar regions in nutrient space (Table 2.3, Figure 2.2A-C). There were no significant differences in the quadratic or correlational effects of nutrient intake on PC1, PC3 and mating success (Table 2.3).

In my other work examining the nutritional regulation of sexual conflict in male *G. sigillatus* (Chapter 3), males were given the choice between alternate pairs of diets to determine how they regulate their intake of P and C. I found that males regulated their intake of nutrients to a mean (\pm SE) P and C intake of 26.39 ± 1.16 mg and 45.79 ± 1.70 mg, respectively, which equates to a P:C ratio of 1:1.74 (Chapter 3). Importantly, this regulated intake point was not well aligned with any of the traits that regulate sexual conflict in *G. sigillatus* (spermatophylax weight, the gustatory appeal of the spermatophylax and ampulla attachment time) suggesting that males are not regulating their intake of nutrients to optimise these traits (Chapter 3). To determine if males regulate their intake of nutrients to optimize CHC expression and/or mating success, I similarly mapped the regulated intake point estimated in Chapter 3, onto the nutritional landscapes for PC1, PC3 and mating success (Figure 2.2). In the case of PC1 and PC3, the regulated intake point was not well aligned with the optima on the nutritional landscapes (Figure 2.2A and B). For both PCs this was due to the fact that the regulated intake point was at a much lower intake of nutrients than the optima, although the optima for PC1 and the regulated intake point are aligned on a similar P:C ratio (Figure 2.2A and B). In contrast, the regulated intake point was well aligned with the optima for mating success on the nutritional landscape (Figure 2.2C) suggesting that when given dietary choice, males regulate their intake of nutrients to optimize this trait.

2.4.2. The effects of CHCs on male mating success

Standardized linear and nonlinear selection gradients for the PCs describing the variation in male CHCs are presented in Table 2.4. There was significant linear sexual selection favouring higher values of PC1 (an increase in all CHC peaks) and lower values of PC2 (an increase in 7,31-C₃₉diene, 9,31-C₄₁diene and two unidentified alkatrienes (C₃₉H₇₄ and C₄₁H₇₈) and a decrease in 5,9-diMeC₃₆, 9,31-C₃₇diene and 7,31-C₃₇diene)(Table 2.4). There was also significant stabilizing selection operating on PC1 and PC3 (Table 2.4). There

was, however, no significant correlational selection targeting the covariance between PC scores (Table 2.4).

Canonical analysis of the γ matrix resulted in two eigenvectors (\mathbf{m}_2 and \mathbf{m}_3) with significant nonlinear sexual selection and in both cases the associated eigenvalues were negative, indicative of multivariate stabilizing selection (Table 2.5, Figure 2.3). The dominant eigenvector of stabilizing selection (\mathbf{m}_3) is negatively weighted to PC3 and positively weighted to PC1, whereas \mathbf{m}_2 is positively weighted to both PC1 and PC3 (Table 2.5). There was also significant linear selection favouring high values of \mathbf{m}_3 , which equates to higher values of PC1 (or higher amounts of all CHCs), and higher values of \mathbf{m}_2 , which equates to higher values of PC1 and PC3 (or higher amounts of the two unidentified alkatrienes, $C_{39}H_{74}$)(Table 2.5).

2.4.3. Does condition-dependent CHC expression exclusively mediate the effect of nutrient intake on mating success?

The partial mediation model provided a good fit to the data ($\chi^2_{12} = 37.57, P < 0.001$, CFI = 0.96, RMSEA = 0.05, 90% CIs = 0.03, 0.07) and accounted for approximately 15% of the observed variation in male mating success. In comparison, the full mediation model did not appear to fit the data as well, ($\chi^2_{16} = 70.86, P < 0.001$, CFI = 0.91, RMSEA = 0.06, 90% CIs = 0.05, 0.08), accounting for only 14% of the observed variation in male mating success. Indeed, a nested model comparison confirmed that the partial mediation model provided a significantly better fit to the data than the full mediation model ($\Delta\chi^2_{2,71} = 24.68, P < 0.001$). This formally demonstrates that the effect of nutrient intake on mating success is not mediated exclusively through CHCs meaning that other trait(s) must also play an important role in mediating this relationship.

SEM parameter estimates for the partial mediation model are presented in Table 2.6. These parameter estimates are largely consistent with the previous response surface and multivariate selection analyses. Consistent with the response surface analysis, the intake of P and C were both positively associated with PC1 and PC3, although the significant negative quadratic terms indicate that these relationships plateaued at higher intakes of both nutrients (Table 2.6). In contrast, PC2 was relatively unaffected by the intake of nutrients (Table 2.6). Consistent with the multivariate selection analysis, mating success increased with higher values of PC1 and the significant negative quadratic term indicates

that this relationship plateaued at higher PC1 values (Table 2.6). In contrast, mating success increased at lower values of PC2 and the significant negative quadratic term for PC3 indicates that mating success peaks at intermediate values of this vector (Table 2.6). The residual linear effects of P and C intake on mating success, after controlling for the variation in CHC expression, were positive and significant (Table 2.6). Furthermore, the residual quadratic effects of P and C intake on mating success were significant and negative indicating that these relationships plateaued at higher intake of both nutrients (Table 2.6). A formal test of the linear mediated effects of P and C intake on mating success through changes in CHC expression using the Monte Carlo method for assessing mediation (Preacher & Selig 2012) indicated that only PC1 significantly mediated the effects of both nutrients on mating success (Table 2.7). However, given the significant negative quadratic relationships between the intake of both nutrients and PC1, and between PC1 and mating success, it is highly likely that the strength of this mediated effect would also plateau at higher nutrient intakes and values of PC1 (Table 2.7).

2.5. DISCUSSION

In this study, I used the GF to examine the effects of P and C intake on CHC expression in *G. sigillatus*, as well as the relative importance of nutrient intake and condition-dependent CHC expression to male mating success. If P and C intake have similar effects on male CHC expression and mating success, I predicted that these traits would be closely aligned in nutrient space. Consistent with this prediction, I found that the nutritional landscapes for these traits occupied similar regions in nutrient space with the dominant vector of CHC expression (PC1) and mating success both being maximised at a high intake of nutrients in a P:C ratio of 1:1.5, while the third vector (PC3) describing CHC variation was maximised at a high nutrient intake with a P:C ratio of 1:1. Furthermore, if the condition dependent expression of CHCs is a key determinant of male mating success, I predicted that female pre-copulatory mate choice will exert significant sexual selection on male CHC expression and that the SEM modelling approach would show that the effect of nutrient intake on male mating success is significantly mediated through CHC expression. Consistent with this prediction, I found significant linear and quadratic sexual selection acting on the condition-dependent variation in male CHC expression. I also found, however, that the

effect of nutrient intake on mating success was not mediated exclusively through CHC expression. This demonstrates that trait(s) other than CHCs must also play an important role in mediating the relationship between nutrient intake and mating success. This finding may also explain why the regulated intake point of P and C (P:C 1:1.74) calculated in previous work for male *G. sigillatus*, did not align well with the nutritional optima for PC1 and PC3 but aligned almost perfectly with the optima for mating success. This suggests that when given dietary choice, males regulate their intake of nutrients to optimise mating success but not CHC expression. Collectively, these findings show that there is a complex interplay between nutrient intake, CHC expression and mating success in male *G. sigillatus* and that this is likely to have important consequences for the operation of sexual selection in this species.

This work combining the GF with a large number of holidic diets shows that both the intake of calories and specific nutrients (P and C) are key to CHC expression in male *G. sigillatus*. Both PC1 (the total abundance of CHCs) and PC3 (the trade-off between two alkatrienes ($C_{39}H_{74}$) and 9,31- C_{41} diene) peaked at a high intake of nutrients, indicating that caloric intake is important to CHC expression. This reliance on a high caloric intake suggests that CHCs are costly produce, a finding that is supported in a number of *Drosophila* species (Blows, 2002; Ferveur, 2005). However, the fact that PC1 and PC3 both peak at a specific P:C ratio demonstrates that it matters what nutrients these calories are coming from. The small angle ($\theta = 9.74^\circ$) between the linear nutritional vectors, the lack of difference in the nutritional gradients from the sequential model and the similar optimal P:C ratio for PC1 and PC3 (1:1.5 and 1:1, respectively) indicate that both vectors of CHC expression are maximised at an almost equal intake of P and C. This work therefore adds to the growing list of studies showing that the balanced intake of specific nutrients are key to the condition-dependent expression of male sexual traits (Maklakov *et al.*, 2008; South *et al.*, 2011; Fedina *et al.*, 2012; Sentinella *et al.*, 2013; Harrison *et al.*, 2014; Cordes *et al.*, 2015; House *et al.*, 2015). This finding is also broadly consistent with earlier work on *G. sigillatus* that found males consuming a high quality diet produced a greater total abundance of CHCs (equivalent to PC1 in my current study) but there was little effect of diet on the trade-off between long and short-chained CHCs (equivalent to PC2 in my study) (Weddle *et al.*, 2012). This latter finding is expected as the relative abundance of long and short-chained CHCs is known to play an important role in preventing evaporative water loss in insects (e.g. Frentiu

& Chenoweth, 2010; Foley & Telonis-Scott, 2011; Ingleby *et al.*, 2013) and this vector of CHC expression is known to be under strong stabilizing natural selection in male *G. sigillatus* (Hunt *et al.*, unpublished data). These studies differ, however, in the effect of diet on PC3. In Weddle *et al.* (2012), males consuming a high quality diet produced more positive PC3 scores which reflects more short-chained alkanes and less unnamed alkadienes (C₃₉H₇₆), whereas in my current study an increase in PC3 reflects more of two unnamed alkatrienes (C₃₉H₇₄) and less 9,31-C₄₁diene. Despite this broad overlap in studies, this current work provides two important advances. First, as the high and low quality diets used by Weddle *et al.* (2012) varied in both nutrient composition and overall caloric content, it is impossible to determine their relative effect on male CHC expression. In contrast, my work unambiguously shows that a large portion of the dietary effects observed in Weddle *et al.* (2012) are due to a balanced intake of P and C. Second, Weddle *et al.* (2012) manipulated the quality of diet provided to males through juvenile development and adulthood. Males reared on a high quality diet were larger at eclosion and produced a greater total abundance of CHCs, which is expected as larger crickets have a greater cuticular surface area covered in CHCs. In contrast, my current experiment only examined the effect of diet on male CHCs after being randomly allocated to diets at eclosion to adulthood. The effects of nutrient intake on male CHCs that I observed are therefore unlikely to be driven by the confounding effect of diet on body size shown in Weddle *et al.* (2012). Indeed, statistically controlling for male pronotum width in the response surface analyses did little to alter the relationship between nutrient intake and male CHC expression (Table 2.8). Consequently, my work shows that the intake of P and C during early adulthood and sexual maturation is sufficient to generate size-independent changes in male CHC expression.

Although it is likely that CHCs first evolved to reduce evaporative water loss in terrestrial arthropods (Hadley, 1981), it is now well documented that male CHCs are also the focus of female mate choice decisions in a diversity of insect species (Wyatt, 2003; Blomquist & Bagnères, 2010). In this regard, crickets and *Drosophila* have become particularly useful insect models for understanding how sexual selection has shaped the evolution of male CHC expression. Even though empirical studies applying formal multivariate selection analysis to male CHC expression are still quite rare, the handful that exist have shown that pre-copulatory mate choice often exerts a complex pattern of linear and nonlinear sexual selection on male CHC expression, but that the exact strength and

form of selection appears to be species-specific. For example, sexual selection on male CHCs is predominantly linear in *D. serrata* (Blows *et al.*, 2004; Chenoweth & Blows, 2005; Gosden & Chenoweth, 2011; Delcourt *et al.*, 2012) and *D. bunnanda* (Van Homrigh *et al.*, 2007; McGuigan, 2009), nonlinear in the field cricket (*Teleogryllus commodus*, Thomas & Simmons, 2009; Simmons *et al.*, 2013) and a mixture of linear and nonlinear in the sagebrush cricket (*Cyphoderris strepitans*, Steiger *et al.*, 2013) and *D. simulans* (Ingleby *et al.*, 2014). Recently, multivariate selection analysis was used to show that pre-copulatory female choice in *G. sigillatus* exerts multivariate stabilizing selection on male CHCs (Steiger *et al.*, 2015). This stabilizing selection was restricted to the two lowest vectors of male CHC expression: PC3 which is positively loaded to two alkadienes (5,9-C₃₇diene and 3,9-C₃₇diene) and two unnamed alkatrienes (C₃₉H₇₄) and PC4 which is positively loaded to 7-C₃₅ene and 3,13-diMeC₃₆ and negatively loaded to two alkadienes (9,31-C₃₈diene and 9,31-C₃₉diene (alkadienes). There was also significant (albeit weak) negative linear selection on PC4 indicating that the curvature of the fitness surface along this dimension is not perfectly symmetrical (see Figure 1 in Steiger *et al.* (2015)). In agreement with this earlier work, I found that stabilizing selection was also the most dominant form of nonlinear sexual selection acting on the condition-dependent variation in male CHC expression, which is expected if the female sensory system is optimally tuned to detect male CHCs (e.g. Baker *et al.*, 1998). In contrast, however, stabilizing selection targeted PC1 (total CHC abundance) and PC3 (positive loading to two unnamed alkatrienes C₃₉H₇₄ and negative loading to 9,31-C₄₁diene) in my current study. Furthermore, linear selection was the dominant form of sexual selection in this current study, favouring higher values of PC1 (a greater total abundance of CHCs) and, to a lesser degree, lower values of PC2 (more longer-chained CHCs). Linear selection is generated whenever the mean phenotype in the population does not reside on the peak of the fitness surface (Lande, 1979; Lande & Arnold, 1983). Consequently, the stronger linear selection observed in my study suggests that condition-dependence has shifted the population mean CHC expression in males away from the peak of the fitness surface (with the peak remaining stationary) and/or the location of the fitness peak itself (with mean CHC expression remaining stationary). Clearly more work is needed, however, to test between these alternatives.

Life-history models have shown that if the variation in total resources acquired is larger than the variation in how these resources are allocated, the expected negative

phenotypic (Van Noordwijk & de Jong, 1986) and genetic (Houle, 1991; de Jong, 1992) covariance between traits competing for these resources (indicating a trade-off) will become positive. That is, rather than traits directly competing for a common pool of resources and being subject to a trade-off, fitness can be optimized by increasing the allocation of resources to both traits simultaneously (Roff & Fairbairn, 2007). Consequently, the fact that males in higher condition have a larger pool of resources to allocate to competing traits (Rowe & Houle, 1996) predicts that they should have a higher fitness (Hunt *et al.*, 2004). Indeed, numerous empirical studies have shown positive effects of diet on important components of male fitness, including mating (Blay & Yuval, 1997; Aluja *et al.*, 2001; Shelly & Kennelly, 2002; Dukas & Mooers, 2003; Engqvist & Sauer, 2003; Holzer *et al.*, 2003; McGuigan, 2009; South *et al.*, 2011) and reproductive (Fedina & Lewis, 2006; McGraw *et al.*, 2007; Bunning *et al.*, 2015; Jensen *et al.*, 2015) success. Few of these studies, however, have examined the specific nutrients responsible for these effects (South *et al.*, 2011; Bunning *et al.*, 2015; Jensen *et al.*, 2015). A notable exception is the work on male cockroaches (*N. cinerea*) showing that pheromone production and attractiveness to females were maximised at a high intake of nutrients in a P:C ratio of 1:8 (South *et al.*, 2011), whereas the number of sperm produced and subsequent fertility (measured as the number of offspring produced by the male's single mating partner) were maximised at a high intake of nutrients in a P:C ratio of 1:2 (Bunning *et al.*, 2015). In addition, the lifetime number of offspring sired by male *D. melanogaster* in a competitive situation is maximised at a P:C ratio of 1:16 (Jensen *et al.*, 2015). In this study, mating success in male decorated crickets is maximised at an intermediate nutrient intake in a P:C ratio of 1:1.5, which is more P biased than mating in male *N. cinerea* (South *et al.*, 2011) and reproductive success in male *D. melanogaster* (Jensen *et al.*, 2015). Furthermore, although the linear nutritional gradients for CHC expression are steeper than for mating success, the optima for these traits are closely aligned in nutritional space ($\sim 16^\circ$) suggesting the potential for the condition-dependence of CHCs to be an important determinant of male mating success. It is important to note that my measure of male mating success, quantified as the ability of a male to gain a mating in a "no-choice" trial, represents a proxy of fitness (Hunt & Hodgson, 2010). Recent work, however, suggests that this measure of mating success is likely to correlate well with male fitness in *G. sigillatus*. Male decorated crickets transfer an externally attached spermatophore, consisting of a sperm-containing ampulla and a gelatinous spermatophylax,

to the female at mating. Immediately after successful transfer of the spermatophore, the female removes the spermatophylax and begins feeding on it, as sperm is evacuated into her reproductive tract from the ampulla. After consuming the spermatophylax, the female immediately removes and consumes the ampulla, thereby terminating sperm transfer (Sakaluk, 1984) and both the size (Sakaluk, 1985) and free-amino acid composition (Gershman *et al.*, 2012) of the spermatophylax have been shown to extend the time the ampulla remains attached to the female. As sperm competition in *G. sigillatus* conforms to a simple “lottery” (Sakaluk and Eggert, 1996; Eggert *et al.*, 2003), greater sperm transfer is expected to increase the number of offspring sired by a given male. I have also used the GF to show that the size and free-amino composition of the spermatophylax, as well as ampulla attachment time, all peak at a P:C ratio of 1:1.3 (Chapter 3). The similarity in the nutritional optima of these traits to that shown for mating success suggests that males consuming an optimal diet will not only be likely to gain more matings but also transfer more sperm at each mating. It remains to be shown, however, whether this diet will increase the number of offspring sired over the lifetime of a male, especially under the more competitive conditions that mating typically occurs.

My work clearly shows that mating success in male *G. sigillatus* is the product of a complex interplay between diet and condition-dependent CHC expression. That is, nutrient intake influenced both CHC expression and mating success in a similar way and the multivariate selection analysis showed that the condition-dependent expression of CHCs has important linear and nonlinear effects on mating success. This raises the obvious question of whether the effect of nutrient intake on mating success is mediated exclusively through condition-dependent CHC expression or whether other trait(s) also play an important role in mediating this relationship? To test this, I used a SEM approach to compare a model where the effect of nutrient intake on mating success was mediated exclusively through condition-dependent CHC expression (full mediation model) to one that examines the residual effect of nutrient intake on mating success after controlling for the effect that condition-dependent CHC expression has on mating success (partial mediation model). I found that the partial mediation model provided a significantly better fit to the data, thereby demonstrating that condition-dependent CHC expression is not the only trait mediating the observed relationship between nutrient intake and male mating success. It is important to note, however, that this does not mean that condition-dependent CHC expression is not an

important mediator of this relationship *per se*, just that other trait(s) must also be involved. This point is illustrated by the fact that within the partial mediation model, PC1 (but not PC2 or PC3) significantly mediated the effects of P and C intake on mating success. The finding that trait(s) other than CHC expression must also mediate the relationship between nutrient intake and mating success also explains why the regulated intake point for male *G. sigillatus* (P:C = 1:1.74, Chapter 3) was aligned almost perfectly with the nutritional optima for mating success but was less well aligned with the optima for PC1 and PC3. This suggests that under dietary choice, males regulate their intake of P and C to optimise mating success rather than CHC expression, an outcome that would not be expected if CHC expression exclusively mediated the relationship between nutrient intake and mating success. The next logical question is: what other traits(s) are likely to mediate this relationship in *G. sigillatus*? When in close proximity, male crickets use a series of stereotypical courtship behaviours to elicit a mating, including contacting the female with his antennae, positioning his body to allow mounting and the production of a courtship call. While successful mating can occur without the first two behaviours, the production of a courtship call is typically essential for mating and studies on a range of cricket species have shown that various properties of the courtship call are targeted by females in pre-copulatory mate choice decisions (e.g. Wagner & Reiser, 2000; Hall *et al.*, 2008; Rebar *et al.*, 2009), including in *G. sigillatus* (Ketola *et al.*, 2007). In contrast, the condition-dependence of the courtship call in crickets has received less attention and existing studies have only used a low number of poorly defined diets in their manipulations. There is little effect of diet on the structure of the courtship call in *Gryllus texensis* (Gray & Eckhardt, 2001) and *G. lineaticeps* (Wagner & Reiser, 2000), but work on *G. sigillatus* (Mallard & Barnard, 2004) shows that males increase the stridulatory rate of their courtship call when consuming a high quality diet. Thus, the courtship call is a prime candidate to simultaneously mediate the relationship between nutrient intake and male mating success, alongside CHCs in *G. sigillatus*, although more work is needed to determine the exact affect that P and C intake has on this male sexual trait. Collectively, my work demonstrates the important insights into condition-dependence that can be gained through measuring both sexual trait expression and mating success in the same experimental design (as advocated by McGuigan, 2009), but also highlights the additional benefits that can also come through measuring nutrient intake.

In conclusion, I show that male CHC expression and mating success are contingent on the balanced intake of P and C and that there are complex linear and nonlinear effects of condition-dependent CHC expression on mating success driven by female pre-copulatory mate choice. However, despite the close alignment of the nutritional landscapes for CHC expression and mating success, I found that CHC expression was not the only factor mediating the effects of nutrient intake on mating success. It is therefore likely that other sexual trait(s) are also condition-dependent and play an important role in mediating this relationship and I propose the male courtship call as a potential candidate. Collectively my work shows that the complex interplay between nutrient intake, CHC expression and mating success in male decorated crickets is likely to have an important effect on the operation of sexual selection in this species. For example, the condition-dependence of male CHC expression that I demonstrate in this study provides an important mechanism promoting the maintenance of genetic variation in this phenotypic trait that is the target of strong sexual selection (Steiger *et al.*, 2015). Furthermore, my work shows that the intake of P and C is subjected to indirect sexual selection, via their effects of sexual trait expression and mating success, and that males are able to actively influence this process by regulating their intake of nutrients through dietary choice. This highlights that feeding behaviour is likely to be a key component of condition-dependence and should be better integrated with sexual selection theory (Morehouse *et al.*, 2010).

Table 2.1. Principal Component (PC) analysis of CHC expression in male *Gryllodes sigillatus*. PCs with eigenvectors exceeding one are presented and used in subsequent analysis. Factor loadings >0.30 (in bold) are interpreted as biological significant (Tabachnick & Fidell, 1989). CHCs are named where known and unnamed CHCs (asterisks) are described by basic chemical structure. CHCs are listed in order of increasing carbon chain length.

| | PC1 | PC2 | PC3 |
|--|-------------|--------------|--------------|
| Eigenvalue | 7.24 | 3.04 | 1.36 |
| % Variance | 45.25 | 18.97 | 8.50 |
| Loadings | | | |
| 7-MeC ₃₃ | 0.93 | 0.06 | -0.15 |
| 5-MeC ₃₃ | 0.84 | 0.14 | -0.27 |
| 3-MeC ₃₃ | 0.85 | 0.03 | -0.27 |
| 3,7-diMeC ₃₃ | 0.85 | 0.12 | -0.12 |
| 7-C ₃₅ ene | 0.40 | 0.28 | -0.23 |
| 3,13-diMeC ₃₆ | 0.64 | 0.28 | -0.03 |
| 5,9-diMeC ₃₆ | 0.57 | 0.62 | -0.05 |
| 9,31-C ₃₇ diene | 0.39 | 0.82 | 0.16 |
| 7,31-C ₃₇ diene | 0.40 | 0.77 | 0.12 |
| 9,31-C ₃₈ diene | 0.74 | -0.20 | 0.17 |
| Alkatriene (C ₃₉ H ₇₄)* | 0.62 | -0.19 | 0.60 |
| Alkatriene (C ₃₉ H ₇₄)* | 0.57 | -0.41 | 0.58 |
| 9,31-C ₃₉ diene | 0.89 | -0.18 | 0.18 |
| 7,31-C ₃₉ diene | 0.71 | -0.46 | 0.15 |
| Alkatriene (C ₄₁ H ₇₈)* | 0.50 | -0.66 | -0.28 |
| 9,31-C ₄₁ diene | 0.49 | -0.51 | -0.48 |

Table 2.2. The linear and nonlinear (quadratic and correlational) effects of protein (P) and carbohydrate (C) intake on the three PCs describing CHC expression, as well as on mating success, in male *G. sigillatus*.

| Response Variable | Linear effects | | Nonlinear effects | | |
|--|----------------|--------------|-------------------|--------------|--------------|
| | P | C | P x P | C x C | P x C |
| PC1 | | | | | |
| Gradient ± SE | 0.16 ± 0.04 | 0.20 ± 0.04 | -0.12 ± 0.03 | -0.10 ± 0.03 | 0.07 ± 0.05 |
| t_{765} | 4.51 | 5.71 | 4.03 | 3.28 | 1.26 |
| <i>P</i> | 0.0001 | 0.0001 | 0.0001 | 0.001 | 0.21 |
| PC2 | | | | | |
| Gradient ± SE | -0.02 ± 0.04 | -0.03 ± 0.04 | 0.01 ± 0.03 | -0.04 ± 0.03 | -0.02 ± 0.06 |
| t_{765} | 0.62 | 0.87 | 0.32 | 1.20 | 0.27 |
| <i>P</i> | 0.54 | 0.39 | 0.75 | 0.23 | 0.79 |
| PC3 | | | | | |
| Gradient ± SE | 0.19 ± 0.04 | 0.20 ± 0.04 | -0.07 ± 0.03 | -0.16 ± 0.03 | -0.06 ± 0.05 |
| t_{765} | 5.44 | 5.59 | 2.33 | 5.42 | 1.02 |
| <i>P</i> | 0.0001 | 0.0001 | 0.02 | 0.0001 | 0.31 |
| Mating success | | | | | |
| Gradient ± SE | 0.08 ± 0.04 | 0.09 ± 0.04 | -0.09 ± 0.03 | -0.12 ± 0.03 | -0.08 ± 0.06 |
| $\beta_{\text{rand}} \geq \beta_{\text{real}}$ | 210 | 68 | 9953 | 9999 | 9197 |
| <i>P</i> | 0.04 | 0.01 | 0.009 | 0.0002 | 0.16 |

Table 2.3. Sequential model comparing the linear and nonlinear effects of protein (P) and carbohydrate (C) intake on PC1 and PC3 that describe the variation in CHC expression and mating success in male *Gryllobates sigillatus*. The angle (θ) and 95% confidence intervals (CI) between the linear nutritional vectors for these traits are also provided.

| | SS_R | SS_C | DF_1 | DF_2 | F | P | θ (95% CI) |
|-------------------------------|---------|---------|--------|--------|------|--------------------|------------------------|
| PC1 vs. PC3 | | | | | | | |
| Linear | 1435.15 | 1434.72 | 2 | 1530 | 0.23 | 0.80 | 9.74° (0.00°, 23.48°) |
| Quadratic | 1383.53 | 1379.94 | 2 | 1526 | 1.98 | 0.14 | |
| Correlational | 1379.91 | 1377.57 | 1 | 1524 | 2.59 | 0.11 | |
| PC1 vs. Mating success | | | | | | | |
| Linear | 1480.40 | 1473.34 | 2 | 1530 | 3.67 | 0.03 ^A | 16.07° (0.00°, 40.83°) |
| Quadratic | 1428.31 | 1427.16 | 2 | 1526 | 0.61 | 0.54 | |
| Correlational | 1427.14 | 1423.82 | 1 | 1524 | 3.56 | 0.06 | |
| PC3 vs. Mating success | | | | | | | |
| Linear | 1485.29 | 1476.19 | 2 | 1530 | 4.71 | 0.009 ^B | 16.11° (0.00°, 40.59°) |
| Quadratic | 1422.29 | 1421.52 | 2 | 1526 | 0.41 | 0.66 | |
| Correlational | 1418.75 | 1418.66 | 1 | 1524 | 0.09 | 0.76 | |

^A P: $F_{1,1530} = 2.71$, $P = 0.10$, C: $F_{1,1530} = 5.03$, $P = 0.03$; ^B P: $F_{1,1530} = 5.29$, $P = 0.02$, C: $F_{1,1530} = 4.69$, $P = 0.03$.

Table 2.4. Vector of standardized linear selection gradients (β) and the γ matrix of standardized nonlinear selection gradients for the three PCs that describe the variation in male CHCs. Significance testing with permutation test: *** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$.

| | β | γ | | |
|------------|-----------------------|-----------------------|-----------------|------------------------|
| | | PC1 | PC2 | PC3 |
| PC1 | $0.21 \pm 0.04^{***}$ | $-0.16 \pm 0.06^{**}$ | | |
| PC2 | $-0.09 \pm 0.04^*$ | 0.04 ± 0.04 | 0.06 ± 0.06 | |
| PC3 | -0.02 ± 0.04 | 0.06 ± 0.04 | 0.01 ± 0.04 | $-0.21 \pm 0.06^{***}$ |

Table 2.5. Canonical analysis to locate the major dimensions of the γ matrix given in Table 2.4. θ_i and λ_i are the linear and nonlinear selection acting on each vector, respectively. Significance tested via permutation test: *** $P < 0.0001$, ** $P < 0.001$.

| | M | | | Selection | |
|-------|--------|--------|--------|------------|-------------|
| | PC1 | PC2 | PC3 | θ_i | λ_i |
| m_1 | -0.187 | -0.980 | -0.070 | 0.046 | 0.067 |
| m_2 | 0.800 | -0.193 | 0.568 | 0.174*** | -0.126** |
| m_3 | 0.570 | -0.050 | -0.820 | 0.138** | -0.254** |

Table 2.6. Structural equation model parameter estimates for protein (P) and carbohydrate (C) intake on the three principal components describing variation in CHCs (PC1, PC2, PC3) and male mating success (MS) taken from the partial mediation model. Significance values: * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

| Predictor | PC1 | PC1 x PC1 | PC2 | PC2 x PC2 | PC3 | PC3 x PC3 | MS |
|-----------|----------|-----------|---------|-----------|----------|-----------|----------|
| P | 0.30*** | -0.10 | -0.04 | 0.08 | 0.26*** | 0.06 | 0.18** |
| P x P | -0.12** | 0.03 | 0.01 | -0.04 | -0.07** | 0.06 | -0.14* |
| C | 0.28*** | -0.21** | 0.01 | 0.16* | 0.36*** | -0.03 | 0.22*** |
| C x C | -0.09*** | 0.14** | -0.04 | -0.05 | -0.16*** | 0.03 | -0.23*** |
| PC1 | - | 0.41*** | - | - | - | - | 0.14** |
| PC1 x PC1 | -0.41*** | - | - | - | - | - | -0.12* |
| PC2 | - | - | - | 0.40*** | - | - | -0.10* |
| PC2 x PC2 | - | - | 0.40*** | - | - | - | 0.03 |
| PC3 | - | - | - | - | - | 0.05** | -0.09 |
| PC3 x PC3 | - | - | - | - | 0.05** | - | -0.15** |

Table 2.7. The linear effects of protein (P) and carbohydrate (C) intake on male mating success mediated through the three principal components (PC1, PC2, PC3) that describe the variation in CHCs. Confidence intervals for the mediated effects have been simulated using the Monte Carlo method described by Preacher & Selig (2012). Values in bold are considered statistically significant (i.e. the 95% CI does not overlap zero).

| Predictor | Mediator | Mediated Effect (95% CI) |
|-----------|------------|--------------------------|
| P | PC1 | 0.01, 0.08 |
| P | PC2 | -0.01, 0.02 |
| P | PC3 | -0.05, 0.001 |
| C | PC1 | 0.01, 0.08 |
| C | PC2 | -0.01, 0.01 |
| C | PC3 | -0.07, 0.002 |

Table 2.8. The effects of protein (P) and carbohydrate (C) intake on male CHC expression (described by PC1, PC2 and PC3) in *G. sigillatus* when correcting for variation in male body size (pronotum width, PW).

| Response Variable | PW | Linear effects | | Nonlinear effects | | |
|-------------------|--------------|----------------|--------------|-------------------|--------------|--------------|
| | | P | C | P x P | C x C | P x C |
| PC1 | | | | | | |
| Gradient ± SE | 0.39 ± 0.03 | 0.16 ± 0.03 | 0.19 ± 0.03 | -0.14 ± 0.03 | -0.14 ± 0.03 | -0.01 ± 0.05 |
| t_{765} | 12.26 | 4.88 | 5.86 | 4.88 | 5.23 | 0.22 |
| <i>P</i> | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.83 |
| PC2 | | | | | | |
| Gradient ± SE | -0.02 ± 0.04 | -0.02 ± 0.04 | -0.03 ± 0.04 | 0.01 ± 0.03 | -0.04 ± 0.03 | -0.01 ± 0.06 |
| t_{765} | 0.51 | 0.61 | 0.85 | 0.33 | 1.15 | 0.23 |
| <i>P</i> | 0.61 | 0.54 | 0.40 | 0.74 | 0.25 | 0.82 |
| PC3 | | | | | | |
| Gradient ± SE | -0.15 ± 0.04 | 0.19 ± 0.04 | 0.20 ± 0.04 | -0.07 ± 0.03 | -0.15 ± 0.03 | -0.03 ± 0.05 |
| t_{765} | 4.38 | 5.52 | 5.79 | 2.23 | 4.99 | 0.59 |
| <i>P</i> | 0.0001 | 0.0001 | 0.0001 | 0.03 | 0.0001 | 0.56 |

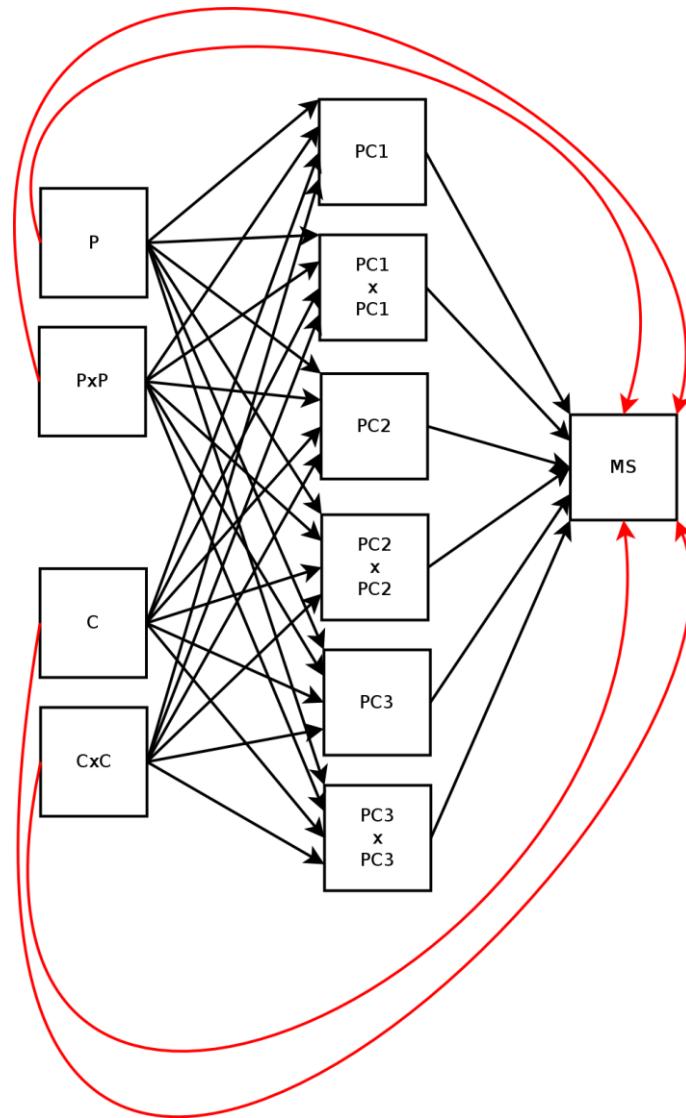


Figure 2.1. Path diagram illustrating the alternate models used to predict male mating success (MS) from the standardized linear and quadratic effects of protein (P) and carbohydrate (C) intake and the three principal components describing the variation in CHC expression (PC1, PC2, PC3). In the *partial mediation model*, I model the effects of nutrient intake as directly influencing MS (red pathways) and indirectly influencing MS through their effects on CHC expression. In the *full mediation model*, I model the effects of nutrient intake as influencing MS exclusively through their effects on CHC expression (i.e. the red pathways are constrained to zero).

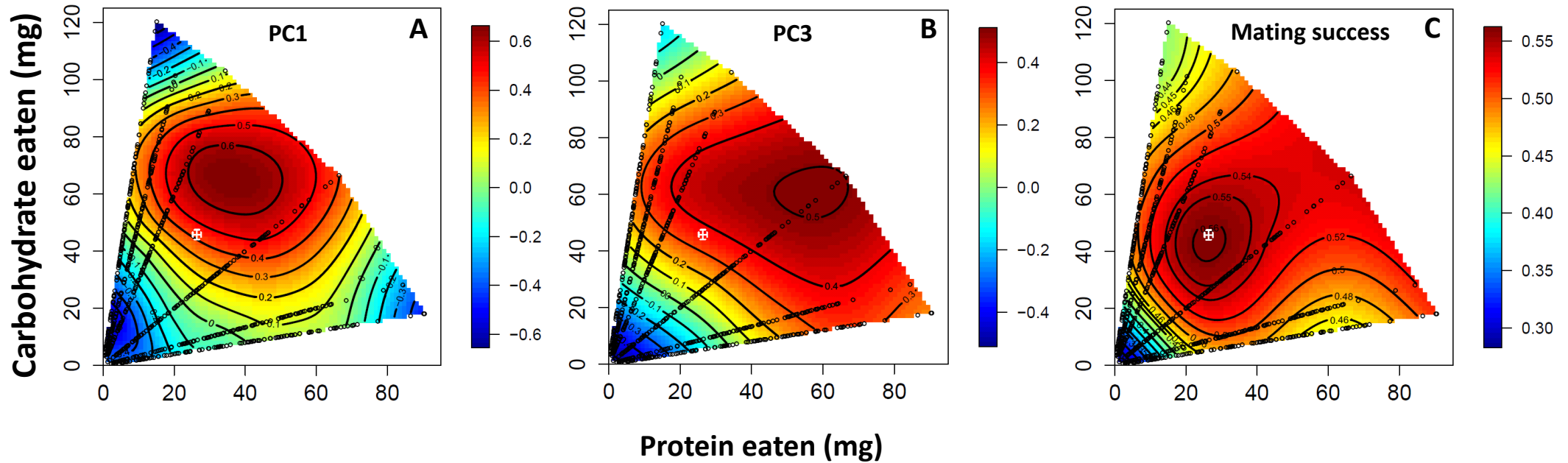


Figure 2.2. Nutritional landscapes illustrating the effects of protein and carbohydrate intake on (A) PC1 and (B) PC3 that describe the variation in CHC expression and (C) mating success in male *Gryllodes sigillatus*. High values of these response variables are given in red and low values in blue. The black dots represent the actual nutrient intake for each male cricket in this experiment and the white cross on each landscape represents the regulated intake point (\pm SE) estimated in Chapter 3 for male *G. sigillatus* when given dietary choice.

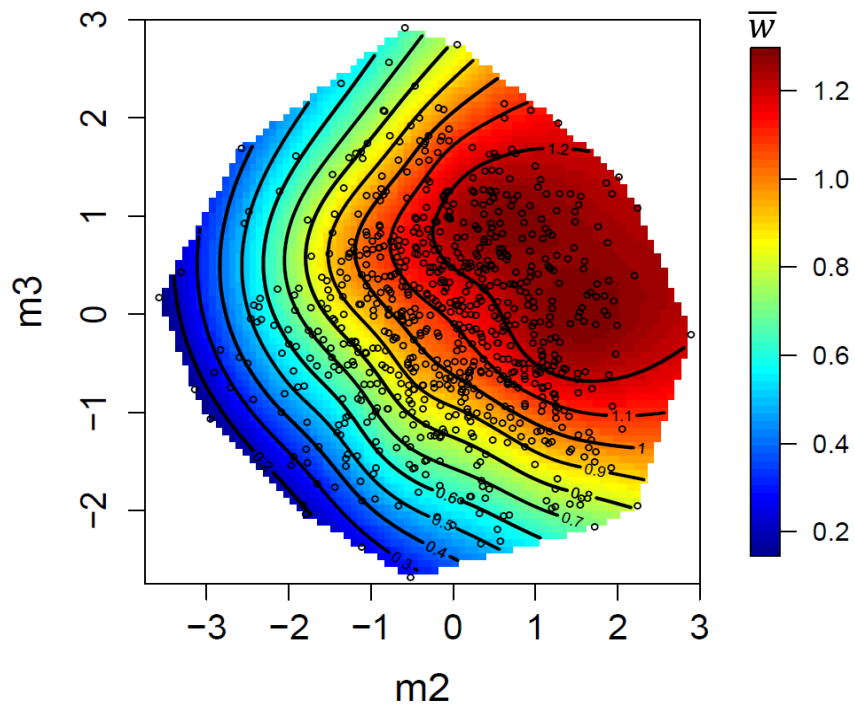


Figure 2.3. Thin-plate spline contour view visualization of the fitness surface on the two major axes of significant nonlinear sexual selection, m_2 and m_3 . The open symbols represent individual data points for each male in this experiment. Colours represent the relative mating success of males (\bar{w}), with red representing the highest relative fitness and blue representing the lowest relative fitness.

CHAPTER 3:
MACRONUTRIENT INTAKE REGULATES SEXUAL CONFLICT IN THE
DECORATED CRICKET *GRYLLODES SIGILLATUS*

3.1. ABSTRACT

Sexual conflict results in a diversity of sex-specific adaptations, including chemical additions to ejaculates. Male decorated crickets (*Gryllodes sigillatus*) produce a gelatinous nuptial gift (the spermatophylax) that varies in size and free amino acid composition, which influences a female's willingness to fully consume this gift. Complete consumption of this gift maximises sperm transfer through increased retention of the sperm-containing ampulla, but hinders post-copulatory mate choice. Here, I examine the effects of protein (P) and carbohydrate (C) intake on the weight and amino acid composition of the spermatophylax that describes its gustatory appeal to the female, as well as the ability of this gift to regulate sexual conflict via ampulla attachment time. Nutrient intake had similar effects on the expression of these traits with each maximised at a high intake of nutrients with a P:C ratio of 1:1.3. Under dietary choice, males actively regulated their nutrient intake but this regulation did not coincide with the peak of the nutritional landscape for any trait. My results therefore demonstrate that a balanced intake of nutrients is central to regulating sexual conflict in *G. sigillatus* but males are constrained from reaching the optima needed to bias the outcome of this conflict in their favour.

Key Words: Carbohydrate, Free amino acids, Geometric Framework, *Gryllodes sigillatus*, Protein, Spermatophylax

3.2. INTRODUCTION

Sexual conflict arises whenever the reproductive interests of males and female do not perfectly coincide (Parker, 1979) and is known to promote the evolution of adaptations that enhance the fitness of individuals of one sex at the expense of the other (Parker, 1979; Arnqvist and Rowe, 2005). These adaptations range from mating behaviours, such as persistent harassment (Córdoba-Aguilar, 2009) and reluctance to mate (Rowe *et al.*, 1994), to morphological structures that make it harder for males to force matings (Arnqvist and Rowe, 1995) or that cause damage to the female during mating (Crudginton and Siva-Jothy, 2000; Stutt and Siva-Jothy, 2001). Conflict between the sexes, however, does not always end at copulation as males may continue to manipulate females long after mating has finished (Arnqvist and Rowe, 2005). A classic example of this occurs in *Drosophila melanogaster* where males transfer seminal fluid proteins (SFPs) in their ejaculate during mating that are known to have a wide range of physiological effects on females including altering sperm storage, decreasing receptivity to additional matings, increasing ovulation and egg production and reducing lifespan (Wolfner, 2002). While SFPs and other chemicals in the ejaculate appear to be taxonomically widespread (Poiani, 2006; Perry *et al.*, 2013), surprisingly little is known about their effects on females in systems other than *D. melanogaster*. Consequently, chemical manipulation continues to be one of the least well understood aspects of sexual conflict (Arnqvist and Rowe, 2005).

Nuptial gifts refer to any material beyond obligatory gametes that is provided by a donor (typically the male) to a recipient (typically the female) during courtship or copulation that acts to improve the fitness of the donor (Lewis *et al.*, 2014) and are taxonomically widespread in insects and spiders (Vahed, 1998, 2007; Gwynne, 2008; Lewis and South, 2012). Males of numerous field cricket and katydid species synthesize their own gifts, including complex spermatophores (e.g. Gwynne, 1997), glandular secretions (e.g. Bussière *et al.*, 2005) and even part of the male's own body (e.g. Eggert and Sakaluk, 1994). Collectively referred to as endogenous oral gifts (Lewis and South, 2012; Lewis *et al.*, 2014), these gifts constitute a major form of reproductive investment and their production has been shown to be costly to the male (e.g. Sakaluk *et al.*, 2004; Leman *et al.*, 2009). In many species, males provision gifts with nutrients or defensive compounds that are otherwise absent or limited in the female's diet (Lewis and South, 2012) and there are many examples

where the female benefits directly and/or indirectly through the fitness of her offspring by consuming these gifts (e.g. Gwynne, 1997; 2008). In other species, however, no such benefits have been detected and nuptial gifts predominantly serve to protect the male ejaculate and cause the female to relinquish some of her control over sperm transfer and eventual paternity (Vahed, 1998; 2007). Consequently, it has been argued that sexual conflict has played a key role in shaping the evolution of nuptial gifts (Vahed, 1998; 2007; Gwynne, 2008; Lewis and South, 2012). For gifts that are produced endogenously, there is the potential for the male to add manipulative substances during production, although it has been noted that such substances may be infrequent in oral gifts because they would be degraded as they pass through the female digestive system (Gwynne, 2008).

In decorated crickets (*Grylloides sigillatus*) males produce an externally attached spermatophore that is transferred to the female during mating. This spermatophore consists of two discrete components: the sperm containing ampulla and the much larger gelatinous spermatophylax. Immediately upon dismounting the male after spermatophore transfer, the female detaches the spermatophylax from the ampulla with her mandibles and commences feeding on it. While the female feeds on this nuptial gift, sperm are evacuated into her reproductive tract from the ampulla. After consuming the spermatophylax, the female immediately removes and consumes the ampulla, thereby terminating sperm transfer. Females vary considerably in the length of time that they feed on the spermatophylax, and the longer that the female is delayed from removing the ampulla, the more sperm are transferred to her sperm storage organ (Sakaluk, 1984; 1985; 1987). The size of the spermatophylax plays an important role in this process, as it takes a female longer to fully consume a larger spermatophylax (Sakaluk, 1985), a pattern that also appears common across bushcricket species (e.g. Wedell & Arak, 1989; Reinhold and Heller, 1993). As females are highly polyandrous, the length of time that a female spends consuming the spermatophylax has profound consequences for the outcome of sperm competition (Sakaluk, 1986; Sakaluk & Eggert, 1996; Eggert *et al.*, 2003). Thus, although producing a spermatophylax comes at a direct cost to the immune function of males (Gershman *et al.*, 2010; Kerr *et al.*, 2010), consumption of this gift by the female clearly enhances male fitness. In contrast, there appears to be little direct benefit to the female in consuming a spermatophylax (Will & Sakaluk, 1994; Kasuya & Sato, 1998; Ivy & Sakaluk, 2005), the one notable exception being a hydration benefit to female lifespan under conditions of limited

water availability (Ivy *et al.*, 1999). In fact, females that accept a nuptial gift from the male are actively prevented from exerting post-copulatory mate choice, which is known to reinforce pre-copulatory mating biases in this species (Ivy & Sakaluk, 2007). Thus, by consuming the spermatophylax, a female relinquishes some of the control over the paternity of her offspring which is unlikely to be in her best “evolutionary” interests. Consequently, it has been argued that sexual conflict has been a major driver of nuptial gift evolution in *G. sigillatus* (Sakaluk, 2000; Sakaluk *et al.*, 2006; Gershman *et al.*, 2012; Gershman *et al.*, 2013).

More recently, research has focused on the chemical composition of the spermatophylax and the potential role this plays in mediating sexual conflict in *G. sigillatus* (Warwick *et al.*, 2009; Gershman *et al.*, 2012; Gershman *et al.*, 2013). The spermatophylax in this species consists mostly of water, as well as a mixture of 19 different free amino acids (Warwick *et al.*, 2009). Many of these amino acids are known phagostimulants in insects (e.g. Cook, 1977). This explains the propensity of females to accept and feed on a gift after mating in *G. sigillatus*, as well as females from numerous other non-gift-giving cricket species (Sakaluk, 2000; Sakaluk *et al.*, 2006). It is important to highlight, however, that up to 25% of female *G. sigillatus* prematurely discard the spermatophylax before it is fully consumed (Sakaluk, 1984; 1987) and there is some evidence to suggest that this behavior is linked to the amino acid composition of the spermatophylax (Warwick *et al.*, 2009; Gershman *et al.*, 2012). By feeding artificial, gelatin-based gels containing the four most abundant amino acids in the spermatophylax (proline, glycine, arginine and alanine) in varying concentration, Warwick *et al.* (2009) showed that female feeding time increased with amino acid concentration, peaking at approximately 14% gelatin dry mass. More recently, Gershman *et al.* (2012) used a multivariate selection analysis to show that specific combinations of amino acids decreased the likelihood that a female would prematurely discard a spermatophylax, most likely by influencing the gustatory appeal of this gift (Warwick *et al.*, 2009). Importantly, there is a positive genetic correlation between this combination of amino acids in the spermatophylax and female feeding duration which indicates that genes expressed in males to produce more manipulative spermatophylaxes are positively linked to genes expressed in females that make them more vulnerable to being manipulated (Gershman *et al.*, 2013). This finding is consistent with an evolutionary history of sexual antagonistic coevolution over the consumption of this nuptial gift in *G.*

sigillatus (Gershman *et al.*, 2013). However, while these studies provide strong evidence that free amino acids are important in mediating sexual conflict in *G. sigillatus*, we currently do not know how these substances are regulated in the spermatophylax.

In general, very little is known about the regulation of chemicals used in sexual conflict, although diet appears to be a strong candidate. In *D. melanogaster*, both the larval and adult diet of males has a significant effect on female remating behavior, most likely by altering SFP expression in the ejaculate (McGraw *et al.*, 2007; Fricke *et al.*, 2008). For example, males fed a diet containing higher levels of protein as a larva (provided in the form of yeast) were more effective at preventing remating in their partner as an adult and had a higher relative transcript abundance of at least one known SFP (*Acp36DE*) (McGraw *et al.*, 2007). Furthermore, males fed a high protein diet as an adult (after being reared on a standard medium diet) were also able to better inhibit remating in their partner (Fricke *et al.*, 2008). Qualitatively similar effects on female remating behaviour have also been shown for male Mediterranean fruit flies (*Ceratitis capitata*) fed a high protein diet (provided as protein hydrolysate) (Blay and Yuval, 1997), a species where SFP expression is known to share many homologies with *D. melanogaster* (Davies & Chapman, 2006). While there is growing evidence showing that the consumption of a nuptial gift influences female remating behavior (e.g. Ortiz-Jimenez & Cueva del Castillo, 2015), surprisingly little is known about the effect of male nutrition on the addition of manipulative chemicals to nuptial gifts.

A limitation shared by all of the above studies examining the relationship between male nutrition and the production of manipulative chemicals is that diet was not manipulated in a controlled manner. For example, although yeast consists mainly of protein, and is therefore used to manipulate this nutrient in experimental diets (McGraw *et al.*, 2007; Fricke *et al.*, 2008), it also contains carbohydrates, lipids, salts and a range of different vitamins. Thus, it is not possible to identify the key nutrient(s) responsible for any observed effects when using such manipulations, or to partition the effects of specific nutrients from the total intake of calories. Consequently, an explicit nutritional framework is needed when examining the effects of nutrition, preferably using chemically defined (holidic) diets. Here, I use a multidimensional nutritional framework, known as the Geometric Framework of Nutrition (GF, Simpson & Raubenheimer 2012), to determine the effects of protein (P) and carbohydrate (C) intake on the regulation of sexual conflict in the decorated cricket, *G. sigillatus*. To test this, I conducted three separate experiments using holidic diets with

precise chemical composition. In my first experiment, I restricted male crickets to feed on one of 24 artificial diets during sexual maturation to quantify the effects of P and C intake on the size and amino acid composition of the spermatophylax that describes its gustatory appeal to females (Experiment 1). Next, I restricted a second group of males to feed on these diets during sexual maturation and then mated them to a randomly allocated virgin female. These females were then observed and the time taken for the female to remove the ampulla (and terminate sperm transfer) was recorded (Experiment 2). In my final experiment, I gave males the choice between alternate diets in four diet pairings to determine if they regulate their intake of P and C to optimize the size and chemical composition of the spermatophylax, as well as ampulla attachment time (Experiment 3). If nutrient intake regulates sexual conflict in *G. sigillatus*, I predict that the intake of P and/or C will influence the size and the amino acid composition that describes the gustatory appeal of the spermatophylax and this will have a similar effect on ampulla attachment time. This should result in the nutritional landscapes for these traits being closely aligned. Moreover, if males are biasing the outcome of this conflict through their intake of nutrients, I predict that the regulated intake of nutrients under dietary choice will coincide with the peak in ampulla attachment time on the nutritional landscape.

3.3. MATERIALS AND METHODS

3.3.1. Experimental Animals

The *G. sigillatus* used in this study are descended from 500 adult crickets collected in Las Cruces, New Mexico in 2001 used to initiate a laboratory culture maintained at a population size of approximately 5000 crickets and allowed to breed panmictially (Ivy & Sakaluk, 2005). Culture crickets are housed in ten 15L plastic containers in an environmental chamber (Percival I-66VL) maintained at $32\pm 1^\circ\text{C}$ on a 14h:10h light/dark cycle and are provided with cat food (Purina Go-Cat Senior[®]) and rat food (SDS Diets) pellets, water *ad libitum* in 60ml glass test tubes plugged with cotton wool and an abundance of cardboard egg cartons to provide shelter. Each generation, nymphs were collected at hatching and randomly allocated across culture containers to enforce gene flow.

Experimental crickets were collected from the laboratory cultures as newly hatched nymphs and housed individually in a plastic container (5cm x 5cm x 5cm). Each container

was provided with a piece of cardboard egg carton for shelter and water in a 2.5ml test tube plugged with cotton wool. Nymphs were fed ground cat food pellets for the first two weeks post hatching and thereafter solid pellets until eclosion to adulthood. Containers were cleaned and food and water replaced weekly. Experimental animals were checked daily for eclosion to adulthood. At eclosion, males were transferred to a larger individual plastic container (20 x 10 x 10cm) and then randomly allocated to an experiment (Experiment 1, 2 or 3)(see below).

3.3.2. Artificial diets and measuring dietary intake

I made 24 artificial, dry diets that varied in P and C, as well as overall nutrition, based on the established protocol outlined in Simpson & Abisgold (1985). The distribution of these diets in nutritional space can be seen in Figure 1.2 and the composition of these diets in Table 1.1.

Each experimental male was given either one (Experiment 1 and 2) or two (Experiment 3) dishes of diet of measured dry weight on their day of eclosion to adulthood and diet was changed every 2 days for a total of 10 days until males were sexually mature. Food and water were provided in feeding platforms constructed by gluing a vial lid (1.6 cm diameter, 1.6 cm deep) upside down onto a petri dish (5.5cm diameter). This design allowed any diet spilled during feeding to be collected in the petri dish. Diet was kept in a drying oven (Binder, model FD 115) at 30°C for 48 hours to remove any moisture prior to weighing. Feeding platforms containing diet were weighed before and after each feeding period, using an electronic balance (Ohaus Explorer Professional, model EP214C). Prior to final weighing, any faeces were removed from the feeding platform using a pair of fine forceps. Diet consumption was calculated as the difference in dry weight of diet before and after feeding. This was converted to a P and C intake following the procedure outlined in South *et al.* (2011).

3.3.3. Experiment 1: The effects of nutrient intake on spermatophylax weight and amino acid composition

To determine the effects of P and C intake on the size and amino acid composition of the spermatophylax, 16 males were established at random on each of the 24 diets (total $n = 384$ males) on their day of eclosion to adulthood and fed for 10 days to quantify their intake

of P and C (as described above). Each male was then mated to a virgin female of the same age (on day 10) and the spermatophylax removed from the female using a pair of fine forceps and stored in an airtight microcentrifuge vial. Spermatophylaxes were dried using a freeze dryer (Heto PowerDry LL3000) and then weighed using an electronic balance (Mettler Toledo, UMX2). A total of 22 free amino acids were extracted from these dried samples and quantified using an EZ:faast reagent kit (Phenomenex, Torrance, CA) optimized for Gas Chromatography-Mass Spectrometry (GCMS) following the protocol outlined in Gershman *et al.* (2012).

Amino acid extraction

Spermatophylax samples were ground using a micro-pestle in the microcentrifuge tube with the addition of 150µl of ethanol. A sample (100µl) was pipetted into a sample vial along with 100µl of internal standard solution (Norvaline 0.2mM, *N*-propanol 10%). This sample was slowly drawn through a sorbent pipette tip using a 1.5ml syringe. 200µl of washing solution (*N*-propanol) was then added to the sample vial and also drawn through the sorbent pipette tip. The syringe was detached and liquid in the syringe was discarded. 200µl of eluting medium (comprised of a 3:2 mix of sodium hydroxide and *N*-propanol) was added to the sample vial. Using a 0.6mm syringe with the piston half way up the barrel, eluting medium was drawn into the pipette tip until the liquid reached the filter at the top of the sorbent particles. The sorbent particles and liquid were then ejected from the tip into the vial. 50µl of chloroform was added using a Drummond Dialamatic Microdispenser. The sample was then emulsified by repeatedly vortexing for 5-8s and left for 1 minute to allow the reaction to proceed. 100µl of *iso*-octane was then added using the Drummond Dialamatic Microdispenser, emulsified for another 5-8s and then left for a further minute to allow the reaction to proceed and for the liquid to separate into two layers. 100µl of the (upper) organic layer was transferred into a new vial and evaporated slowly to almost dry under a gentle stream of nitrogen. The amino acid derivatives were re-dissolved in 100µl of re-dissolution solvent (80% *iso*-octane, 20% chloroform), emulsified for 5s and then transferred into an auto-sampler vial for analysis by GC-MS.

Quantification of amino acid composition in the spermatophylax

I injected 2µl of the extracted amino acid sample into a GC-MS (Agilent 7890A gas chromatograph coupled with an Agilent 5975B mass spectrometer and an Agilent CTC PAL autosampler chilled to 5°C) fitted with a ZB-AAA GC column of 10m x 0.255mm internal diameter, using helium as a carrier gas. The inlet was set at 325°C, and the injection was in pulsed splitless mode. Separation of the extract was achieved following the method supplied with the EZ:faast kit, which used a column profile starting at 110°C and rising at 30°C per minute to 320°C, where it was held for 1 min. The MS transfer line was set at 300°C. Data were analysed using MSD CHEMSTATION software (v.E.02.00.493, Agilent Technologies) and amino acids were quantified based on standard solutions provided in the EZ:faast kit. A range of standard solutions varying in concentration were prepared and calibration curves created for each amino acid, enabling us to measure the absolute quantity of each amino acid (measured in nanomols per millilitre of internal standard) present in a spermatophylax.

Using the quantification method of Gershman *et al.* (2012) I measured the following 22 amino acids using the EZ:faast kit: alanine (ALA), glycine (GLY), α-aminobutyric acid (AAA), valine (VAL), leucine (LEU), isoleucine (ILE), threonine (THR), serine (SER), proline (PRO), asparagine (ASN), aspartic acid (ASP), methionine (MET), 4-hydroxyproline (HYP), glutamic acid (GLU), phenylalanine (PHE), glutamine (GLN), orthonine (ORN), glycyl-proline (GPR), lysine (LYS), histidine (HIS), tyrosine (TYR) and tryptophan (TRP). An example chromatograph of the 22 quantified amino acids is presented in Figure 3.1 with peak numbers corresponding to the amino acids in Table 3.1. As in Gershman *et al.* (2012), three of these amino acids (AAA, ORN and GPR) were excluded from further analysis as they were not present in all spermatophylaxes examined.

Assigning multivariate scores of gustatory appeal

Previously, a multivariate selection analysis has shown that a specific combination of amino acids in the spermatophylax is a significant predictor of whether females prematurely discard the spermatophylax after mating (Gershman *et al.*, 2012). It is clear from this work that multivariate combinations of amino acids in the spermatophylax are a far better predictor of this female behaviour than the amount of specific amino acids. I, therefore, used the results of this selection analysis to define the multivariate gustatory appeal of the

spermatophylax based on its amino acid composition. Following the approach of Gershman *et al.* (2013), I employed the following steps to calculate a multivariate score of gustatory appeal for each spermatophylax. First, I excluded three amino acids from my samples (α -aminobutyric acid, orthinine and glycyl-proline) as these were not present in all spermatophylaxes and were therefore not included in the selection analysis of Gershman *et al.* (2012). Second, Gershman *et al.* (2012) used Principal Component (PC) analysis to describe the variation in amino acid composition of the spermatophylax and then analysed the first three extracted principal components (PCs) in their selection analysis. It was therefore necessary to project the amino acid composition of male spermatophylaxes in my study into the same multivariate space examined in the selection analysis. This was done by substituting the amount of each amino acid present in the spermatophylax into the linear equations (i.e. eigenvectors) describing the three PCs in the selection analysis. These equations can be found in Table 1 of Gershman *et al.* (2012). Third, Gershman *et al.* (2012) showed in their selection analysis that the equation best describing the effect of the amino acid composition of the spermatophylax on the gustatory appeal of the spermatophylax (w , measured as the acceptance or rejection of the spermatophylax by a female) was given by the vector of linear selection gradients (β) as:

$$w = (-0.034PC1) + (-0.177PC2) + (-0.181PC3)$$

By substituting my PC scores into this equation, I calculated a unique multivariate attractiveness score for each spermatophylax in this experiment, whereby spermatophylaxes with a higher score have a greater gustatory appeal to females (i.e. not prematurely discarded) and therefore promote greater sperm transfer (Gershman *et al.*, 2013). A similar approach has been used by Jia & Greenfield (1997) to define the multivariate attractiveness of male acoustic calls in waxmoths to females during mate choice. I quantified the effect of P and C intake on this multivariate score rather than focussing on the amount of specific amino acids present in the spermatophylax.

3.3.4. Experiment 2: The effects of nutrient intake on ampulla attachment time

To determine the effects of P and C intake on ampulla attachment time, 16 males were established at random on each of the 24 diets (total $n = 384$ males) on their day of eclosion to adulthood and fed for 10 days to quantify the intake of P and C measured (as described above). Each male was then mated to a virgin female of the same age and the time taken for

the female to remove the ampulla (and therefore terminate sperm transfer) was recorded under red lighting. The male was removed immediately after spermatophore transfer using fine forceps to ensure that he did not influence female behaviour.

3.3.5. Experiment 3: Measuring nutrient intake under dietary choice

To determine if males actively regulate their intake of nutrients when given dietary choice, a total of 160 males were assigned at random on their day of eclosion to adulthood to one of four possible diet pairings ($n = 40$ per diet pair). These diet pairs differ in both the P to C ratio, as well as total nutritional content (P:C ratio(total nutrition): Pair 1: 5:1(36%) versus 1:8(36%), Pair 2: 5:1(36%) versus 1:8(84%), Pair 3: 5:1(84%) versus 1:8(36%) and Pair 4: 5:1(84%) versus 1:8(84%). This corresponds to diets 2, 4, 22 and 24 in Table 1.1 and is marked by red symbols in Figure 1.2. Diet consumption and the intake of P and C were measured every 2 days for a total of 10 days for each cricket using the protocol outlined above.

3.3.6. Statistical Analysis

In Experiments 1 and 2, I used a multivariate response-surface approach (South *et al.*, 2011) to estimate the linear and nonlinear effects of P and C intake on my response variables (spermatophylax weight, multivariate attractiveness of the spermatophylax and ampulla attachment time). Nonparametric thin-plate splines were used to visualize the nutritional landscapes for each response variable and were constructed using the *Tps* function in the 'FIELDS' package of R (version 2.15.1, www.r-project.org).

I used a sequential model building approach (South *et al.*, 2011) to determine whether the linear and nonlinear (quadratic and correlational) effects of P and C intake differed across the response variables (South *et al.*, 2011; Appendix 2). Inspection of the individual interaction terms in these models were used to determine which nutrient(s) contributed to any overall effects (South *et al.*, 2011). As my response variables were measured in different units, I standardized each response variable to a mean of zero and a standard deviation of one using a Z-transformation prior to analysis. Although this approach statistically tests for differences in the magnitude of linear and nonlinear gradients between the response variables, it does not provide information on the direction of this difference in nutritional space. It is therefore possible that the response variables show differences in the

magnitude of gradients but are optimized in similar regions on the nutritional landscape. I therefore also calculated the angle (θ) between the linear vectors for the two response variables being compared using trigonometry and the 95% confidence intervals for θ using a Bayesian approach implemented in the 'MCMCglmm' package of R (version 2.15.1, www.r-project.org). When $\theta = 0^\circ$ the vectors are perfectly aligned and the optima for the two response variables reside in the same location in nutrient space, whereas $\theta = 180^\circ$ represents the maximum possible divergence between vectors. Full details of these calculations and accompanying R code are provided in Appendix 3.

I used paired *t*-tests to compare the consumption of diets in each diet pair and a multivariate analysis of variance (MANOVA) to compare the total intake of P and C across diet pairs (Experiment 3). Univariate ANOVAs were used to determine which nutrients contributed to the overall multivariate difference across diet pairs and Tukey HSD tests to contrast the total intake of nutrients across each of the diet pairs. I calculated the regulated intake point, defined as the point in nutrient space that individuals actively defend when given dietary choice, as the mean intake of P and C across diet pairs (Simpson and Raubenheimer, 1993).

3.4. RESULTS

The intake of both P and C had clear linear effects on the weight and gustatory appeal of the spermatophylax, as well as ampulla attachment time (Table 3.2, Figure 3.2). All traits increased with the intake of these nutrients, with the intake of P and C having roughly similar effects on the expression of each trait (Table 3.2, Figure 3.2). There were significant quadratic effects of P intake on the weight and gustatory appeal of the spermatophylax and ampulla attachment time (Table 3.2) and inspection of the nutritional landscapes (Figure 3.2A-C) reveals peaks centred around a P:C ratio of approximately 1:1.3 for each trait. There were also significant positive correlational gradients for the gustatory appeal of the spermatophylax and ampulla attachment time (Table 3.2), providing further evidence that the expression of these traits increases with the intake of both nutrients.

Formal statistical comparisons showed that the linear and nonlinear effects of P and C intake on spermatophylax weight did not differ significantly from the effects of these nutrients on the gustatory appeal of the spermatophylax and ampulla attachment time

(Table 3.2). Consequently, the angle (θ) between the linear vectors for the weight and gustatory appeal of the spermatophylax and between spermatophylax weight and ampulla attachment time were small, being 15.47° (95% CIs: 7.25° , 26.57°) and 8.06° (3.92° , 13.69°), respectively. The linear effect of P and C intake on the gustatory appeal of the spermatophylax and ampulla attachment time differed significantly but the quadratic and correlational effects of these nutrients did not (Table 3.3). The difference in linear effects was due to the fact that ampulla attachment time was more responsive to the intake of P and C than the gustatory appeal of the spermatophylax rather than P and C intake having contrasting effects on these traits (Table 3.2). As a result, θ was also small between these two traits at 14.27° (6.65° , 24.38°). Collectively, these analyses demonstrate that there is little divergence in the effects of P and C intake on the weight and gustatory appeal of the spermatophylax and ampulla attachment time in *G. sigillatus*.

In each diet pair, males consumed significantly more of the high C diet than the high P diet (Figure 3.3). Not surprisingly, there was a significant difference across the diet pairs in the intake of nutrients (MANOVA: Pillai's Trace = 0.97, $F_{6,312} = 48.61$, $P = 0.0001$) and univariate ANOVAs showed that this effect was driven by the intake of both P ($F_{3,156} = 34.43$, $P = 0.0001$) and C ($F_{3,156} = 66.83$, $P = 0.0001$). Tukey HSD tests (at $P < 0.05$) showed that the order of diets pairs for P intake was $1 = 2 < 4 < 3$ and for C intake was $3 < 1 < 2 = 4$ (Figure 3.4). The regulated intake point was estimated at an intake of 26.39 ± 1.16 mg of P and 45.79 ± 1.70 mg of C, which corresponds to a P:C ratio of 1:1.74 (Figure 3.4). Importantly, this regulated intake point does not correspond with the peaks for the weight and gustatory appeal of the spermatophylax or ampulla attachment time (Figure 3.2) and therefore demonstrates that the regulation of P and C intake is not optimal for the expression of these traits.

3.5. DISCUSSION

In this study, I used the GF to examine the effects of P and C intake on the weight and gustatory appeal of the male spermatophylax in *G. sigillatus*, as well as the role this plays in mediating sexual conflict via ampulla attachment time. If the intake of these nutrients regulates sexual conflict in *G. sigillatus*, I predicted that nutrient intake would have similar effects on the weight and gustatory appeal of the spermatophylax, as well as ampulla

attachment time. Consistent with this prediction, I found that the nutritional landscapes for these traits were all closely aligned, with each trait being maximised at a high intake of nutrients in a P:C ratio of 1:1.3. Furthermore, if males are able to regulate their intake of nutrients to bias the outcome of sexual conflict in their favour, I predicted that when given dietary choice males would regulate their intake of nutrients to coincide with the peak in ampulla attachment time on the nutritional landscape. In agreement with this prediction, I found that males regulated their intake of nutrients at a P:C ratio of 1:1.74, which is close (but not identical) to the P:C ratio maximising ampulla attachment time and the weight and gustatory appeal of the spermatophylax. This regulated intake point, however, did not fall on the optima for ampulla attachment time or for the weight and gustatory appeal of the spermatophylax. Collectively, my findings show that a balanced intake of P and C plays a crucial role in regulating sexual conflict in *G. sigillatus* but males are unable to completely bias this process in their favour by regulating the intake of these nutrients.

The intake of P and C had clear effects on both the weight and gustatory appeal of the spermatophylax and the nature of these nutritional effects provide two important insights into how this endogenous gift is produced in *G. sigillatus*. First, I show that the weight of the spermatophylax and the amino acid composition that increases the likelihood that a female will fully consume this gift increased with the overall intake of nutrients (and therefore caloric or energy intake). This finding is consistent with the weight and gustatory appeal of the spermatophylax being energetically costly to produce, as only males with the highest intake of P and C have sufficient nutritional resources available to allocate to maximizing these traits. Supporting this view are studies on *G. sigillatus* showing that immune function is traded against spermatophylax weight (Gershman *et al.*, 2010) and males that have their immune function challenged with lipopolysaccharides (Kerr *et al.*, 2010) or sexually transmitted nematodes (Luong and Kaya, 2004) produce smaller spermatophylaxes. We currently do not know if similar trade-offs exist for the combination of amino acids that enhances the gustatory appeal of the spermatophylax. Second, I show that the weight and gustatory appeal of the spermatophylax was maximised at a P:C ratio of 1:1.3. Thus, it is not only a high intake of nutrients that is important for maximising these traits in *G. sigillatus* but the intake of P and C must also be balanced. This P:C ratio is strikingly similar to the ratio known to optimize egg production in a range of female insect species (e.g. P:C = 1:2 in *D. melanogaster*, (Lee *et al.*, 2008; Reddiex *et al.*, 2013; Jensen *et*

al., 2015); 1:2.3 and 1:1 in Queensland fruit flies, (Fanson *et al.*, 2009; Fanson & Taylor, 2012); 1:1 and 1:3 in field crickets, (Maklakov *et al.*, 2008; Harrison *et al.*, 2014)), as well as a number of male traits, including sperm production in cockroaches (P:C = 1:2, Bunning *et al.*, 2015) and competitive ability in *D. melanogaster* (P:C = 1:2, Reddiex *et al.*, 2013; Jensen *et al.*, 2015) . It contrasts, however, the P:C ratio commonly found to maximise male traits used in pre-copulatory sexual selection, including calling effort in crickets (P:C = 1:8, Maklakov *et al.*, 2008) and pheromone production in cockroaches (P:C = 1:8, South *et al.*, 2011), where a relatively higher intake of C is needed to fuel these energetically costly traits. Just as female insects require a higher intake of P than males to manufacture eggs (Maklakov *et al.*, 2008; Jensen *et al.*, 2015), there is good reason to expect that producing a heavy spermatophylax with an enhanced gustatory appeal also has a relatively high demand for P. Over 90% of the dry mass of the spermatophylax in *G. sigillatus* consists of P with only 7% of this representing the free amino acids that influence gustatory appeal (Warwick *et al.*, 2009). Consequently, my measure of dry spermatophylax weight largely reflects sources of P in the gift other than free amino acids and it has been speculated that this may represent elastin-like structural proteins that contribute to the gummy consistency of the spermatophylax (Heller *et al.*, 1998). Casein (bovine milk) and albumin (egg yolk) which comprise 80% of the P used in my artificial diets, contain high quantities of essential amino acids, including lysine which plays a key role in elastin synthesis (Cohen, 2015). Furthermore, these sources of P are also known to be abundant in many of the essential (e.g. lysine, phenylalanine and valine) and some non-essential (e.g. glycine, histidine, leucine and 4-hydroxy-proline) amino acids (Cohen, 2015) known to contribute heavily to the gustatory appeal of the spermatophylax (Gershman *et al.*, 2012). More work is needed, however, to determine how these sources of P are degraded to free amino acids during digestion in *G. sigillatus* and how these amino acids are absorbed and possibly converted before they are incorporated into the spermatophylax.

Importantly, I found that the intake of P and C that maximised the weight and gustatory appeal of the spermatophylax were closely aligned with the intake of nutrients needed to maximise ampulla attachment time. Indeed, the largest angle between the vectors describing the linear effects of nutrient intake on these traits was only 15.47°. This close alignment provides support for the proposed causal relationship linking the properties of the spermatophylax to ampulla attachment time in *G. sigillatus*. That is, a high intake of

nutrients with a P:C ratio of 1:1.3 enables males to produce a heavy spermatophylax with high gustatory appeal, both of which are known to prolong female feeding on the spermatophylax and increase subsequent ampulla attachment time (Sakaluk, 1984; Gershman *et al.*, 2012). As the number of sperm transferred to a female increases linearly with ampulla attachment time (up to a maximum of 55 minutes, (Sakaluk, 1984) and sperm competition conforms to a simple lottery system (Eggert *et al.*, 2003), this intake of nutrients is likely to enhance the number of offspring sired by a male *G. sigillatus* when mating in competitive situations. This argument assumes, however, that the intake of nutrients that maximise the weight and gustatory appeal of the spermatophylax also enables males to produce a large number of viable sperm. While the intake of nutrients that maximises sperm number and viability is currently not known for *G. sigillatus*, a recent study on the cockroach *Nauphoeta cinerea* found that sperm number was maximised at a high intake of nutrients in a P:C ratio of 1:2, whereas the intake of these nutrients did not influence sperm viability (Bunning *et al.*, 2015). Therefore, while more work is needed to confirm this, it appears likely that a male is able to capitalize on producing a heavy and appealing spermatophylax by also producing a large number of viable sperm.

While the peaks for ampulla attachment time and the gustatory appeal of the spermatophylax were closely aligned, with an angle of only 14.27° between the linear effects of nutrient intake, there was a significant difference in the magnitude of the linear effects of both P and C intake on these traits. More specifically, ampulla attachment time was more responsive to the intake of these nutrients than the gustatory appeal of the spermatophylax. This suggests that factors other than the appeal of the spermatophylax may also influence a female's decision to terminate mating by removing the ampulla. It is possible that females prolong attachment time as a form of post-copulatory mate choice because males with this intake of nutrients are more attractive. This is unlikely, however, as post-copulatory mate choice is known to reinforce pre-copulatory mate choice in *G. sigillatus* (Ivy & Sakaluk 2007) and male traits known to be attractive to females before mating, such as calling effort and cuticular hydrocarbon expression, are optimized at a much higher intake of C (P:C ratio of 1:8, Chapters 2 and 4). It is also possible that substances (other than free amino acids) transferred in the spermatophylax and/or ejaculate influence this female behaviour, especially if substances are influenced by the intake of P and C. A prime candidate is SFPs which have been detected in both the ejaculate of field crickets

(e.g. Andres *et al.*, 2008; Simmons *et al.*, 2013) and the spermatophylax of bushcrickets (Marchini *et al.*, 2009) and recent work (Pauchet *et al.*, 2015) has shown that SFPs are also present in the spermatophylax of *G. sigillatus*. SFPs in insects are known to influence many aspects of female reproduction and behaviour, with examples of the latter including an increase in feeding and general activity levels (Avila *et al.*, 2011). Given that most identified insect SFPs represent numerous classes of proteins (e.g. peptides, proteases)(Avila *et al.*, 2011), it is not surprising that their expression is influenced by the amount of dietary P consumed by the male (McGraw *et al.*, 2007). It is more difficult to envisage how the intake of C would influence SFP expression, although it is important to note that seminal fluid in insects also contains a variety of non-protein molecules (i.e. steroids, prostaglandins) that also influence female behaviour (Avila *et al.*, 2011) and may be influenced by the intake of this nutrient. More work is clearly needed, however, to determine if the intake of P and C influences SFP expression in the spermatophylax of male *G. sigillatus* and whether this influences the ampulla removal behaviour of females.

My work shows that males can clearly optimize the size and gustatory appeal of the spermatophylax, as well as subsequent effects on ampulla attachment time, by consuming a high intake of nutrients in a P:C ratio of 1:1.3. Yet, when provided with dietary choice males regulated their intake of nutrients towards a slightly higher intake of C than is optimal (a P:C ratio of 1:1.74) and at a much lower overall intake of nutrients. Consequently, this regulated intake point did not coincide with the optima for ampulla attachment time or for the weight and gustatory appeal of the spermatophylax (Figure 3.2A-C) and therefore provides clear evidence that male *G. sigillatus* do not optimally regulate their intake of these nutrients to bias the outcome of sexual conflict in their favour. Indeed, inspection of the nutritional landscape in Figure 3.2C shows that this observed pattern of nutrient regulation reduces ampulla attachment time by approximately 23 minutes (or 35%). This reduction in ampulla attachment time is biologically important as comparison to the sperm transfer curve for this species (see Figure 2 in Sakaluk, 1984) reveals that 4×10^{-3} fewer sperm would be transferred to the female compared to the optimal intake of P and C. Given the substantial costs to the suboptimal regulation of nutrient intake shown here, it is surprising that optimality does not appear to be the norm more generally: GF studies on *D. melanogaster* (Jensen *et al.*, 2015), field crickets (Maklakov *et al.*, 2008; Harrison *et al.*, 2014) and cockroaches (South *et al.*, 2011; Bunning *et al.*, 2015) all show that males do not optimally

regulate the intake of P and C even though this comes at a substantial cost to trait expression.

There are a number of possible explanations for why male *G. sigillatus* do not optimally regulate their intake of P and C. First, males may regulate their intake of nutrients to maximise the expression of other, more heavily prioritised traits. The regulated intake point shown here for *G. sigillatus* is similar to that observed in male *D. melanogaster* (P:C = 1:4; Lee *et al.*, 2013; Jensen *et al.*, 2015), field crickets (P:C = 1.3, Maklakov *et al.*, 2008; 1:4.1, Harrison *et al.*, 2014) and cockroaches (P:C = 1:3.2, South *et al.*, 2011; 1:4.95, Bunning *et al.*, 2015) where a C biased intake is also preferred. In all of these species, important fitness-related traits in males, such as pheromone expression, competitive ability, calling effort and lifespan, are also maximised at a high intake of C biased diets. Similarly, CHC expression, calling effort and lifespan in male *G. sigillatus* are all maximised at a high intake of nutrients in a P:C ratio of approximately 1:8 (Chapter 5). It is therefore possible that males bias their relative C intake for the expression of these traits, although given the proximity of the regulated intake point to the optima for traits used in sexual conflict, this appears unlikely to have a large impact on the regulated intake point. Alternatively, males may be regulating their nutrient intake to balance the expression of multiple, competing traits. For example, male cockroaches regulate their intake of nutrients at a P:C ratio of 1:4.95 which is midway between the ratio maximizing sperm production (P:C = 1:2, Bunning *et al.*, 2015) and pheromone production (P:C = 1:8, South *et al.*, 2011). However, it is currently unclear exactly what traits male *G. sigillatus* may be balancing, although I have shown that immune function in males is maximised at a P:C ratio of approximately 5:1 (Chapter 4). If males are balancing immunity against calling effort this would produce a regulated intake of nutrients close to my observation in the present study. Second, males may not optimally regulate the intake of P and C because they are constrained from doing so. One constraint that is likely to explain the reduced intake of nutrients at the regulated intake point compared to the optima for traits involved in sexual conflict are physiological constraints on feeding behaviour. It is well known that dietary assimilation, digestion, absorption and utilization can all constrain feeding behaviour in animals (Henson & Hallam, 1995) and that the efficiency of these processes is often contingent on gut morphology (e.g. Penry & Jumars, 1990; McWhorter & del Rio, 2000). If the choice between complex diets limits the efficiency of one or more of these processes and/or is limited by gut morphology,

it may prevent males from regulating their intake of nutrients optimally. Another possibility is that male nutrient intake under dietary choice is genetically constrained. Males and females share most of their genome, including the genes that underlie nutrient regulation and this generates strong positive genetic correlations between the intakes of nutrients across the sexes (r_{MF}). For example, there is a strong positive r_{MF} for C intake across the sexes in *D. melanogaster* but this relationship is much weaker for P intake (Reddiex *et al.*, 2013). In *G. sigillatus*, these positive r_{MF} s are much stronger for the intake of both nutrients (Hunt *et al.*, unpublished data) as is also the case in *T. commodus* (Chapter 5). In theory, positive r_{MF} s can prevent nutrient regulation from evolving independently in the sexes, thereby constraining the evolution of sexual dimorphism in this trait (Bonduriansky and Chenoweth, 2009). Thus, if the sexes have different optima for nutrient intake, as is known to occur for lifespan and reproduction in *G. sigillatus* (Hunt *et al.*, unpublished data), intralocus conflict over nutrient regulation can occur and one or both sexes can be displaced from their optimal nutrient intake. Although the evidence for intralocus conflict over optimal nutrient intake is not strong in *D. melanogaster* (Reddiex *et al.*, 2013), recent work suggests that it plays a more significant role in the evolution of the regulated intake point in male *G. sigillatus* (Hunt *et al.*, unpublished data). In general, understanding the constraints that shape the evolution of nutrient regulation remains one of the major unanswered questions in nutritional ecology and is clearly a topic that deserves more attention.

In conclusion, I show that nutrition is a key determinant of the weight and gustatory appeal of the spermatophylax, as well as the subsequent effects of consuming this gift on ampulla attachment time, in male *G. sigillatus*. This relationship was complex, however, with a high intake of P and C in a specific balance being required to maximise these traits. Although well-defined nutritional optima for these traits illustrates that males have the potential to bias the outcome of sexual conflict in their favour by regulating their intake of nutrients, males did not do so despite coming at a substantial cost to sperm transfer. Collectively my work shows that sexual conflict is regulated by the intake of these important macronutrients in *G. sigillatus* and highlights the value of using a standardized framework, such as the GF, to reveal the complexity of this process.

Table 3.1. The amino acids that correspond to the peak numbers found on the example chromatograph in Figure 3.1.

| Peak No. | Amino Acid Abbreviation | Amino Acid |
|----------|-------------------------|-----------------------------|
| 1 | ALA | Alanine |
| 2 | GLY | Glycine |
| 3 | AAA | α -aminobutyric acid |
| 4 | VAL | Valine |
| 5 | STANDARD | Norvaline |
| 6 | LEU | Leucine |
| 7 | ILE | Isoleucine |
| 8 | THR | Threonine |
| 9 | SER | Serine |
| 10 | PRO | Proline |
| 11 | ASN | Asparagine |
| 12 | ASP | Aspartic Acid |
| 13 | MET | Methionine |
| 14 | HYP | 4-hydroxyproline |
| 15 | GLU | Glutamic Acid |
| 16 | PHE | Phenylalanine |
| 17 | GLN | Glutamine |
| 18 | ORN | Ornithine |
| 19 | GPR | Glycyl-proline |
| 20 | LYS | Lysine |
| 21 | HIS | Histidine |
| 22 | TYP | Tyrosine |
| 23 | TRP | Tryptophan |

Table 3.2. The effect of protein (P) and carbohydrate (C) intake on spermatophylax (SPHYLAX) weight, the gustatory appeal of the SPHYLAX and ampulla (AMP) attachment time in *Gryllobates sigillatus*.

| Response variable | Linear effects | | Nonlinear effects | | |
|----------------------------|----------------|-------------|-------------------|--------------|-------------|
| | P | C | P x P | C x C | P x C |
| SPHYLAX weight | | | | | |
| Coefficient ± SE | 0.22 ± 0.05 | 0.17 ± 0.05 | -0.12 ± 0.04 | -0.01 ± 0.04 | 0.09 ± 0.07 |
| t_{378} | 4.50 | 3.50 | 2.75 | 0.25 | 1.24 |
| <i>P</i> | 0.0001 | 0.001 | 0.006 | 0.81 | 0.22 |
| SPHYLAX appeal | | | | | |
| Coefficient ± SE | 0.11 ± 0.05 | 0.11 ± 0.05 | -0.10 ± 0.04 | -0.02 ± 0.04 | 0.17 ± 0.08 |
| t_{378} | 2.13 | 2.06 | 2.25 | 0.52 | 2.24 |
| <i>P</i> | 0.03 | 0.04 | 0.03 | 0.60 | 0.03 |
| AMP attachment time | | | | | |
| Coefficient ± SE | 0.32 ± 0.05 | 0.26 ± 0.05 | -0.14 ± 0.05 | -0.00 ± 0.04 | 0.20 ± 0.08 |
| t_{381} | 6.63 | 5.42 | 3.05 | 0.02 | 2.66 |
| <i>P</i> | 0.0001 | 0.0001 | 0.002 | 0.98 | 0.008 |

Table 3.3. Sequential model comparing the linear and nonlinear effects of protein (P) and carbohydrate (C) intake on spermatophylax (SPHYLAX) weight, the gustatory appeal of SPHYLAX and ampulla (AMP) attachment time in *Gryllobates sigillatus*.

| | SS_R | SS_C | DF_1 | DF_2 | <i>F</i> | <i>P</i> | θ (95% CI) |
|---|--------|--------|--------|--------|----------|----------|------------------------|
| SPHYLAX weight vs. appeal | | | | | | | |
| Linear | 731.08 | 727.79 | 2 | 762 | 1.72 | 0.18 | 15.47° (7.25°, 26.57°) |
| Quadratic | 718.42 | 718.20 | 2 | 758 | 0.12 | 0.89 | |
| Correlational | 712.46 | 711.94 | 1 | 756 | 0.56 | 0.46 | |
| SPHYLAX weight vs. AMP attachment time | | | | | | | |
| Linear | 683.10 | 680.17 | 2 | 762 | 1.64 | 0.19 | 8.06° (3.92°, 13.69°) |
| Quadratic | 665.80 | 665.44 | 2 | 758 | 0.21 | 0.81 | |
| Correlational | 659.17 | 658.20 | 1 | 756 | 1.12 | 0.29 | |
| SPHYLAX appeal vs. AMP attachment time | | | | | | | |
| Linear | 713.29 | 701.33 | 2 | 762 | 6.50 | 0.002* | 14.27° (6.65°, 24.38°) |
| Quadratic | 690.22 | 689.14 | 2 | 758 | 0.59 | 0.55 | |
| Correlational | 678.51 | 678.42 | 1 | 756 | 0.10 | 0.75 | |

Univariate test: * *P*: $F_{1,762} = 8.96$, *P* = 0.003; *C*: $F_{1,762} = 4.91$, *P* = 0.027.

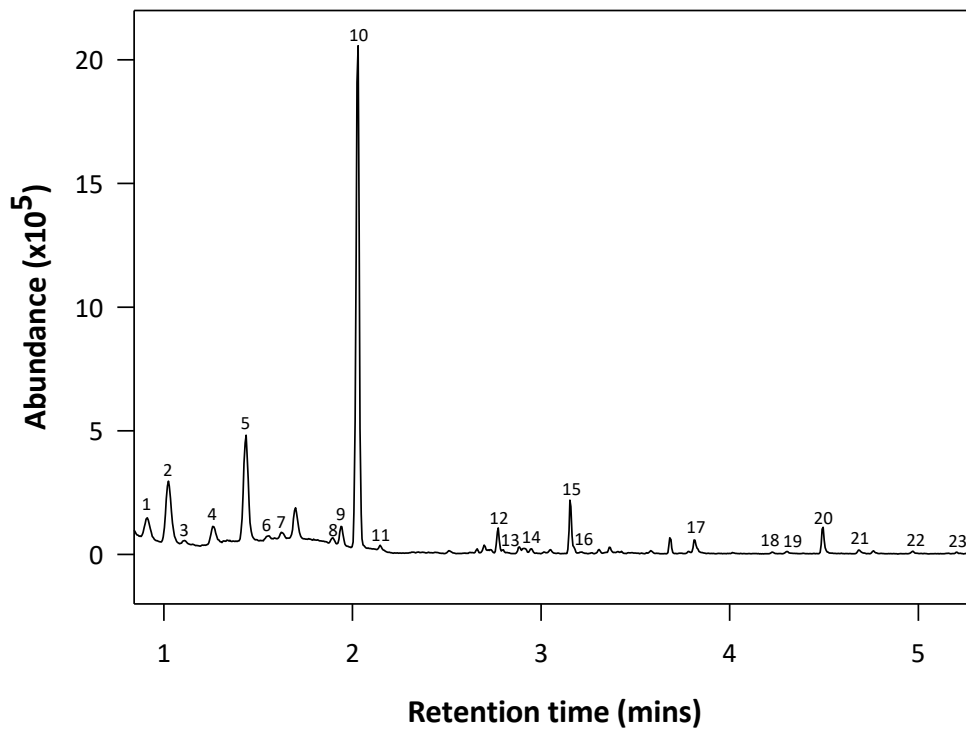


Figure 3.1. A typical chromatograph showing the amino acids contained in the spermatophylax of male *Gryllodes sigillatus*. The x-axis shows the retention time for each amino acid and the y-axis shows the abundance of each amino acid, measured as the area under the peak and expressed in actual quantities based on standard curves. The numbers above the amino acid peaks correspond to the peak numbers provided in the Table 3.1.

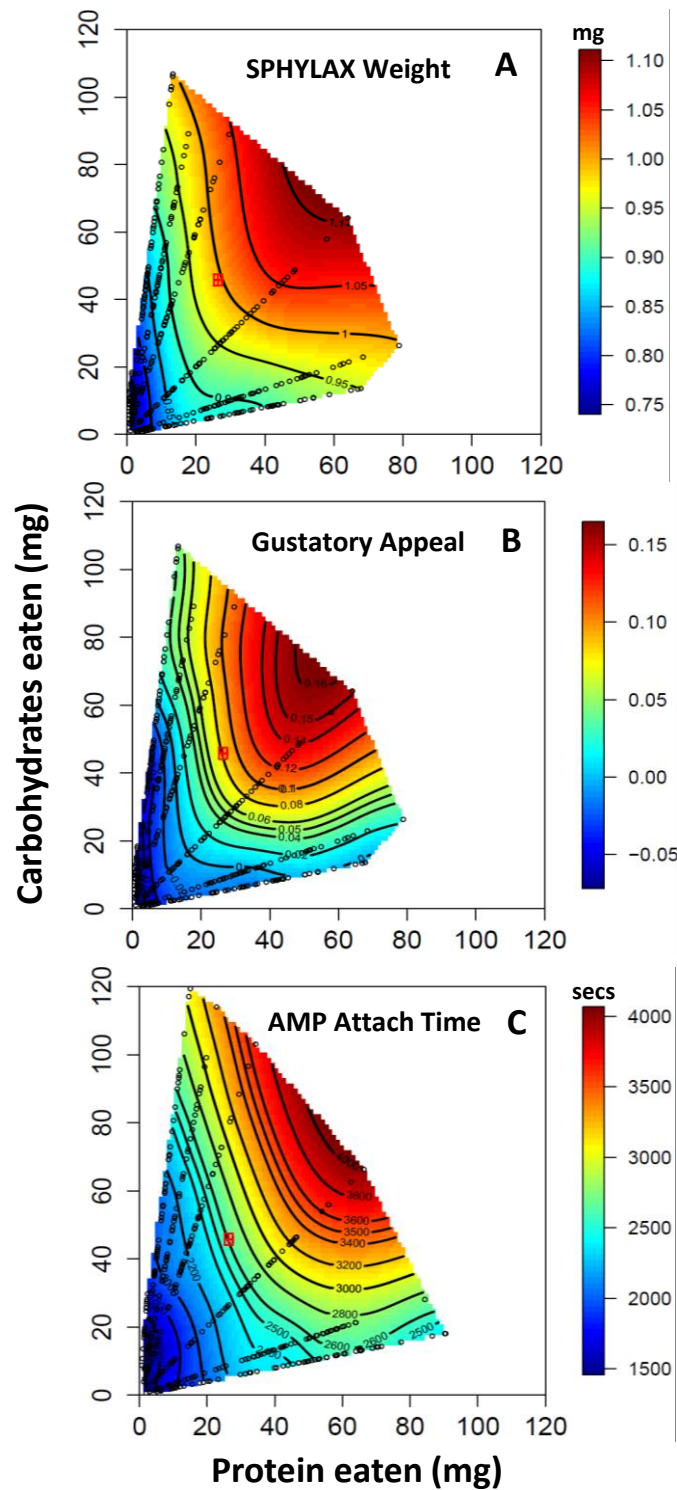


Figure 3.2. Nutritional landscapes illustrating the effects of protein and carbohydrate intake on (A) spermatophylax weight, (B) the gustatory appeal of the spermatophylax and (C) ampulla attachment time in *Gryllodes sigillatus*. High values of these traits are given in red and low values in blue. The black dots represent the actual nutrient intake data for each individual cricket and the red cross on each landscape represents the regulated intake point (\pm SE) that is presented in Figure 3.4 and derived from the choice experiment (Experiment 2).

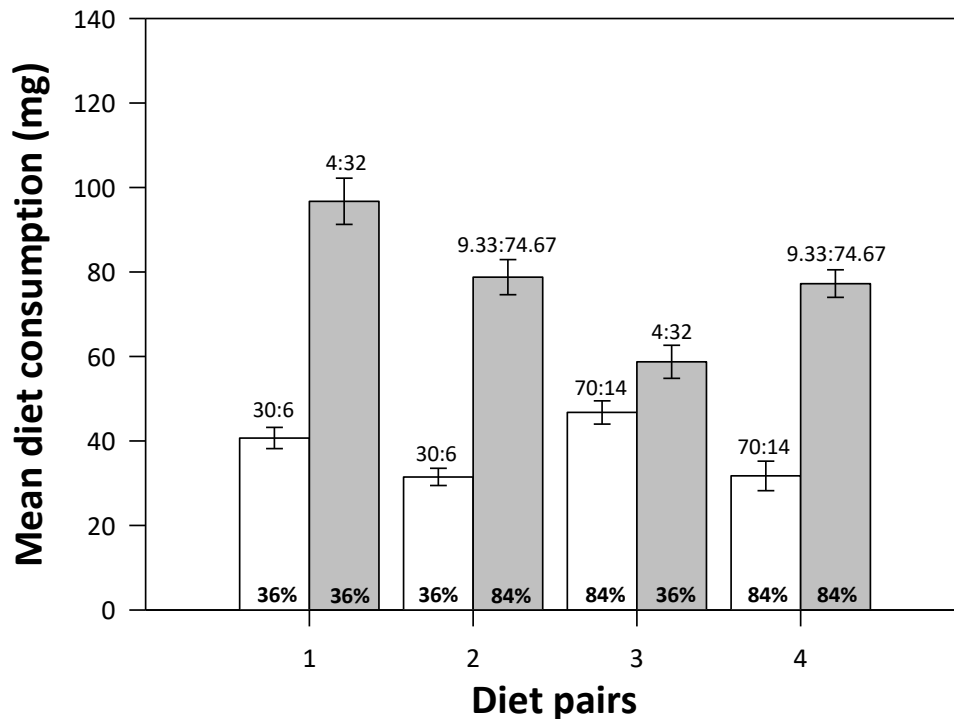


Figure 3.3. The mean (\pm SE) consumption of diets in each diet pair. White bars represent the high P diet and grey bars the high C diet in each diet pair. The P:C ratio of the diet is provided above the bar and the total nutritional content of the diet (%) is provided within the bar in bold. In each diet pair, males consumed significantly more of the high C diet than the high P diet (Diet pair 1: $t_{39} = 9.58$, $P = 0.0001$; Diet pair 2: $t_{39} = 10.32$, $P = 0.0001$; Diet pair 3: $t_{39} = 2.42$, $P = 0.020$; Diet pair 4: $t_{39} = 9.70$, $P = 0.0001$).

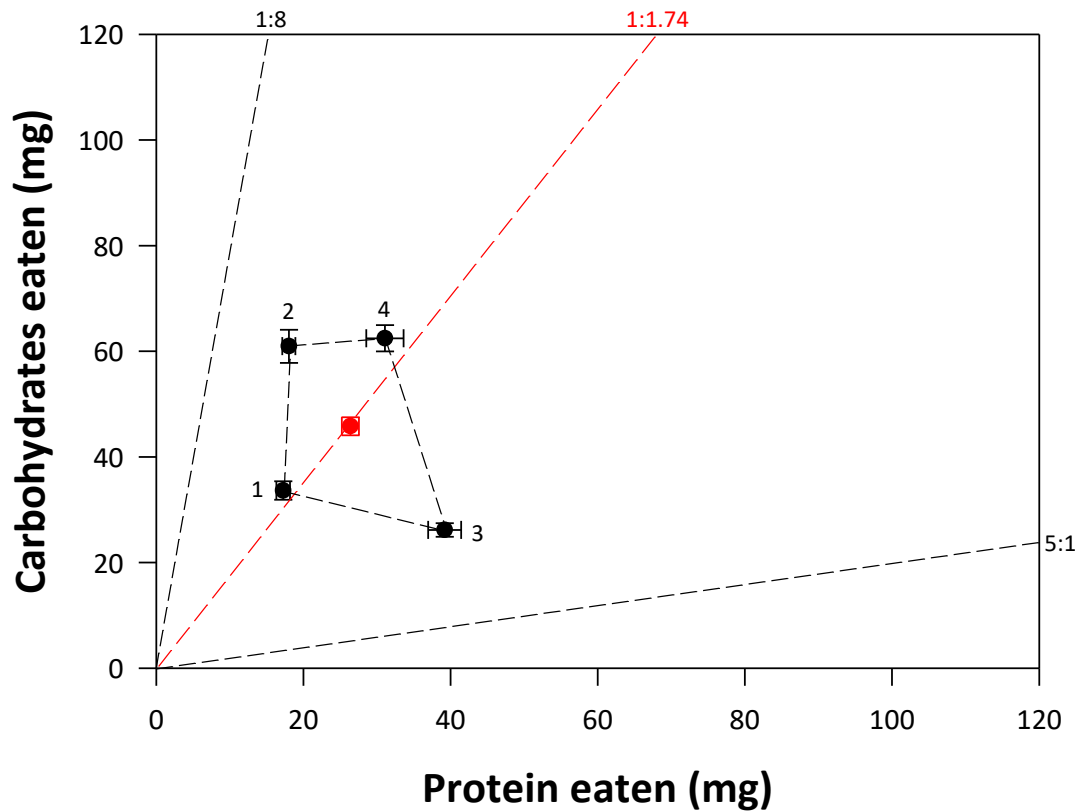


Figure 3.4. The mean (\pm SE) intake of protein (P) and carbohydrates (C) for the 4 diet pairs (black symbols, labelled by number) and the regulated intake point (\pm SE, red symbol), calculated as the mean P and C intake across diet pairs. The black dashed lines represent the outer boundaries of the choice experiment design (P:C ratios of 5:1 and 1:8) and therefore crickets are able to feed to any point in nutritional space within these rails. The red dashed line represents the P:C ratio that passes through the regulated intake point, estimated at a P:C ratio of 1:1.74. This is the P:C ratio that crickets actively defend when given dietary choice.

CHAPTER 4:

THE GEOMETRY OF NUTRITIONALLY BASED LIFE-HISTORY TRADE-OFFS: SEX DIFFERENCES IN THE EFFECT OF MACRONUTRIENT INTAKE ON THE TRADE-OFF BETWEEN IMMUNE FUNCTION AND REPRODUCTIVE EFFORT IN DECORATED CRICKETS

4.1. ABSTRACT

Life history theory is based on the assumption that resources are finite so that traits competing for this common pool of resources will experience a trade-off. The shared resource most commonly studied is food and studies typically manipulate resource acquisition by varying diet quantity or quality without considering the specific nutrients involved. Recent studies using the Geometric Framework (GF), however, suggest that life-history trade-offs are often regulated by the intake of specific nutrients. Despite this, a robust framework documenting the existence and quantifying the strength of nutritionally based trade-offs currently does not exist for studies using the GF. Here, I provide a conceptual framework showing that such trade-offs occur when life-history traits are maximised in different regions of nutrient space and that this divergence can be quantified by the overlap in the 95% confidence region (CR) of the global maxima, the angle (θ) between the linear nutritional vectors and the Euclidean distance (d) between the global maxima for each trait. I then empirically tested this framework by examining the effects of protein (P) and carbohydrate (C) intake on the trade-off between reproduction and immune function in male and female decorated crickets (*Gryllodes sigillatus*). Encapsulation ability and egg production in females increased with the intake of both nutrients, being maximised at a P:C ratio of 1.04:1 and 1:1.17, respectively. In contrast, encapsulation ability in males only increased with the intake of P being maximised at a P:C ratio of 5.14:1, whereas calling effort increased with the intake of C but decreased with the intake of P and was maximized at a P:C ratio of 1:7.08. Consequently, the trade-off between reproduction and encapsulation ability is much larger in males than females, a view supported by the non-overlapping 95% CRs on the global maxima for these traits in males and the larger estimates of θ and d . The sexes regulated their intake of nutrients in a similar way under dietary choice, at a P:C ratio of 1:2 and 1:1.84 in males and females, respectively. Although this ratio was more closely aligned with the optima for immune function and reproduction in females than males, neither sex optimally regulated their nutrient intake. Collectively, my study highlights that greater consideration should be given to the intake of specific nutrients when examining nutritionally based life-history trade-offs and how this varies across the sexes.

Key Words: Dietary Choice, Geometric Framework, *Grylodes sigillatus*, Immune Function, Reproductive Effort, Trade-offs.

4.2. INTRODUCTION

Life-history traits are often negatively correlated with each other (Reznick, 1985; Stearns, 1992; Roff, 2002). These negative correlations, referred to as life-history trade-offs, form the cornerstone of life-history theory and are central to predicting the optimal life-history of an organism in a given environment (Stephen & Krebs 1986; Charnov, 1989; Stearns, 1992; 2000; Houston & McNamara, 1999; Reznick *et al.*, 2000; Roff, 1993; 2002; Roff & Fairbairn, 2007). Phenotypic trade-offs exist because different life-history traits compete for a finite pool of resources, so that the allocation of resources to one trait necessarily means there is less available to allocate to other traits (Stearns, 1992; Roff, 2002). In this way, trade-offs have the potential to bias or constrain the evolution of life-history strategies as both traits involved in the trade-off cannot be simultaneously maximised (Roff & Fairbairn, 2007; 2012).

While the existence of trade-offs between life-history traits appear taxonomically widespread, positive phenotypic correlations are also commonly found in both laboratory and natural populations (Stearns, 1992; Reznick *et al.*, 2000; Roff, 2002). One of the most prominent models explaining this variation in the sign of phenotypic correlations between life-history traits is the acquisition-allocation model (more commonly known as the “Y-model”) of Van Noordwijk & de Jong (1986). This model (and subsequent extensions) posits that the sign of the covariance between life-history traits depends critically on the relative variances in the acquisition and allocation of resources (Van Noordwijk & de Jong, 1986; Roff & Fairbairn, 2007). More specifically, if the sum of the variances in resource allocation to the two life-history traits ($\sigma_{x1}^2 + \sigma_{x2}^2$) exceeds the variance in resource acquisition (σ_T^2) then a negative covariance between life-history traits will occur, whereas if $\sigma_T^2 > \sigma_{x1}^2 + \sigma_{x2}^2$ then a positive covariance will occur (Roff & Fairbairn, 2007). Despite the elegance of this theoretical prediction, direct empirical tests have proven challenging due, in part, to the difficulties associated with quantifying resource acquisition (Stearns, 1989; Zera & Harshman, 2001; Roff, 2002; Roff & Fairbairn, 2007). Although a number of different measures have been used as proxies for resource acquisition, including body size at a given age (e.g. Biere, 1995; Dudycha & Lynch, 2005) and lipid stores (e.g. Chippindale *et al.*, 1998), a more powerful approach to examine trade-offs in life-history studies is to experimentally alter resource acquisition ability through dietary manipulation (Reznick, 1985; Reznick *et al.*,

2002; Roff & Fairbain, 2007). Indeed, empirical studies on a range of animal taxa have shown that manipulating the quantity and/or quality of the available diet can have a profound effect on the trade-off between different life-history traits (e.g. invertebrates: Hunt *et al.*, 2004; fish: Kolluru & Grether, 2005; amphibians: Lardner & Loman, 2003; reptiles: Brown & Shine, 2002; birds: Karell *et al.*, 2007; mammals: Hill & Kaplan, 1999).

Variance in reproduction and immune function are major determinants of fitness and are therefore central to the life-history of most organisms (Reznick, 1985; Stearns, 1992; Roff, 2002). Both processes are energetically demanding and have been shown to trade-off in a range of animal taxa (e.g. invertebrates: Ahtianinen *et al.*, 2004, Schwenke *et al.*, 2016; fish: Kalbe *et al.*, 2009; amphibians: McCallum & Trauth, 2007; reptiles: French *et al.*, 2007; birds: Nordling *et al.*, 1998; mammals: Mills *et al.*, 2009). This energetic requirement of reproduction and immune function suggests that the competitive allocation of limiting resources is likely to be the basis for the trade-off between these traits (Sheldon & Verhulst, 1996; Zara & Harshman, 2001; Schwenke *et al.*, 2016). Indeed, numerous studies have shown that this trade-off is “facultative”, being less pronounced or absent when individuals have *ad libitum* access to food (e.g. Shoemaker *et al.*, 2006; French *et al.*, 2007; Xu *et al.*, 2012) or only during energetically taxing reproductive periods (e.g. Adamo *et al.*, 2001). For example, female tree lizards (*Urosaurus ornatus*) provided with unlimited food are able to sustain both reproduction and immune function whereas individuals maintained on a restricted diet showed a trade-off between these traits (French *et al.*, 2007). In addition, prior to being sexually mature the immune function of male crickets (*Gryllus texensus*) is similar to that of females but this is dramatically reduced when males reach sexually maturity and start producing a metabolically demanding acoustic signal (Adamo *et al.*, 2001). However, the trade-off between reproduction and immune function may also be “obligate” whereby physiological changes that occur during reproduction directly impact immune function or *vice versa*. For example, it is well established in vertebrates that sex steroids (such as testosterone and oestrogen) can bind to immune cells and suppress their function (Schuurs & Verheul, 1990). Furthermore, phenoloxidase is a key enzyme of the invertebrate immune system but can induce self-damage that may reduce overall reproductive performance (Sadd & Siva-Jothy, 2006). In such cases, the trade-off between reproduction and immune function should be independent of resource acquisition. To evaluate whether the trade-off between reproduction and immune function is “facultative”

or “obligate” therefore requires having some insight into what the limiting resource(s) might be and a mechanistic understanding of how that resource(s) is allocated (Zara & Harshman, 2001; Schwenke *et al.*, 2016).

In most species, the sexes possess different optimal reproductive strategies and this is expected to promote the evolution of sex differences in the trade-off between reproduction and immune function (Rolff, 2002; Zuk & Stoehr, 2002; Zuk, 2009). Females typically invest heavily in reproduction, producing nutrient-rich eggs and often providing the most care to offspring, and their reproductive success is therefore limited by the number of offspring that can be produced and reared (Bateman, 1948; Trivers, 1972). In contrast, males typically invest relatively little in reproduction so that their reproductive success is limited by the number of mates that can be fertilized (Bateman, 1948; Trivers, 1972). Consequently, the variance in reproductive success (and therefore the intensity of sexual selection, Arnold, 1994) is expected, on average, to be greater in males than females (Bateman, 1948; Trivers, 1972). It has been argued that males will be selected to pursue a “live hard, die young” strategy, whereby more resources are allocated to current reproduction at the expense of immune function and lifespan (Rolff, 2002; Zuk & Stoehr, 2002; Zuk, 2009). Conversely, females can produce more offspring by living longer and therefore are expected to invest less in current reproduction and more in immune function (Rolff, 2002; Zuk & Stoehr, 2002; Zuk, 2009). Formal testing of this view with mathematical models, however, have shown that the magnitude and direction of sex differences in the allocation of resources to immune defences are far more variable than this single outcome and that any number of possible outcomes can be favoured under certain conditions (Medley, 2002; Stoehr & Kokko, 2006; Restif & Amos, 2010). For example, Stoehr & Kokko (2006) showed that if both the impact of parasites on condition and the condition-dependence of reproductive effort is the same for the sexes and if these effects are not particularly strong, then males should have a weaker immune response than females as the intensity of sexual selection increases. However, if parasites are very detrimental to condition and/or reproductive effort shows strong condition-dependence, then males cannot afford to sacrifice immune function to enhance mating success even when sexual selection is strong and both sexes will invest equally in immune function. Finally, if the impact of parasites on condition is greater for males than females, then males should invest

more of their resources to immune function than females, despite stronger sexual selection on males.

Empirical studies largely agree with the variability of outcomes highlighted by theoretical models, showing little consensus in the direction of sex differences in immune function across taxa. In birds and mammals, males tend to suffer more from parasitic infections and have a reduced immune response relative to females (Poulin, 1996; Zuk & McKean, 1996; Moore & Wilson, 2002) but this pattern is far from clear in arthropods (Sheridan *et al.*, 2000). Male-biased parasitism is positively associated with the intensity of sexual selection on males, as measured indirectly by the mating system (monogamous or polygynous) and the degree of sexual size dimorphism, across mammal species (Moore & Wilson, 2002). Similarly, males have been shown to evolve a reduced immune function relative to females when the strength of sexual selection acting on them is experimentally increased (i.e. polyandry or a male-biased sex ratio) in some arthropod species (Hosken, 2001; Hangartner *et al.*, 2015) but not others (McKean & Nunney, 2007; Hangartner *et al.*, 2013; McNamara *et al.*, 2013). More empirical work is therefore needed to resolve this issue, especially studies comparing how the sexes allocate resources to reproduction and immune function within a species (Zuk, 2009).

A limitation of most studies using diet to manipulate resource acquisition in life-history studies is that typically only a few diets of poorly defined nutritional composition (e.g. “good” versus “bad” diets) are used and diet consumption is rarely measured (e.g. Holzer *et al.*, 2003; Hunt *et al.*, 2004). This approach makes it difficult (if not impossible) to statistically partition the effects of specific nutrients from calories or to examine any effect that the interaction between nutrients may have on the relationship between life-history traits. Furthermore, not measuring dietary intake ignores any effects of compensatory feeding. Compensatory feeding, the ability of an individual to increase consumption to compensate for reduced food quality (Simpson & Raubenheimer, 2012), appears widespread in animals (Behmer, 2009) and means that it is possible for individuals on poorer quality diets to consume as many calories or nutrients as on a high quality diet. Compensatory feeding therefore has the potential to undermine the use of diet to manipulate resource acquisition in life-history studies. These limitations can be resolved by using chemically defined (holidic) diets within the Geometric Framework (GF) for nutrition (Simpson & Raubenheimer, 2012). The GF is a multidimensional nutritional approach, within

which the effects of the intake of multiple nutrients (n) can be separated in n -dimensional nutritional space by restricting individuals to a geometric array of diets that differ in known nutrient composition and concentration (i.e. calories)(Simpson & Raubenheimer, 2012). When combined with precise measurements of diet consumption (allowing nutrient intake to be calculated), the GF provides a powerful way to partition the effects of the intake of specific nutrients and calories on the expression of different life-history traits. Furthermore, demonstrating that life-history traits have different nutritional optima can be taken as evidence for a nutrient based trade-off, as neither trait can be maximized on a single diet. Indeed, empirical studies using the GF have been used to show that the trade-offs between lifespan and reproduction (Lee *et al.*, 2008a; Maklakov *et al.*, 2008; Jensen *et al.*, 2015) and growth and immune function (Cotter *et al.*, 2011) are mediated by a balanced intake of nutrients. A robust framework, however, formally documenting the existence and strength of such nutritionally based trade-offs is currently lacking.

The decorated cricket (*Gryllodes sigillatus*) provides an excellent model to examine the nutritional basis of the trade-off between reproduction and immune function in the sexes. Unlike most insect species, male and female reproductive effort can be easily measured in *G. sigillatus*: female reproductive effort can be measured as the number of eggs produced in a given time period (Head *et al.*, 2005; Hunt *et al.*, 2006, Zajitschek *et al.*, 2007; Archer *et al.*, 2012a; 2012b), whereas male reproductive effort can be measured as the time spent calling each night to attract a mate (Hunt *et al.*, 2004; 2006; Judge *et al.*, 2008; Zajitschek *et al.*, 2007; Archer *et al.*, 2012a; 2012b). Producing a call is metabolically costly (Kavanagh, 1987) and the number of females attracted increases with amount of time spent calling in male *G. sigillatus* (Sakaluk, 1987), as well as in a range of other cricket species (e.g. Bentsen *et al.*, 2006, Jacot *et al.*, 2008; Rodriguez-Munoz *et al.*, 2010). Males also manufacture a large, gelatinous nuptial gift (the spermatophylax) that is transferred to the female at mating which can represent up to 2.2% of his body weight (Sakaluk, 1985). The female feeds on this gift during mating which prevents her from removing the sperm-containing ampulla, therefore enabling more sperm to be transferred to her reproductive tract (Sakaluk, 1984). Diet has sex-specific effects on age-dependent reproductive effort in *G. sigillatus* (Houslay *et al.*, 2015). Females consuming a poor quality diet produce fewer eggs and invest in egg production later in life compared to those consuming a high quality diet, whereas male calling effort increased with age irrespective of diet quality (Houslay *et*

al., 2015). Furthermore, the weight and amino acid composition of the spermatophylax and ampulla attachment time (Sakaluk, 1987; Gershman *et al.*, 2012) are all maximised at a high intake of protein and carbohydrate in a ratio of approximately 1:1.3 (Chapter 3). Immune function is sexually dimorphic in *G. sigillatus* with females having higher phenyloxidase activity and a greater encapsulation ability than males (Gershman *et al.*, 2010; Galicia *et al.*, 2014). The reverse pattern, however, appears true for lytic activity (Galicia *et al.*, 2014; but see Gershman *et al.*, 2010). Although the effect of diet on immune function has not been thoroughly investigated in *G. sigillatus*, diet quality does not appear to alter phenyloxidase or lytic activity in the sexes (Galicia *et al.*, 2014). Immune function in males shows a clear trade-off with the weight (Gershman *et al.*, 2010; Kerr *et al.* 2010) but not the amino acid composition (Duffield *et al.*, 2015) of the spermatophylax. For example, Gershman *et al.* (2010) found a negative correlation between spermatophylax weight and lytic activity and Kerr *et al.* (2010) showed that males injected with lipopolysaccharides produced lighter spermatophylaxes compared to control males. In contrast, males injected with heat-killed *E. coli* do not alter the amino acid composition that increases the gustatory appeal of the spermatophylax, whereas control males produce a less appealing combination of amino acids after a sham injection (Duffield *et al.*, 2015). We currently do not know, however, if immune function is traded against other forms of reproductive effort in males (such as calling effort), whether similar trade-offs exist in females or the role (if any) that diet plays in mediating the trade-off between immune function and reproduction.

In this study, we provide a conceptual framework to formally document the existence and quantify the strength of nutritionally based life-history trade-offs when using the GF for nutrition. We then test this conceptual framework by using the GF to determine the effect of protein (P) and carbohydrate (C) intake on the trade-off between reproduction and immune function in male and female *G. sigillatus*. We started by restricting male and female crickets to 24 different holidic diets differing in P:C ratio and total nutritional content. This created a nutritional landscape with six nutritional rails along which male and female crickets could vary their intake of P and C by eating more or less of a given diet (Table 1.1, Figure 1.2). We measured the intake of nutrients during sexual maturation and examined how this influenced reproductive effort (calling effort and egg production in males and females, respectively) and immune function (inactive and activated phenyloxidase activity and encapsulation ability) in the sexes and the extent to which these traits are

subject to a nutritionally based trade-off. In our second experiment, we examined how the sexes regulate their intake of P and C when given dietary choice. Males and females were given the choice between diets that differed in both the P:C ratio and total nutritional content in four diet pairings (Table 1.1, Figure 1.2). The total intake of P and C was again measured for crickets in each diet pairing during sexual maturation and the average intake of these nutrients across diet pairs was used to estimate the regulated intake point, defined as the point in nutrient that individuals actively defend when given choice (Simpson & Raubenheimer, 2012). We mapped the regulated intake point for each sex onto their nutritional landscapes for reproduction and immune function to determine if the sexes optimally regulate their intake of nutrients to maximise these traits.

4.3. MATERIALS AND METHODS

4.3.1. Conceptual framework for quantifying the nutritional basis of life-history trade-offs

The GF for nutrition is a state-space modelling approach that examines how an individual solves the problem of balancing the need for multiple nutrients in a complex, multidimensional nutritional environment (Simpson & Raubenheimer, 2012). To determine the intake of nutrients that is optimal, individuals are constrained to feed on a range of diets of precise nutritional composition. These diets are typically arranged in a geometric array (Figure 4.1A), being positioned along discrete nutritional rails where the ratio of nutrients is fixed (solid lines, Figure 4.1A) and on isocaloric lines across different nutritional rails where the nutrient ratio differs but the caloric content of the diets are the same (dashed lines, Figure 4.1A). Consequently, diets differ in both the ratio of nutrients and overall caloric content. Individuals are allowed to feed for a given period of time and the consumption of diets (and therefore nutrients) is precisely measured: as individuals are constrained to a single diet in this array, they can only feed along the length of a given nutritional rail (Figure 4.1B). A variety of different life-history traits can then be measured, mapped onto this nutrient intake data and response surface methodologies (Lande & Arnold, 1983; Box & Draper, 1987) used to determine the linear and nonlinear effects of nutrient intake on the life-history traits (see South *et al.* (2011) for a direct application of this approach to nutrient data). Life-history optima can be formally demonstrated by the presence of significant

negative quadratic terms in this analysis and non-parametric thin-plate splines (Green & Silverman, 1994) can be used to help visualize these optima by constructing nutritional landscapes (Figure 4.1C).

A separate set of individuals can also be provided with a choice of diets to determine how they actively regulate their intake of nutrients (Simpson & Raubenheimer, 2012). Alternate diets are typically provided in pairs differing in both the nutrient ratio and overall caloric content: in the example provided (red circles, Figure 4.1A), diets vary in the ratio of nutrient A to B (i.e. 1:8 or 8:1) and total caloric content (i.e. 40% or 80%) and diet pairs can be created by matching diets across nutritional rails and caloric contents (e.g. diets 1 versus 3, 1 versus 4, 2 versus 3 and 2 versus 4). The consumption of each diet in these pairs is precisely measured over a given time period and a paired *t*-test used to determine any dietary preference within each pair. Furthermore, multivariate analysis of variance (MANOVA) can be used to determine if there is significant variation in the intake of nutrients across diet pairs. The average intake of nutrients across diet pairs is used to estimate the regulated intake point which can then be mapped onto the nutritional landscape (white cross, Figure 4.1C) to determine whether individuals are regulating their intake of nutrients to maximise certain life-history traits. A close alignment of the regulated intake point with the nutritional optima for a given life-history trait is taken as evidence for optimal nutrient regulation (Simpson & Raubenheimer, 2012).

Although the above framework has provided many important insights into how animals balance their nutrient intake in a complex nutritional environment (Simpson & Raubenheimer, 2012), many conclusions are reached by a visual inspection of the nutritional landscape which can be subjective. For example, the location of the nutritional optima for a given trait and the extent to which the regulated intake point aligns with this optimum are typically derived by visualizing the nutritional landscape. As is clear from the hypothetical example in Figure 4.1C, this can be difficult to ascertain with any precision. This issue is even more striking when considering trade-offs between different life-history traits. A nutritional trade-off will exist when the optimal expression of two life-history traits occur in different regions of the nutritional landscape, meaning that both traits cannot be optimized at the same intake of nutrients. Consequently, characterizing nutritionally based trade-offs requires formally locating the nutritional optima for each life-history trait and quantifying

the extent of any differences that exist. There is, therefore, a clear need for a more robust conceptual framework to study nutritionally based life-history trade-offs.

I consider that three steps are essential to providing such a framework. First, the linear and nonlinear effects of nutrient intake on life-history traits need to be estimated in a standardized way. While response surface methodologies offer a simple way to estimate these effects, any resulting parameters will only be comparable across different life-history traits, the sexes or different species if nutrient intake and life-history traits are provided in the same scale. This is because nutrient intake often differs markedly across the sexes and species and life-history traits are frequently measured on different scales (e.g. days, growth rate and size). While many different approaches exist to standardize biological data (Houle *et al.*, 2011), I advocate standardizing nutrient intake and life-history traits to a z-score (z) by subtracting the population mean (μ) from each data point (x_i) and dividing by the standard deviation (σ) of the population ($z = (x_i - \mu) / \sigma$). Z-scores are therefore provided in units of standard deviations above (positive scores) and below (negative scores) the population mean and have the useful property of altering the scale but not the underlying distribution of the traits being standardized.

Second, when nutritional optima are formally detected on the nutritional landscapes for a given life-history trait, it is necessary to locate the position of the global maxima in nutrient space and also to estimate the 95% confidence region (CR) for this maxima. Locating the position of the global maxima is essential for determining the exact intake of nutrients that maximises the expression of a life-history trait. As both the intake of nutrients and life-history traits are measured with error, the 95% CR is needed to determine how this uncertainty influences the position of the global maxima. Furthermore, it allows a direct test of whether individuals optimally regulate their intake of nutrients under dietary choice: the regulated intake of nutrients can be considered optimal if the regulated intake point resides within the 95% CR for the global maxima. Second order (quadratic) parametric models are typically used to locate the global maxima on a response surface and estimating the 95% CR of this point (Peterson *et al.*, 2002; Box & Draper, 2007). This approach assumes, however, that the data is normally distributed and that the global maximum is best located by a second-order quadratic approximation (Peterson *et al.*, 2002). These assumptions are often not tenable for many nutritional and life-history data sets. These limitations can be overcome by using nonparametric bootstrap methods that are not reliant on a normal

distribution and incorporates a much more flexible regression spline model. I develop a new R package (“OptimaRegion”, del Castillo *et al.*, 2016) to perform this analysis with nutritional data (see below).

Finally, the nutritional landscapes for different life-history traits must be formally compared and the magnitude of any differences accurately quantified. A sequential model-building approach (Draper & John, 1988) offers a well-established protocol for comparing the linear, quadratic and correlational regression coefficients for different response surfaces (Draper & John, 1988) and this approach has already been used extensively to formally compare nutritional landscapes for different life-history traits within (Chapter 3; South *et al.*, 2011; Bunning *et al.*, 2015; House *et al.*, 2015) and between (Jensen *et al.*, 2015; Bunning *et al.*, 2016) the sexes. A limitation of this approach, however, is that only sign and magnitude of the regression coefficients are compared. Thus, it is possible that the two life-history traits being compared show different linear and/or nonlinear regression coefficients but the global maxima actually occupy the same region in nutrient space. The simplest way to formally demonstrate that the global maxima for two life-history traits occupy different regions in nutrient space is to visually compare the 95% CRs for the global maxima: if the 95% CRs are non-overlapping, this provides clear evidence that the global maxima are located in different regions. This approach, however, is subjective and does not quantify the exact magnitude of any difference. Using geometric principles, I propose two additional measures that will quantify any difference in the location of the global maxima in simple metric form. First, the divergence between the global maxima in nutrient space can be measured as the angle (θ) between the linear nutritional vectors as:

$$\theta = \cos^{-1} \left(\frac{a \cdot b}{\|a\| \|b\|} \right) \quad (1)$$

where a is the linear effects of nutrient intake on the first life-history trait being compared, b is the linear effects of these nutrients for the second life-history trait, $\|a\| = \sqrt{a \cdot a}$ and $\|b\| = \sqrt{b \cdot b}$. Larger values of θ indicate a greater separation of the optima in nutrient space and therefore a larger trade-off between life-history traits (Figure 4.2A & B), whereas as θ approaches 0° the optima become more aligned in nutrient space indicative of a weaker trade-off between life-history traits. This approach has already been applied to

nutritional data throughout this thesis (but see also, Bunning *et al.*, 2015; Bunning *et al.*, 2016). Second, the divergence between the global maxima in two-dimensional nutrient space can be measured as the Euclidean distance (\mathbf{d}) between the global maxima for each nutritional landscape as:

$$\mathbf{d} = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2} \quad (2)$$

where x_1, y_1 and x_2, y_2 are the two nutrient coordinates for the global maxima of the first and second life-history traits being compared, respectively. When the nutritional optima for the two life-history traits are located on similar iso-caloric lines, measures of θ and \mathbf{d} will be closely related: a larger θ will be associated with a longer \mathbf{d} (Figure 4.2A-C). However, when the nutritional optima are on different iso-caloric lines, θ may under-estimate the true degree of divergence between optima and \mathbf{d} will provide a better metric of this difference (Figure 4.2D). To provide measures of θ and \mathbf{d} that are comparable across studies, these parameters can be expressed as a percentage of their maximum value. As θ measures the divergence between linear nutritional vectors, the maximum separation is always 180° . In the case of \mathbf{d} , the maximum value will depend on the range of nutrient rails included in the geometric design of diets and how much individuals feed along these rails. Thus, the maximum \mathbf{d} will vary between experiments and I propose that the highest intake of nutrients on the two most extreme nutritional rails be used in Equation (2) to calculate the maximum \mathbf{d} .

In summary, nutritionally based trade-offs occur when the global maxima for two life-history traits occur in different regions in nutrient space meaning that both traits cannot be maximised at a single intake of nutrients. I provide a conceptual framework that provides an easy way to formally document the existence and quantify the strength of such nutritionally based trade-offs when using the GF. I show that two life-history traits will exhibit a large nutritionally based trade-off when the 95% CRs for the global maxima are not overlapping in nutrients space, there is a large angle (θ) between the linear nutritional vectors for the two traits and/or there is a large Euclidean distance (\mathbf{d}) between the global maxima for each trait. If applied, this framework enables the strength of nutritionally based

trade-offs to be estimated in a standardized way that facilitates direct comparison across different life-history traits and GF studies.

4.3.2. Experimental Animals

G. sigillatus used in my experiments were taken from a mass colony which are housed in twelve 15L plastic containers in an environmental chamber (Percival I-66VL) maintained at $32 \pm 1^\circ\text{C}$ on a 14h:10h light/dark cycle. The crickets in the mass culture are the descendants of approximately 500 adults collected in Las Cruces, New Mexico in 2001, and used to initiate a laboratory culture maintained at a population size of approximately 5000 crickets and allowed to breed panmictically (Ivy & Sakaluk, 2005). Crickets were provided with commercial cat pellets (Purina Go-Cat Senior[®]) and water in 60ml glass test tubes plugged with cotton wool *ad libitum*, as well as an abundance of egg cartons to provide shelter. As soon as adults were detected, moistened cotton wool was provided in a petri-dish (10cm diameter) as an oviposition substrate. Each generation, crickets were randomly mixed between containers and were maintained at a density of approximately 300 crickets per container.

Experimental animals were collected as newly hatched nymphs from the oviposition pads, housed individually in a plastic container (5cm x 5cm x 5cm) and provided with a piece of cardboard egg carton for shelter and water in a 2.5ml test tube plugged with cotton wool. Crickets were fed dry cat pellets and their enclosure cleaned once a week. Experimental animals were checked daily for newly eclosed adults and these were then randomly allocated to an experiment and diet treatment (see below).

4.3.3. Artificial Diets and Measuring Dietary Intake

24 artificial, dry and granular foods that varied in protein and carbohydrate content were made following the procedure outlined in Simpson & Abisgold (1985). Proteins consisted of a 3:1:1 mixture of casein, albumen and peptone with digestible carbohydrates consisting of a 1:1 mixture of sucrose and dextrin. All diets contained Wesson's salts (2.5%), ascorbic acid (0.28%), cholesterol (0.55%) and vitamin mix (0.18%). After the appropriate dry weight of protein and carbohydrate had been added to the mixture, the remainder of the mixture was made up to the appropriate dilution with crystalline cellulose. The diets

used in each experiment are presented in Table 1.1 and Figure 1.2 shows their distribution of the diet treatments in nutritional space.

Each experimental cricket was given either one (Experiment 1) or two (Experiment 2) containers of food of measured dry weight on their first day of adulthood and food was changed every 2 days for a total of 16 days. Food and water were provided in feeding platforms created by gluing the upturned plastic lid of a vial (1.6cm diameter, 1.6 cm deep) in the centre of a plastic petri dish (5.5cm diameter). The materials and design of the feeding platforms ensured that experimental animals could not consume anything other than the artificial diet(s) and water and that food could be collected (in the petri dish) if spilled during feeding. Food was kept in a drying oven at 30°C for 72 hours to remove moisture prior to weighing. Prior to weighing, any dried faeces were removed from the feeding platform using a pair of fine forceps.

Diet consumption was calculated as the difference in dry weight before and after feeding and converted to a weight of P and C ingested by multiplying by the proportion of that nutrient in the diet (e.g. 5mg of 15P:45C ingested equals 0.75mg of protein and 2.25mg of carbohydrates). The total nutrient content of each diet is the percentage P plus the percentage C, with the remainder of the diet being indigestible crystalline cellulose and micronutrients (e.g. the 15P:45C diet has a total nutrient content of 60%).

4.3.4. Experiment 1: No Choice of Diet on Six Nutritional Rails

Experimental Design

On the day of eclosion to adulthood (day 0), a total of 15 males and females were randomly allocated to one of the twenty four artificial diets for 16 days ($n= 720$). Each experimental individual was weighed at eclosion using an Ohaus electronic balance (Explorer[®] Pro model EP214C) and their pronotum width measured under a dissection microscope (Leica MZ6) fitted with an eyepiece graticule. There was no significant difference in the body size (male: $F_{23,336} = 1.02$, $P = 0.44$; female: $F_{23,336} = 0.54$, $P = 0.96$) or weight (male: $F_{23,336} = 0.89$, $P = 0.61$; female: $F_{23,336} = 1.02$, $P = 0.44$) of the sexes across diets, thereby confirming random allocation. On the night of day 7 post-eclosion, each experimental cricket had their diet(s) removed and were placed with a virgin cricket taken at random from the stock population and allowed to mate overnight. On day 8, the mating

partners were removed and the experimental individuals provided with their specific diets as normal. I measured reproductive effort in the sexes on day 8 and 9 post-eclosion and immune function between days 14 to 16 post-eclosion (see below).

Measuring Reproductive Effort

I measured female reproductive effort as the number of eggs produced by a female on days 8 and 9. Each female was provided with a small petri dish (5.5cm diameter) full of moist sand for oviposition. Females had access to this oviposition substrate continuously for this two day period, after which it was removed and replaced with a fresh petri dish every night until the end of the experiment (day 16). This was done to ensure that both sexes had the same potential for reproductive effort between days 8 and 9 and the end of the experiment (see below). To count eggs, the content of each petri dish was emptied into a round container of water (10cm diameter, 12cm height), swirled in a circular fashion for 20 seconds and the eggs removed with fine forceps as they moved to the surface of the sand and counted.

Male reproductive effort was measured as the amount of time a male spent calling to attract a mate each night, as this has been shown to provide a good measure of mating success in both the laboratory and the field (Bentsen *et al.*, 2006; Rodriguez-Munoz *et al.*, 2010). I measured the amount of time each male spent calling on the nights of days 8 and 9 using a custom-built electronic monitoring device (see Hunt *et al.*, 2004) for a full description of this device). Each male was placed in an individual recording chamber (5cm x 5cm x 5cm), with a condenser microphone (c1163, Dick Smith[®]) embedded in the lid, on each night of sampling. Each recording chamber was placed inside a larger foam container (15cm x 15cm x 15cm) to ensure each male was acoustically isolated. Each microphone was connected via acoustic leads to a data acquisition unit (DaqBook 120, IO-Tect, Cleveland) and computer (Dell™ OptiPlex™580). The data acquisition unit activates a single microphone at a time, which then relays the sound level to the PC board, where it is compared to background noise. If the received signal is ≥ 10 dB louder than background noise, this is recorded as a call. The microphone is then deactivated and the next one in the series is activated, with each recording chamber being sampled and recorded 10 times per second. The number of seconds a male called each night between 18:00 to 09:00. During this sampling period, males were not given access to diet. Therefore, to ensure that access to

diet was consistent across the sexes, the female's diet was covered during this period to prevent feeding.

Measuring Immune Function

Invertebrates (especially insects) have become an important model in the study of immune function because their immune system is much simpler than vertebrates in that it lacks an acquired immune response (Rolff & Reynolds, 2009; Schmid-Hempel, 2011). Despite lacking an acquired immune response, insects are still capable of protecting themselves against a diverse array of parasites and pathogens by having a well-developed innate immune system that allows general and rapid responses to invading microbes (i.e. bacteria, fungi, viruses)(Rolff & Reynolds, 2009; Schmid Hempel & Schmid-Hempel, 2011). The insect immune response consists of two tightly interconnected components: the cellular and humoral responses (Tsakas & Marmaras, 2010). Cellular responses are performed by hemocytes and include processes such as phagocytosis, modulation and encapsulation (Tsakas & Marmaras, 2010) that typically target larger microbial invaders. The humoral response includes the production of antimicrobial peptides (AMPs), as well as the activation of enzymatic cascades that regulate coagulation and melanisation of the hemolymph (Tsakas & Marmaras, 2010). One enzyme cascade that is known to play a particularly important role in the insect immune system is the pro-phenoloxidase (PO) cascade (González-Santoyo & Córdoba-Aguilar, 2012). PO is a copper containing enzyme that catalyzes the oxygenation of mono-phenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones (González-Santoyo & Córdoba-Aguilar, 2012). These are the key steps in the synthesis of melanin; a pigment found in the insect cuticle (e.g. Ashida & Brey, 1995) and is also used to encapsulate foreign bodies (Gotz, 1986). Both endogenous and exogenous signals are known to trigger the activation of PO in insects (e.g. Bidla *et al.*, 2008) and this activation occurs rapidly (i.e. minutes to hours) (e.g. Korner & Schmid-Hempel, 2004) and provides protection to a range of pathogens, including nucleopolyhedroviruses (NPVs), fungi, nematodes and parasitoids (e.g. Ourth & Renis, 1993; Hagen *et al.*, 1994; Washburn *et al.*, 1996; Bidochka & Hajek, 1998; Reeson *et al.*, 1998).

In this study, I focus the examination of the immune function on the PO cascade in *G. sigillatus*. I start by measuring the amounts of PO circulating in the haemolymph before and after the insert of a foreign body: I refer to these as inactive and activated PO, respectively. I

finish by measuring the ability of crickets to encapsulate this foreign body via it being engulfed by hemocytes and melanization. Thus, I examine PO from the start to end of the cascade (Tsakas & Marmaras, 2010). It is important to note, however, that my estimates of PO activity are “potential” responses because they measure in vitro the amount of the enzyme present, providing information about the potential of an individual to mount an immune response (Gershman, 2008). In contrast, my estimate of encapsulation ability measures the response of an individual to a novel immune challenge and thus represents a “realized” immune response (Gershman, 2008). The immune function of male and female crickets was measured between days 14 and 16 post-eclosion following the protocol outlined below.

Encapsulation ability

At 14 days of age, crickets were cold-anesthetized at 6°C in a refrigerator for 10 minutes. During this period, a 2mm long segment of nylon monofilament fishing line with a diameter of 0.3mm with the surface abraded with sandpaper (referred to as an implant), and a hypodermic needle from a 30.5 gauge syringe were sterilized in 70% ethanol. A small hole was made ventrally between the fifth and sixth abdominal segments of the cricket using the needle and the implant was inserted into the wound with dissection forceps until it was completely contained within the abdominal cavity of the cricket. After implantation, crickets were returned to their individual containers and provided with fresh diet and water. Previous work using this approach has shown that the greatest variation in melanisation occurs 2 days after implantation (Gershman *et al.*, 2010). Therefore, 2 days after each cricket was implanted, it was placed in a 3ml microcentrifuge tube and frozen at -20°C.

Implants were dissected from frozen crickets and any clumps of tissue removed with paper towel. Each implant was photographed using PixeLink Capture SE software (Version 2.2) three times from three different sides next to a clean implant control using a camera (PixeLink Megapixel Firewire Camera) mounted on a dissecting microscope (Leica MZ6). Each implant and control was outlined using the ImageJ (<http://rsbweb.nih.gov/ij/>) polygon tool. The darkness of each implant and control was measured as the average grayscale value of all the pixels within each image using ImageJ. The darkness score for each individual was calculated as the average grayscale of the three implants’ darkness scores subtracted from the average grayscale of the three control implants’ darkness scores. Therefore, darker

implants yielded higher darkness scores. A pilot study where two implants were inserted into opposite sides for each of 20 crickets and levels of melanisation assessed after 2 days following the above protocol showed that the repeatability (R) of this method was high ($R = 0.93, 95\% \text{ CI: } 0.89, 0.97$) (Wolak *et al.*, 2012).

Pre- and activated phenoloxidase activity

During implantation, a $3\mu\text{l}$ sample of haemolymph was collected with a glass pipette from the wound and mixed with $40\mu\text{l}$ of 1 X Phosphate Buffered Saline solution (PBS) in a 1.5ml microcentrifuge tube. This was used to measure pre-PO activity. After 2 days just before each cricket was frozen, a further $3\mu\text{l}$ of haemolymph was extracted and mixed with $40\mu\text{l}$ of 1 X PBS in a 1.5ml microcentrifuge tube. This was used to measure activated PO activity. Haemolymph samples were frozen and stored in a -80°C freezer to induce cell lysis and to prevent enzymatic reactions from proceeding.

To measure the PO activity of my haemolymph samples, 58mg of 3, 4-Dihydroxyl-L-phenylalanine (L-Dopa) was dissolved in 20 ml of 1 X PBS in a 50 ml conical flask, which was capped with parafilm and immediately covered in aluminium foil due to this solution being photosensitive. The haemolymph samples were defrosted, vortexed and spun down while the L-Dopa was mixing. A total of $5\mu\text{l}$ of each haemolymph sample was pipetted into a separate well of a 96 well plate, with the last row of the plate filled with $5\mu\text{l}$ of PBS for control. Each sample was mixed three times with the pipette before pipetting and the plate was filled rapidly to minimize evaporation and avoid air bubbles. Using a multipipetter, $90\mu\text{l}$ of L-Dopa solution was added to each well of the 96 well-plate, with each sample mixed twice with the pipette. The optical density (OD) was then recorded at 490nm using a spectrophotometer (SpectraMax M2) and the programme SoftMax Pro 5.4 (MDS Analytical Technologies). This method estimates the total change in OD over the course of the reaction, with OD readings taken every 10 minutes over a 210 minute period. The average slope of the change in OD over time for the control row was calculated and subtracted from the change in OD slope of a given haemolymph sample to extract the corrected OD slope, with a larger slope indicating more PO activity. Each haemolymph sample was tested twice to calculate an average OD slope for analysis and all samples were randomized within and across plates before measuring average OD. A pilot study where I measured pre- and activated PO activity from two independent haemolymph samples for each of 20 cricket

using the above protocol showed that the repeatability of this method was high (Pre-PO: $R = 0.96$, 95% CI: 0.93, 0.99, Activated-PO: $R = 0.93$, 95% CI: 0.86, 0.97).

4.3.5. Experiment 2: Measuring Nutrient Intake Under Dietary Choice

A total of 20 crickets of each sex were assigned at random to one of four diet pairs (total $n = 160$) on their day of eclosion to adulthood. The diets used in these pairs varied in both the P:C ratio and total nutrition (P:C ratio (total nutrition %)): Pair 1: Diet 2 (5:1 (36%)) versus Diet 22 (1:8 (36%)), Pair 2: Diet 2 (5:1 (36%)) versus Diet 24 (1:8 (84%)), Pair 3: Diet 4 (5:1 (84%)) versus Diet 22 (1:8 (36%)) and Pair 4: Diet 4 (5:1 (84%)) versus Diet 24 (1:8 (84%)) (Table 1.1)). This choice of diet pairs provides a wide coverage in nutrient space (Figure 1.2). Using the protocol outlined above for Experiment 1, the consumption of both diets was measured every two days for a total of 16 days post-eclosion, with a random mate being assigned to each experimental cricket overnight on day 7.

4.3.6. Statistical Analyses

Characterizing the linear and nonlinear effects of nutrient intake

I used a multivariate response-surface approach to estimate the linear and nonlinear effects of P and C intake on my response variables (i.e. reproductive effort, encapsulation ability, pro-PO activity and activated PO activity)(South *et al.*, 2011). Prior to analysis, I standardized my nutrient intake and life-history data to a z-score (i.e. a mean of zero and standard deviation of one) to ensure that any differences in nutritional gradients were not driven exclusively by scale (South *et al.*, 2011). Non-parametric thin-plate splines (TPS) were used to visualize the nutritional landscapes for each life-history trait that was significantly influenced by nutrient intake. Thin-plate splines were constructed using the *Tps* function in the 'FIELDS' package of R (R Core Team, version 2.15.1, Vienna, Austria. www.r-project.org). To aid interpretation, all TPS were constructed using unstandardized data. For each nutritional landscape, I used the lambda value that minimized the generalized cross-validation (GCV) score (Green & Silverman, 1994).

Estimating the location of the nutritional optima and its 95% CR

To estimate the location of the nutritional optima and its 95% CR, I develop a novel nonparametric bootstrapping method that does not make any distributional assumptions and uses a more flexible regression spline model. This approach is provided in the R package “OptimaRegion” (del Castillo *et al.*, 2016). In brief, this package fits a two-dimensional thin-plate spline model to the experimental data using a penalized roughness approach and the *Tps* function in the ‘FIELDS’ package of R, where the user is able to define the smoothness parameter λ (for example, obtained by cross-validation and the *Tps* function on the experimental data). This function yields the vector of predictions \hat{y} at all the experimental points using the Kimeldorf-Wahba predictor $\hat{y} = T \hat{d} + \Sigma \hat{c}$ where $\hat{\psi} = (\hat{d}, \hat{c})$ are fitted parameters, T is a matrix of polynomial basis functions (up to cubic degree) and Σ is a matrix of radial basis functions computed at all pairs of points x_i (see (Nychka, 2000) for further details). I compute residuals r_i adjusted for small sample bias (Kauermann *et al.*, 2012). I then applied bootstrapping to these residuals to create bootstrapped realizations $y^*(x) = \hat{y}(x) + r^*$ for each experimental data point (x) in my data set. For each simulated set of $y^*(x)$, I fit a TPS(λ) model and found parameter estimates ψ^* . Following Yeh and Sing (1997), I repeated this procedure 1,000 times and computed Tukey’s data depth for each generated ψ^* vector, keeping the $100(1 - \alpha)$ % deepest (where in this case $\alpha = 0.95$). This provides an approximate nonparametric bootstrap confidence region for the Tps coefficients ψ . The responses $y^*(x)$ that correspond to the parameter vectors ψ^* inside of their CR were then maximized numerically using the “nloptr” package in R (Johnson, 2014; Ypma, 2014) with respect to the regressors (x_1, x_2) yielding the bootstrapped response global maxima (x^*) . The nonparametric bootstrapped CR for the location of the global maximum of the fitness function is approximated and displayed as the convex hull of all the bootstrapped peaks (x^*) . I use the centroid (average) of all the maxima located as the point estimate of the global maxima (or nutritional optima) of the nutritional landscape. This constitutes a bagging estimate of the location of the maxima (Hastie *et al.*, 2001).

This procedure for computing the resulting CR is justified by recent work by Woutersen and Ham (2013) who have recently shown that better coverage of bootstrapped CRs of parametric functions (in this case, the vector function $g(x; \psi) = \arg \max_{x \in C} y(x)$ where C is the experimental region) can be obtained if the bootstrapped values of the function $g(x; \psi)$ are generated from parameters ψ inside their $100(1 - \alpha)$ %

CR, rather than directly generating an empirical distribution of $g(x; \psi)$ and trimming it to get the desired CR. The rationale behind the Wourtesen and Ham (2013) method is that it is better to sample only from “good” parameters ψ (those inside their CR) and using these for generating the values of $g(x; \psi)$ rather than using both “good” and “bad” generated values of ψ .

Comparing the sign and magnitude of the linear and nonlinear nutritional gradients

I used a sequential model-building approach (Draper & John, 1988) to determine whether the linear and nonlinear (quadratic and correlational) effects of nutrient intake differed across my response variables. Full details of this analysis, as applied to nutritional data, are provided in South *et al.* (2011)(but see also Appendix 2). In brief, I started the sequential model building approach by first fitting a linear model to the data, including a dummy variable (response type or sex) as a fixed effect, P and C intake as covariates and my response measures as the dependent variable. From this reduced model I extracted the residual sums of squares (SS_r). I then ran a second linear model that included all the interactions between the dummy variable and the covariates and again extracted the residual sums of squares for this complete model (SS_c). A partial F -test was then used to statistically compare SS_r and SS_c , where the number of terms added to the complete model and the error degrees of freedom from the complete model are used as the numerator and denominator degrees of freedom, respectively. A significant reduction in SS_c compared to SS_r indicates that the complete model significantly increases the amount of variance explained and, therefore, demonstrates that the nutritional gradients differ significantly across the dummy variable used. This model was repeated by sequentially adding the quadratic terms for nutrient intake (P x P and C x C) and then the correlational term (P x C). In instances where an overall significant difference was detected in the complete model, univariate analyses were used to determine which nutrient (P, C or both) contributed to this effect.

Calculating the angle and 95% CIs between linear nutritional vectors

I used a Bayesian approach implemented in the “MCMCglmm” package of R (Hadfield, 2010) to determine the magnitude of θ and the degree of certainty associated with this estimate, measured as the 95% CIs. For each life-history trait being compared, I ran

a separate linear model ($R \sim \beta_1 P + \beta_2 C + \varepsilon$) using 400,000 Markov chain iterations with a burn-in of 20,000, a thinning interval of 25 and a relatively uninformative prior ($\nu = 1$, $nu = 0.02$), to create a posterior distribution of β for each nutrient. I used these distributions in Equation 1 to generate 15,200 values for θ . The median of these values was used as my point estimate of θ and the 95% CIs were estimated using the “HPDinterval” function. The associated R code for this procedure is presented in Appendix 3.

Calculating the Euclidean distance and 95% confidence intervals between global maxima

To determine the mean and median Euclidean distance between the locations of the maxima of two response surfaces, I developed a custom program “CRcompare.R” in the R package “OptimaRegion” (del Castillo *et al.*, 2016). The program starts by calling the function “OptRegionTps.R” from the “OptimaRegion package” (del Castillo *et al.*, 2016) twice, once for each of the two life-history traits being compared, to compute a CR on the maxima of each response surface. These CRs are a set of points, obtained by repeatedly bootstrapping the residuals of the original TPS model, creating sample response data to which new TPS models are fitted and then optimized to obtain the location of their maxima. This program then computes all possible pairwise Euclidean distances between the response maxima in each CR. Finally, it bootstraps the mean and median of these distances using R’s package boot (Canty & Ripley, 2016) to obtain 95% bias-corrected and accelerated (BCA, see (Efron *et al.*, 1998) bootstrapped confidence intervals on the mean and median distance.

Testing for dietary choice and the non-random intake of nutrients

To determine whether male and female crickets preferentially consumed one of the diets over the other in each diet pair contained in Experiment 2, I compared the absolute consumption of each diet using a paired *t*-test. However, this approach does not account for the fact that my choice diets have different concentrations of P and C meaning that crickets may actually eat more of a less concentrated diet (i.e. compensatory feeding) to increase their intake of P and/or C. I therefore investigated the non-random intake of nutrients in the sexes in two ways. First, I calculated the total intake of P and C for each diet pair and subtracted the expected intake of these nutrients if crickets fed at random. This difference was compared to a mean of zero (i.e. expected if crickets were feeding at random on diets) using a one-sample *t*-test. A value greater than zero therefore means that a cricket has

consumed significantly more P or C than expected, a value less than zero means that a cricket has consumed significantly less than expected, whereas a value that does not differ significantly from zero means that crickets have consumed nutrients equally from both diets. Second, I used a multivariate analysis of variance (MANOVA) to compare the total intake of P and C across the sexes and diet pairs; sex, diet pair and their interaction were included as fixed effects and P and C intake as dependent variables. Univariate ANOVAs were used to determine which nutrients contribute to the overall multivariate effect. As there are four diet pairs, Tukey's HSD contrasts were used to determine how the intake of P and C differed across diet pairs for each sex.

Estimating and comparing the regulated intake point across the sexes

I estimated the regulated intake point in each sex, defined as the point in nutrient space that individuals actively defend when given dietary choice, as the mean intake of P and C across diet pairs (Simpson & Raubenheimer, 2012). I used an analysis of covariance (ANCOVA) including sex as a fixed effect, P intake as a covariate, the interaction between sex and P intake as a fixed effect and C intake as the dependent variable to determine if the regulated intake point differed significantly across the sexes. Significance of the sex by P intake interaction term demonstrates that the sexes have different regulated intake points.

Determining if nutrient regulation is optimal for trait expression in the sexes

To determine whether males and females optimally regulate their intake of nutrients to maximise trait expression, I mapped the regulated intake point for each sex onto the nutritional landscape containing the 95% confidence region of the peak (global maxima) for each trait (see Figure 4.3). I consider nutrient regulation to be optimal for a given trait if the regulated intake point overlaps the 95% confidence region of the peak (global maxima) on the nutritional landscape.

4.4. RESULTS

Experiment 1: No Choice of Diet on Six Nutritional Rails

There was a clear significant linear effect of the intake of both nutrients on male reproductive effort, with the amount of calling increasing with the intake of C but decreasing with the intake of P (Table 4.1, Figure 4.3A). There was also a significant negative quadratic effect of C intake on calling effort and inspection of the nutritional landscape (Figure 4.3A) reveals a peak in calling effort at a high intake of C and low intake of P centred around a P:C ratio of 1: 7.08 (global maxima: P = 17.87 mg, C= 126.57 mg, Figure 4.3A). A significant negative correlational gradient for calling effort (Table 4.1), provides further evidence that calling effort increases with the intake of C and decreases with the intake of P. The encapsulation ability of males increased with the intake of P but was not influenced by the intake of C (Table 4.1, Figure 4.3B). There was also a significant negative quadratic effect of P intake on encapsulation ability (Table 4.1) and inspection of the nutritional landscape (Figure 4.3B) reveals a peak in encapsulation ability at a high intake of P and low intake of C centred around a P:C ratio of 5.14:1 (global maxima: P = 104.24 mg, C= 20.29 mg, Figure 4.3B). The significant negative correlational gradient further demonstrates that encapsulation ability is maximized on a high intake of high P and low C diets (Table 4.1). In contrast to calling effort and encapsulation ability, the intake of P and C did not significantly influence inactive and activated PO activity (Table 4.1).

Female reproductive effort increased linearly with the intake of P and C, with egg production being equally responsive to the intake of both nutrients (Table 4.1, Figure 4.3C). There were also significant negative quadratic effects of P and C intake on egg production (Table 4.1) and inspection of the nutritional landscape (Figure 4.3C) shows a peak in egg production at a high intake of P and C centred around a P:C ratio of 1:1.17 (global maxima: P = 126.09 mg, C= 148.09 mg, Figure 4.3C). The significant positive correlational gradient provides further evidence that egg production is maximised on a high intake of high P, high C diets (Table 4.1). Female encapsulation ability also increased linearly with the intake of both nutrients, although this trait was slightly more responsive to the intake of P than C (Table 4.1). There were also significant negative quadratic effects of P and C intake on encapsulation ability in females (Table 4.1) and inspection of the nutritional landscape

(Figure 4.3D) shows a peak in encapsulation activity at a high intake of P and C centred around a P:C ratio of 1.04:1 (global maxima: P = 129.66 mg, C= 124.70 mg, Figure 4.3D). There was, however, no significant correlational effect on nutrients on female encapsulation ability (Table 4.1). As shown for males, the intake of P and C did not significantly influence inactive and activated PO activity (Table 4.1).

Formal statistical comparison using a sequential model building approach showed significant differences in the linear, quadratic and correlational effect of nutrient intake on male calling effort and encapsulation ability (Table 4.2). The difference in linear effects was due to the fact that encapsulation ability increased with P intake but calling effort decreased with the intake of this nutrient and also because calling effort increased with C intake but encapsulation ability did not (Table 4.2). The difference in quadratic effects was driven by the fact that there was a peak in encapsulation ability but not calling effort with P intake and there was a peak in calling effort but not encapsulation ability with C intake (Table 4.2). Finally, the difference in the correlational effect exists because the effect of the negative covariance between P and C intake is stronger on encapsulation ability than calling effort (Table 4.2). This pattern of nutritional effects on calling effort and encapsulation ability leads to nutritional optima that are located in different regions in nutrient space (Figure 4.3A & B), as evidence by the large angle between the linear nutritional vectors ($\theta = 107.20^\circ$, 95% CI: 93.26° , 120.53°) and the large Euclidean distance between the global maxima for calling effort and encapsulation ability ($d = 145.05$ mg, 95% CI: 143.60 mg, 146.30 mg) which represents 78.41% and 59.56% of the maximum differences, respectively (maximum $\theta = 180^\circ$, maximum $d = 185.00$). Furthermore, the comparison of Figures 4.4A & B shows that there is no visible overlap in the 95% confidence regions for optimal calling effort and encapsulation ability. Collectively, this provides clear evidence of a trade-off between calling effort and encapsulation ability in males, a view that is confirmed by a significant negative correlation between these traits across diets in my geometric design ($r = -0.104$, 95% CI = -0.154, -0.055, $n = 360$, $P = 0.048$).

In contrast to males, a sequential model building approach showed a significant difference in the linear effects of nutrient intake on female egg production and encapsulation ability but no difference in the quadratic or correlational effects (Table 4.2). The difference in linear effects was due to the fact that egg production was more responsive to the intake of both nutrients than encapsulation ability (Table 4.2). This pattern of

nutritional effects on egg production and encapsulation ability in females leads to nutritional optima that are located in similar regions in nutrient space (Figure 4.3C & D). Consequently, there was a small angle between the linear nutritional vectors ($\theta = 16.71^\circ$, 95% CI: 0.00° , 34.64°) and a short Euclidean distance between the global maxima for calling effort and encapsulation ability ($d = 33.83$ mg, 95% CI: 29.90 mg, 37.29 mg) which represents 9.28% and 13.64% of the maximum differences, respectively (maximum $\theta = 180^\circ$, maximum $d = 248.02$). Furthermore, the 95% confidence regions for egg production and encapsulation ability are completely overlapping (Figure 4.4C & D). Together, these results suggest that egg production and encapsulation ability in females do not trade-off and this view is supported by the positive correlation between these traits across diets ($r = 0.260$, 95% CI = 0.156, 0.361, $n = 360$, $P = 0.0001$).

Formal statistical comparison using a sequential model building approach also showed significant differences in the linear, quadratic and correlational effects of nutrient intake on reproductive effort and encapsulation ability across the sexes (Table 4.2). For reproductive effort, the sex difference in the linear effects of nutrient intake is driven exclusively by P intake, having a negative effect on calling effort but a positive effect on egg production (Table 4.2). The sex difference in the quadratic effect of nutrients is due to the fact that there is a peak in egg production but not calling effort with P intake and also because the peak in egg production with C intake has a more pronounced curvature than for calling effort (Table 4.2). The sex difference in the correlational effect of nutrients is due to the fact that egg production increased but calling effort decreased with the covariance between P and C intake (Table 4.2). For encapsulation ability, the sex difference in the linear effects of nutrient intake is due to the fact that C intake increases this trait in females but not males and because encapsulation ability is more responsive to the intake of C in males than females (Table 4.2). The sex difference in the quadratic effects of nutrient intake is due to the fact that there is a peak in encapsulation ability with C intake in females but not males and also because the curvature of the peak in encapsulation ability with P intake is more pronounced in females than males (Table 4.2). The sex difference in the correlational effect of nutrient intake is due to the fact that encapsulation ability decreased with the covariance between P and C intake in males but not in females (Table 4.2). This divergence in the effects of nutrient intake on reproductive effort and encapsulation ability across the sexes leads to nutritional optima that are located in different regions in nutrient space

(Figure 4.1). This results in a large angle between the linear nutritional vectors for reproductive effort ($\theta = 55.58^\circ$, 95% CI: 41.59°, 69.98) and encapsulation ability ($\theta = 35.00^\circ$, 95% CI: 14.81°, 56.53°) in the sexes, as well as large Euclidean distances between the global maxima for reproductive effort ($d = 119.17$ mg, 95% CI: 118.60, 119.6) and encapsulation ability ($d = 117.72$ mg, 95% CI: 115.50, 119.60) in the sexes (Figure 4.4).

Experiment 2: Measuring Nutrient Intake under Dietary Choice

MANOVA revealed that sex, diet pair and the interaction between these fixed effects all significantly influenced the intake of nutrients when *G. sigillatus* is given dietary choice (Table 4.3). On each diet pair, females consumed significantly more diet than males (Figure 4.5A & B) resulting in a higher intake of both P and C (Figure 4.6, Table 4.3). Both sexes consumed significantly more of the high C diet than the high P in each diet pair (Figure 4.5A & B) and this resulted in a significantly higher intake of C and a significantly lower intake of P than expected if individuals fed indiscriminately on diets (Figure 4.5C & D). Despite this clear dietary preference, the significant difference across diet pairs suggests that the intake of nutrients was not tightly regulated in the sexes (Table 4.3, Figure 4.6). Indeed, Tukey's HSD pairwise contrasts showed that the order of diet pairs was 1<2<4<3 for P intake and 3<1<4=2 for C intake in males and 1=2<4<3 for P intake and 3<1<4<2 for C intake in females (Figure 4.6). The significant interaction term between sex and diet pair in my MANOVA model reflects this different ordering of diet pairs across the sexes (Table 4.3, Figure 4.6).

The regulated intake point was estimated at an intake of 29.51 ± 1.30 mg of P and 59.12 ± 1.96 mg of C in males and 55.06 ± 2.35 mg of P and 101.12 ± 3.17 mg of C in females, which corresponds to a P:C ratio of 1:2.00 and 1:1.84 for males and females, respectively (Figure 4.6). ANCOVA showed that the intake of C differed significantly across the sexes ($F_{1,156} = 29.96$, $P = 0.0001$) but was not related to P intake ($F_{1,156} = 1.85$, $P = 0.18$), nor was there a significant interaction between sex and P intake ($F_{1,156} = 0.78$, $P = 0.38$). The fact that this interaction term is not significant formally demonstrates that the regulated intake point does not differ across the sexes (Figure 4.6). In Figure 4.4, I map the regulated intake points for the sexes onto the nutritional landscapes containing the 95% confidence region of the peak (global maxima) to determine if males and females are regulating their intake of nutrients to optimize reproductive effort and/or encapsulation ability. In general,

the P:C ratio of the regulated intake point was more closely aligned with the P:C ratio of the global maxima for reproductive effort and encapsulation ability in females than in males (Figure 4.4). However, with the exception of female encapsulation ability (Figure 4.3D), the regulated intake point for all other traits in both sexes did not overlap the 95% confidence region of the global maxima on the nutrient landscape indicating suboptimal nutrient regulation (Figure 4.4A,B & C). The optimal regulation of nutrients by females to maximize encapsulation ability should be interpreted with a degree of caution, however, due to the large 95% confidence region associated with the peak expression of this trait (Figure 4.4D).

4.5. DISCUSSION

A core assumption of life history theory is that resources are finite, causing traits competing for this common pool of resources to experience a trade-off (Stearns, 1992; Roff, 2002; Roff & Fairbairn, 2007; 2012). Diet is one of the most widely examined “shared resources” and an extensive literature exists showing that the strength of trade-offs (or in fact whether trade-offs exist) can be altered dramatically through dietary manipulation (e.g. Hill, 1999; Brown & Shine, 2002; Lardner & Loman, 2003; Hunt *et al.*, 2004; Kolluru & Grether, 2005; Karell *et al.*, 2007). The vast majority of empirical studies manipulating diet, however, use diets that are poorly defined with regards to nutrient content and do not precisely measure dietary consumption. This makes it impossible to determine the effect that specific nutrients may have on regulating any nutritionally based trade-offs or whether changes in feeding behaviour, such as compensatory feeding, alter this relationship. In this regard, the development and application of the GF has been instrumental in demonstrating the important role that the intake of specific nutrients has on the expression of key life-history traits (e.g. Chapters 2 & 3; South *et al.*, 2011; Fanson *et al.*, 2013; Bunning *et al.*, 2015; House *et al.*, 2015; Bunning *et al.*, 2016). However, a robust framework for quantifying the existence and strength of nutritionally based trade-offs between life-history traits is currently lacking, with the existing approach based on visually comparing the nutritional landscapes for two traits (e.g. Lee *et al.*, 2008a; Maklakov *et al.*, 2008; Harrison *et al.*, 2014; Jensen *et al.*, 2015). To help bridge this gap, I develop a new conceptual framework to formally demonstrate the existence and quantify the strength of nutritionally based trade-offs when using the GF. I argue that nutritionally based trade-offs will occur

whenever life-history traits are maximised in different regions of nutrient space, as demonstrated by non-overlapping 95% CRs of the global maxima for the two traits. Moreover, I show that the magnitude of this trade-off can be quantified by the angle (θ) between the linear nutritional vectors and the Euclidean distance (d) between the global maxima for each trait. I then empirically test my framework by examining the effects of protein (P) and carbohydrate (C) intake on the trade-off between reproduction and immune function in male and female decorated crickets (*Gryllodes sigillatus*). The intake of P and C had significant but divergent effects on reproduction and encapsulation ability in both sexes but did not influence inactive or activated PO activity. However, the divergence in the nutritional optima for these traits was much larger in males than females as illustrated by the non-overlapping 95% CRs on the global maxima for these traits in males and the larger estimates of θ and d . Collectively, this work suggests that the trade-off between reproduction and encapsulation ability is larger in males than females. Furthermore, it demonstrates the utility of my conceptual framework and the important role that specific nutrients play in regulating life-history trade-offs.

I found that the linear and nonlinear effects of P and C intake on reproduction in *G. sigillatus* were highly divergent across the sexes. Male calling effort was maximized at high intake of C and low intake of P in a P:C ratio of 1:7.08, whereas female egg production was maximised at a high intake of both nutrients in a P:C ratio of 1:1.17. Furthermore, this divergence was confirmed by significant differences in the linear, quadratic and correlational effects of P and C intake on reproduction in the sexes, a large angle (55.58°) between the nutritional linear vectors and a large Euclidean distance ($d = 119.17\text{mg}$) between the global maxima for reproduction in the sexes. These differences in the optimal nutritional requirements for reproduction in *G. sigillatus* are reflective of the divergent reproductive strategies of the sexes. In most sexually reproducing organisms, males are subject to more intense sexual selection than females because they typically contribute far less to each offspring than females (Trivers, 1972). As a result, males typically face intense competition for access to mates and those with the most elaborate sexual trait or display are often most successful (Andersson, 1994). A major determinant of mating success in male crickets is the amount of time spent producing an advertisement call (e.g. Bentsen *et al.*, 2006; Jacot *et al.*, 2008; Rodriguez-Munoz *et al.*, 2010) which is known to be metabolically costly (e.g. Kavanagh, 1987). To fuel this energetic activity, males require a high intake of C

to provide an abundant source of energy that can be rapidly utilized after ingestion (Bonduriansky *et al.*, 2008). In contrast, females typically do not have to compete for mating and their reproductive success is determined by the number of eggs they produce (Trivers, 1972). Females therefore require a relative higher intake of P than males, as this macronutrient plays a key role in stimulating oogenesis and regulating vitellogenesis in insects (Wheeler, 1996). The sex differences in the nutritional optima for reproduction that I document for *G. sigillatus* are consistent with other studies on field crickets (*Teleogryllus commodus* (Chapter 5; Maklakov *et al.*, 2008), *Gryllus veletis* (Harrison *et al.*, 2014) and *Drosophila* (*Drosophila melanogaster* (Reddiex *et al.*, 2013; Jensen *et al.*, 2015)). It contrasts, however, with a recent study on the ovoviparous cockroach (*Nauphoeta cinerea*) that found both sexes maximise reproductive success at a high intake of C and low intake of P (P:C ratio of 1:8; (Bunning *et al.*, 2016). The relatively low P requirement for female clutch size in this species is likely to reflect the unique action of endosymbionts that help recycle stored nitrogen for P synthesis (Bunning *et al.*, 2016).

I also found that the linear and nonlinear effects of P and C intake on encapsulation ability were divergent across the sexes in *G. sigillatus*. While a high intake of P was important to encapsulation ability in both sexes, this trait was maximised at a relatively higher intake of P to C in males (P:C = 5.14:1) than females (P:C = 1.04:1). As shown for reproduction, this divergence was statistically confirmed by significant differences in the linear, quadratic and correlational effects of P and C intake on encapsulation ability in the sexes. The degree of this divergence in nutrient effects, however, was not as large as documented for reproduction, with a smaller angle between the linear nutritional vectors (35.00°) and a shorter Euclidean distance ($d = 117.72\text{mg}$) between the global maxima for encapsulation ability than reproduction in the sexes. While the link between diet and encapsulation ability is well established in a range of insects (e.g. Ojala *et al.*, 2005; Anagnostou *et al.*, 2010; Saastamoinen & Rantala, 2013; Kelly, 2016), including crickets (e.g. Simmons, 2011), the specific nutrients responsible for this relationship are seldom identified.

In the few existing studies that have used well-defined diets of known composition, there appears to be a reliance of encapsulation ability (as well as other measures of immune function) on P intake (Lee *et al.*, 2006; Lee *et al.*, 2008b; Cotter *et al.*, 2011). It is important to note, however, that these studies are restricted to larvae of a single species: the cotton

leafworm (*Spodoptera littoralis*). For example, Lee *et al.* (2006) found that encapsulation ability, antimicrobial activity and PO activity in *S. littoralis* were all higher on P biased (P:C ratios of 5:1 and 2:1) than C biased diets (P:C ratios 1:2 and 1:5). Likewise, Cotter *et al.* (2011) found that both lysozyme activity and cuticular melanism (a proxy for the encapsulation process) increased with P intake and Lee *et al.* (2008b) found that antibacterial activity and cuticular melanism were increased on higher quality (casein) than lower quality P (zein). My work therefore adds much needed support for the relationship between P and encapsulation ability in another insect species, as well as showing that this relationship can vary across the sexes. As I provide the first study, to my knowledge, of documented sex differences in the effect of P intake on encapsulation ability it is impossible to determine if this is a general pattern or one that is specific to *G. sigillatus*. It also makes it difficult to explain why this difference exists. While any explanation would be speculative at this stage, it is possible that the higher intake of P needed to maximise encapsulation ability in males than females reflects the sexual dimorphism in immune function in this species. Males, on average, have a lower PO activity and encapsulation ability than females in *G. sigillatus* (Gershman *et al.*, 2010) and it is possible that they therefore require more P to activate the PO cascade than females.

In contrast to encapsulation ability, I found that P and C intake had little effect on inactive or activated PO activity in *G. sigillatus*. Previous studies using the GF have also found broadly similar results. For example, in *S. littoralis*, PO activity was not influenced by either the quality of P contained in the diet (casein vs zein, (Lee *et al.*, 2008b) or the absolute intake of P (Cotter *et al.*, 2011); but see (Lee *et al.*, 2006). In the closely related African armyworm (*S. exempta*), however, PO activity was shown to increase with P intake (Povey *et al.*, 2009). The general lack of nutrient effects on PO activity may reflect the different and varied physiological functions performed by PO. In addition to their role in the immune system, PO is also involved in cuticular melanisation after moulting (e.g. Hiruma & Riddiford, 1988), which is known to play an important role in key processes such as evaporative water loss (e.g. King & Sinclair, 2015; Rajpurohit *et al.*, 2016) and thermoregulation (e.g. Fielding & Defoliart, 2005; Yin *et al.*, 2016) in insects. Furthermore, activation of the PO cascade in the haemocoel of insects also results in the production of quinones and reactive oxygen species that do not discriminate self from non-self and hence are also known to destroy self-matter (Saul & Sugumaran, 1989; Nappi & Vass, 1993). Given

the importance of such processes to fitness, PO activity may be tightly regulated and insensitive to nutritional effects to help conserve their function. Clearly more work is needed to empirically validate this idea.

The large divergence that I document in the nutritional landscapes for calling effort and encapsulation ability, suggests that males are unable to maximise both of these traits on the same diet. Whereas the smaller divergence between the nutritional landscapes for egg production and encapsulation suggests that females are able to maximise these traits on a single diet. Traditionally, studies using the GF have taken the inability to maximise two life-history traits on a single diet as evidence of a nutritionally based trade-off (e.g. Lee *et al.*, 2008a; Maklakov *et al.*, 2008; Cotter *et al.*, 2011; Jensen *et al.*, 2015). While we agree with this logic, the problem with this view is that it does not formally demonstrate that a nutritionally based trade-off exists nor does it quantify the strength of any trade-off that may exist. My new novel framework posits that a nutritionally based trade-off can be formally demonstrated if the 95% CRs for the global maxima on the nutritional landscapes for the two traits are not overlapping. Furthermore, I show that the strength of the nutritionally based trade-off between two traits can be quantified using a sequential model-building approach, as well as the angle between the linear nutritional vectors (θ) and the Euclidean distance (d) between the global maxima on each nutritional landscape. I show that the 95% CRs on the global maxima for reproduction and encapsulation ability overlap in females but not in males. Furthermore, I found significant differences in the linear, quadratic and correlational effects of nutrients on reproduction and encapsulation ability in males, but only a difference in the linear effects in females that was driven by the fact that egg production was more responsive to the intake of both P and C than encapsulation ability. Finally, I found that θ and d were both significantly larger in males than females, as demonstrated by their non-overlapping 95% CIs. Collectively, this provides strong evidence for the presence of a nutritionally based trade-off between reproduction and encapsulation ability in males but not females, a finding that is confirmed by the negative correlation between these traits in males and a positive correlation in females. This result is consistent with sexual selection theory that predicts that males should invest more in sexual advertisement at the expense of immune function (Kokko, 1997; 1998; Getty, 1998; Kokko *et al.*, 2002; Zuk & Stoehr, 2002). While my study is the first to use the GF to examine the nutritional basis of differences in the trade-off between reproduction and immune function

in the sexes, a number of studies have used this approach to document the trade-off between lifespan and reproduction (Maklakov *et al.* 2008; Harrison *et al.* 2014; Jensen *et al.* 2015). In all three of these studies, however, the opposite pattern was found with the nutritionally based trade-off between lifespan and reproduction being larger in females than males.

Optimal foraging theory predicts that animals should forage to maximise their fitness (Stephens & Krebs, 1986). While traditional models focussed on animals optimizing their rate of energy intake (Stephens & Krebs, 1986), there is a growing appreciation that animals can also optimally balance their intake of specific nutrients (e.g. Jensen *et al.*, 2012). I show that male and female *G. sigillatus* clearly regulate their intake of P and C when given dietary choice. Furthermore, even though the regulated intake of P and C was 87% and 71% higher in females than males, respectively, both sexes regulated their intake of nutrients at the same P:C ratio (males: 1:2.00 and females 1:1.84). Despite this regulation, it is clear from mapping the RIP for each sex onto the nutritional landscapes for reproduction and encapsulation ability that this regulated intake of nutrients was not optimal for either sex. That is, the RIP did not overlap the 95% CR for the global maxima for reproduction or encapsulation ability. The obvious exception to this was for encapsulation ability in females but it is important to note that this trait also had the largest 95% CR (Figure 4.3D). In each instance, the RIP was markedly lower than the intake of nutrients needed to maximise these traits and the P:C ratio of the RIP also did not align particularly with the optimal P:C ratio for reproduction and encapsulation ability, especially in males. There are a number of possible reasons to explain these patterns. First, the lower than optimal absolute intake of P and C may represent physiological constraints on feeding behaviour. It is well established that dietary assimilation, digestion, absorption and utilization can all constrain feeding behaviour in animals (Henson & Hallam, 1995) and that the efficiency of these processes is influenced by gut morphology (e.g. Penry & Jumars, 1990; McWhorter & del Rio, 2000). It is therefore possible that gut morphology and its effects on these physiological processes are preventing the sexes from ingesting a higher intake of nutrients. Second, it is possible that the sexes in *G. sigillatus* are regulating the relative intake of P to C to maximise other, more heavily prioritised traits. In male *G. sigillatus*, mating success and CHC expression are maximised at a P:C ratio of 1:1.5 (Chapter 2) and the weight and amino acid composition of the spermatophylax and ampulla attachment time are maximised at a P:C ratio of 1:1.3 (Chapter

3). It is therefore possible that males regulate their intake of nutrients to maximise these traits, at the expense of calling effort and encapsulation ability. By comparison, LS is maximised at a P:C ratio of 1:8 in female *G. sigillatus* (Hunt, unpublished data) so it is possible that females prioritise this trait over egg production and encapsulation ability when regulating their intake of nutrients. Third, both sexes may be regulating their intake of nutrients to balance the expression of both reproduction and encapsulation ability. In support of this view, the RIP I estimate for males and females is close to midway between the optimal P:C ratio for reproduction and encapsulation ability at 1:1.32 and 1:1.06, respectively. Finally, it is possible that nutrient regulation under active dietary choice is genetically constrained. If the genes for nutrient regulation are positively genetically correlated across the sexes and the effects of nutrients on life-history traits are divergent across the sexes, this will generate intralocus sexual conflict (ISC, Bonduriansky & Chenoweth, 2009) over the optimal intake of nutrients. That is, even though selection will be pulling the RIP for males and females towards the sex-specific nutritional optima, the positive genetic correlation across the sexes prevents males and females from evolving independently (Bonduriansky & Chenoweth, 2009). ISC can therefore, in theory, prevent the RIP in males and females from evolving to the optima for reproduction and encapsulation ability. There is currently, however, little support for this process in the species where this has been correctly examined (*D. melanogaster* (Reddiex *et al.*, 2013), *Teleogryllus commodus*, Chapter 5) and further work is needed to empirically validate this possibility in *G. sigillatus*.

In conclusion, my work provides a novel conceptual framework for studying nutritionally based life-history trade-offs, as well as statistical tools to do so. Largely due to recent developments in the GF for nutrition, we now have a much better understanding of how the intake of specific nutrients influences life-history traits. We are now in the unique position where the integration of the GF with existing life-history theory is very much needed if we are to further progress our understanding of how nutrition regulates the trade-off between life-history traits. A particular strength of my conceptual approach is that θ and d are measured in standardized units and can be expressed as a percentage of the maximum value that is set by geometric design of the diets used. For example, the estimates of θ and d were 78.14% and 59.56% of their maximum values in males suggesting that the nutritionally based trade-off between reproduction and encapsulation ability is

actually much stronger than suggested by the phenotypic correlation between these traits ($r = -0.104$). This approach also facilitates the direct comparison of the strength of nutritionally based trade-offs across different species and studies using different designs and thereby sets the challenge for future empiricists examine the nutritional basis of life-history trade-offs.

Table 4.1. The linear and nonlinear effects of protein (P) and carbohydrate (C) intake on early life reproductive effort (calling effort and egg production) and immune function (encapsulation, pro-phenyloxidase (PO) activity and activated phenyloxidase activity) in male and female *Gryllodes sigillatus*.

| Response variable | Linear effects | | Nonlinear effects | | |
|------------------------------|----------------|--------------|-------------------|--------------|--------------|
| | P | C | P x P | C x C | P x C |
| Male | | | | | |
| <i>Calling effort</i> | | | | | |
| Coefficient ± SE | -0.10 ± 0.05 | 0.43 ± 0.05 | 0.00 ± 0.05 | -0.08 ± 0.03 | -0.19 ± 0.07 |
| t_{359} | 2.11 | 9.00 | 0.05 | 2.74 | 2.54 |
| <i>P</i> | 0.04 | 0.0001 | 0.96 | 0.006 | 0.01 |
| <i>Encapsulation ability</i> | | | | | |
| Coefficient ± SE | 0.75 ± 0.04 | -0.05 ± 0.04 | -0.10 ± 0.03 | 0.02 ± 0.02 | -0.36 ± 0.05 |
| t_{359} | 21.71 | 1.52 | 3.00 | 0.93 | 7.21 |
| <i>P</i> | 0.0001 | 0.31 | 0.003 | 0.35 | 0.0001 |
| <i>Inactive PO activity</i> | | | | | |
| Coefficient ± SE | -0.07 ± 0.05 | 0.05 ± 0.05 | 0.06 ± 0.05 | -0.04 ± 0.03 | -0.10 ± 0.08 |
| t_{359} | 1.35 | 0.99 | 1.20 | 1.37 | 1.21 |
| <i>P</i> | 0.18 | 0.32 | 0.23 | 0.17 | 0.23 |
| <i>Activated PO activity</i> | | | | | |
| Coefficient ± SE | 0.05 ± 0.05 | -0.09 ± 0.05 | -0.08 ± 0.05 | -0.02 ± 0.03 | -0.06 ± 0.08 |
| t_{359} | 0.95 | 1.77 | 1.55 | 0.49 | 0.70 |
| <i>P</i> | 0.35 | 0.08 | 0.12 | 0.63 | 0.49 |
| Female | | | | | |
| <i>Egg production</i> | | | | | |
| Coefficient ± SE | 0.44 ± 0.04 | 0.48 ± 0.04 | -0.69 ± 0.14 | -0.57 ± 0.13 | 0.45 ± 0.09 |
| t_{359} | 10.18 | 11.14 | 4.96 | 4.49 | 4.74 |
| <i>P</i> | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 |
| <i>Encapsulation ability</i> | | | | | |
| Coefficient ± SE | 0.24 ± 0.05 | 0.15 ± 0.05 | -0.47 ± 0.18 | -0.42 ± 0.16 | 0.20 ± 0.12 |
| t_{359} | 4.65 | 2.80 | 2.70 | 2.57 | 1.68 |
| <i>P</i> | 0.0001 | 0.005 | 0.007 | 0.011 | 0.09 |
| <i>Inactive PO activity</i> | | | | | |
| Coefficient ± SE | -0.03 ± 0.05 | 0.03 ± 0.05 | 0.07 ± 0.18 | 0.05 ± 0.17 | 0.04 ± 0.13 |
| t_{359} | 0.59 | 0.47 | 0.38 | 0.28 | 0.28 |
| <i>P</i> | 0.56 | 0.64 | 0.71 | 0.78 | 0.78 |
| <i>Activated PO activity</i> | | | | | |
| Coefficient ± SE | -0.07 ± 0.05 | 0.03 ± 0.05 | -0.11 ± 0.19 | -0.11 ± 0.17 | 0.02 ± 0.13 |
| t_{359} | 1.22 | 0.60 | 0.56 | 0.67 | 0.15 |
| <i>P</i> | 0.22 | 0.55 | 0.58 | 0.51 | 0.88 |

Table 4.2. Differential linear and nonlinear effects of protein and carbohydrate intake on male and female reproductive effort and encapsulation ability in *Gryllobates sigillatus*.

| Model term | SS_R | SS_C | DF_1 | DF_2 | F | P |
|---|--------|--------|--------|--------|--------|---------------------|
| Male calling effort vs. male encapsulation ability | | | | | | |
| Linear | 627.99 | 436.57 | 2 | 714 | 156.53 | 0.0001 ^A |
| Quadratic | 430.09 | 424.18 | 2 | 710 | 4.94 | 0.007 ^B |
| Correlational | 402.68 | 400.46 | 1 | 708 | 3.93 | 0.05 |
| Female egg production vs. female encapsulation ability | | | | | | |
| Linear | 589.62 | 566.33 | 2 | 714 | 14.68 | 0.0001 ^C |
| Quadratic | 529.85 | 528.71 | 2 | 710 | 0.77 | 0.46 |
| Correlational | 515.65 | 513.82 | 1 | 708 | 2.52 | 0.11 |
| Male calling effort vs. female egg production | | | | | | |
| Linear | 568.20 | 517.05 | 2 | 714 | 35.31 | 0.0001 ^D |
| Quadratic | 504.95 | 484.08 | 2 | 710 | 15.30 | 0.0001 ^E |
| Correlational | 484.08 | 466.81 | 1 | 708 | 26.20 | 0.0001 |
| Male vs. female encapsulation ability | | | | | | |
| Linear | 545.07 | 485.85 | 2 | 714 | 43.52 | 0.0001 ^F |
| Quadratic | 477.70 | 468.81 | 2 | 710 | 6.73 | 0.0013 ^G |
| Correlational | 461.25 | 447.47 | 1 | 708 | 21.79 | 0.0001 |

Univariate tests: ^A $P: F_{1,714} = 209.64, P = 0.0001, C: F_{1,714} = 66.85, P = 0.0001$; ^B $P \times P: F_{1,710} = 3.87, P = 0.05, C \times C: F_{1,710} = 5.93, P = 0.02$; ^C $P: F_{1,714} = 8.607, P = 0.003, C: F_{1,714} = 24.686, P = 0.0001$; ^D $P: F_{1,714} = 70.436, P = 0.0001, C: F_{1,714} = 0.634, P = 0.426$; ^E $P: F_{1,710} = 23.915, P = 0.0001, C: F_{1,710} = 7.791, P = 0.005$; ^F $P: F_{1,714} = 67.423, P = 0.0001, C: F_{1,714} = 10.103, P = 0.002$; ^G $P: F_{1,710} = 9.499, P = 0.0001, C: F_{1,710} = 3.802, P = 0.023$

Table 4.3. Multivariate Analysis of Covariance (MANCOVA) examining differences in the protein (P) and carbohydrate (C) intake by male and female *Gryllobates sigillatus* when given the choice between alternate diets. Univariate ANOVAs was used to determine how each of the nutrients contributed to the overall multivariate effect. As *G. sigillatus* is sexually size dimorphic, body weight was included as a covariate in both the multivariate and univariate models.

| | MANCOVA | | | |
|---------------|----------------|----------|-----------|----------|
| Model term | Pillai's Trace | <i>F</i> | <i>df</i> | <i>P</i> |
| Sex (A) | 0.71 | 181.38 | 2,151 | 0.0001 |
| Diet Pair (B) | 1.04 | 55.36 | 6,304 | 0.0001 |
| A x B | 0.21 | 5.91 | 6,304 | 0.0001 |
| | ANCOVA | | | |
| | Nutrient | <i>F</i> | <i>df</i> | <i>P</i> |
| Sex (A) | P | 199.66 | 1,152 | 0.0001 |
| | C | 291.24 | 1,152 | 0.0001 |
| Diet Pair (B) | P | 60.86 | 3,152 | 0.0001 |
| | C | 66.35 | 3,152 | 0.0001 |
| A x B | P | 5.13 | 3,152 | 0.002 |
| | C | 3.87 | 3,152 | 0.011 |

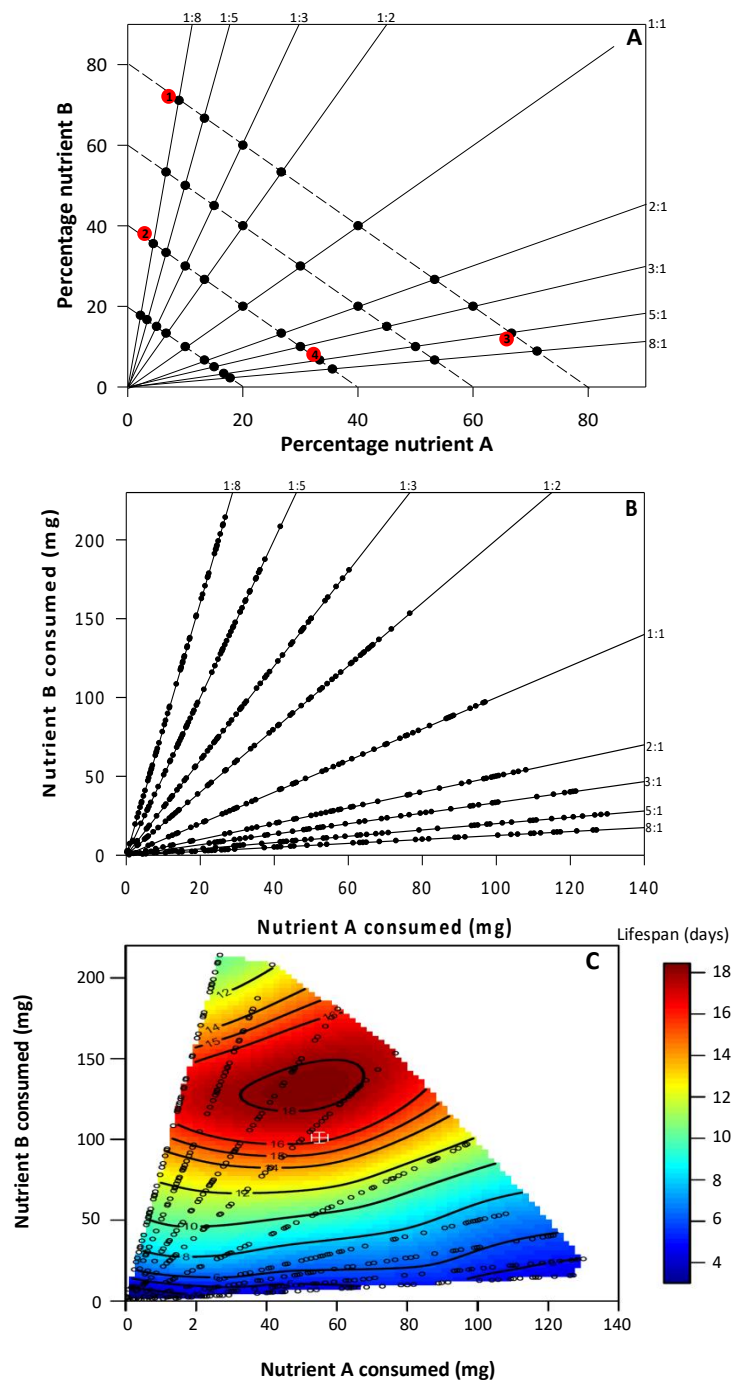


Figure 4.1. A hypothetical example demonstrating the typical protocol of the Geometric Framework (GF) of nutrition to study nutrient effects on lifespan. (A) The geometric array of diets presented in nutrient space. This particular geometric array consists of 9 different nutritional rails, each with a different fixed ratio of nutrient A to nutrient B (moving left to right: 1:8, 1:5, 1:3, 1:2, 1:1, 2:1, 3:1, 5:1 and 8:1). There are four different diets (black dots) located along each nutritional rail that have the same nutrient ratio but increase in total nutrition (i.e. caloric content) as they move away from the origin. Across different nutritional rails the four diets are arranged so that they are connected by iso-caloric lines

(dashed lines). These iso-caloric lines connect diets with different A:B ratios but the same caloric content. This hypothetical array therefore consists of 36 unique diets that differ in both the A:B ratio and total nutrition. (B) The distribution of actual feeding data (small black dots) recorded from animals restricted to each of the 30 unique diets. The consumption of diet by each animal is precisely measured over a defined feeding period and because the nutritional composition of the diets is known, this consumption of diet can be easily converted to an intake of nutrient A and B. As each animal is restricted to a single diet, they can only feed along the length of the nutritional rail by eating more or less of the diet (thereby ingesting more or less nutrients and calories). (C) An example of a nutritional landscape for lifespan. For each animal where the intake of nutrient A and B has been measured, the researcher also measures lifespan. This enables lifespan to be superimposed on the nutrient intake data and the linear and nonlinear effects of nutrient intake on lifespan can be quantified statistically using response surface methodologies. The relationship between nutrient intake and lifespan can also be visualized using thin-plate splines to plot the nutritional landscape in contour view. In the hypothetical example provided, the nutritional landscape is provided in contour view where regions in red represent increased lifespan and regions in blue represent reduced lifespan. The peak in lifespan appears to be centred at 50mg of nutrient A and 125 mg of nutrient B, which represents an A:B ratio of 1:2.5. To test whether animals are “optimally” regulating their intake of nutrients to maximise lifespan, a researcher can present animals with alternate pairs of diets differing in the ratio of A to B and total nutrition. A typical dietary choice design might pair diets 1 and 3 (diet pair 1), 1 and 4 (diet pair 2), 2 and 4 (diet pair 3) and 2 and 3 (diet pair 4) (red dots, panel A) and measure the consumption of both diets and the subsequent total intake of A and B over a predefined time period. The average intake of nutrient A and B across these diet pairs is referred to as the regulated intake point (RIP) and represents the point in nutrient space that individuals actively defend when given dietary choice. The RIP (white cross, panel C) can be mapped onto the nutritional landscape and its proximity to the peak used to determine whether dietary choice is optimal for lifespan.

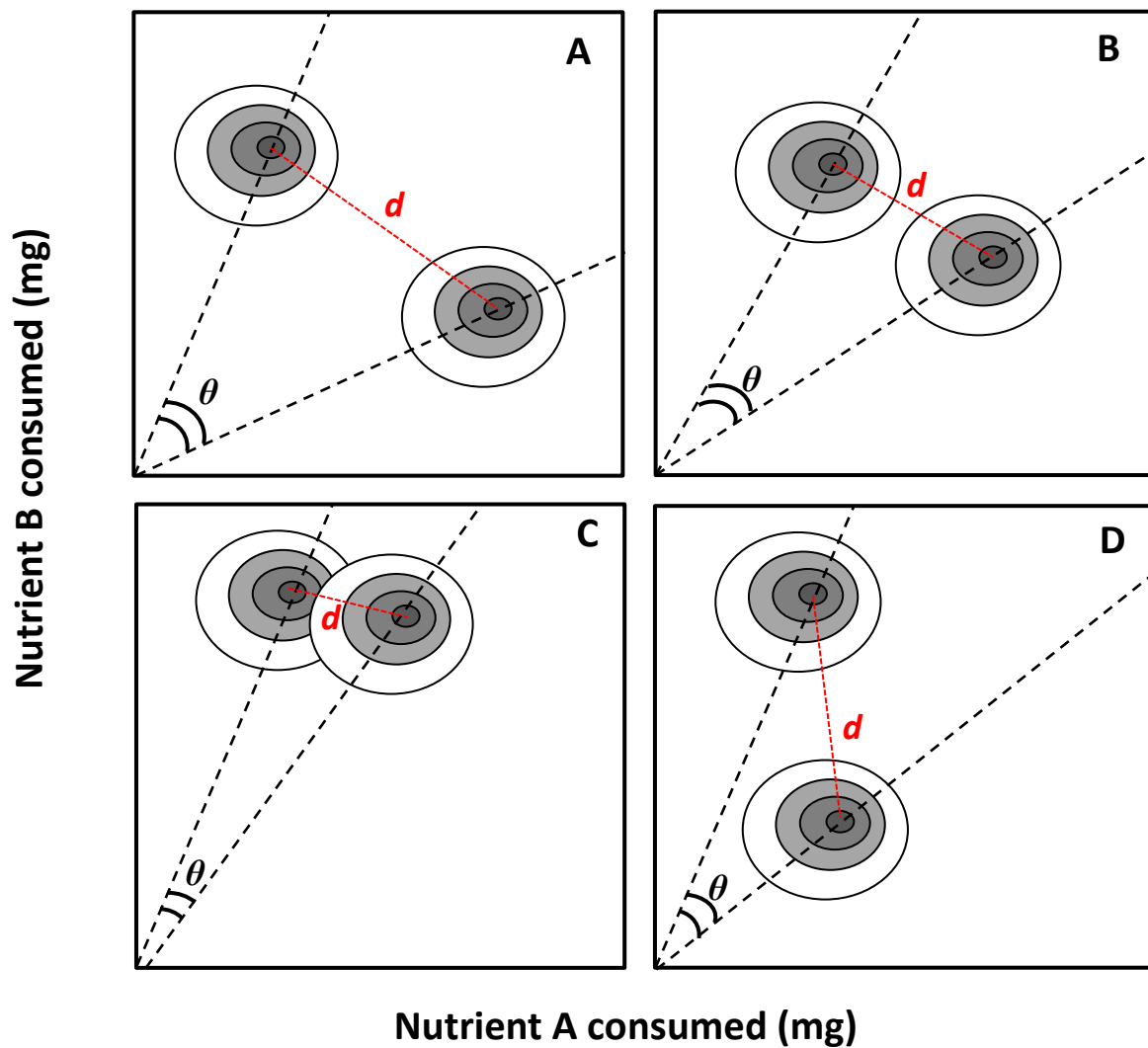


Figure 4.2. A hypothetical example illustrating how to quantify differences in the strength of nutritionally based trade-offs between two life-history traits. In each panel, the nutritional landscape of two competing life-history traits is provided in contour view where the darker shading represents an increased expression of the trait and light shading a decreased expression of the trait. The dashed black lines in each panel are the A:B nutritional rail that passes through the nutritional optima for each trait. The pair of curved, solid black lines that connect the nutritional rails passing through the optima represents the angle (θ) between these rails and the red dashed line represents the Euclidean distance (d) between the global maxima for each life-history trait. Panels A to C represent the case where the nutritional optima for both life-history traits occur at the same (or very similar) caloric intake. Consequently, both θ and d provide an accurate measure of how divergent the nutritional optima are for the two life-history traits and therefore the strength of the nutritionally

based trade-off between these traits. In moving from panel A to C, the nutritional optima for the two life-history traits move closer together (ending with overlap in panel C) and both θ and d get smaller indicating the strength of the nutritionally based trade-off between these traits is getting weaker. Panel D represents the case where the nutritional optima for the two life-history traits are located at two different caloric intakes. Consequently, θ and d provide different measures of the extent of the nutritionally based trade-off between life-history traits. In this instance d provides a better estimate of the divergence between optima and the strength of the life-history trade-off than θ .

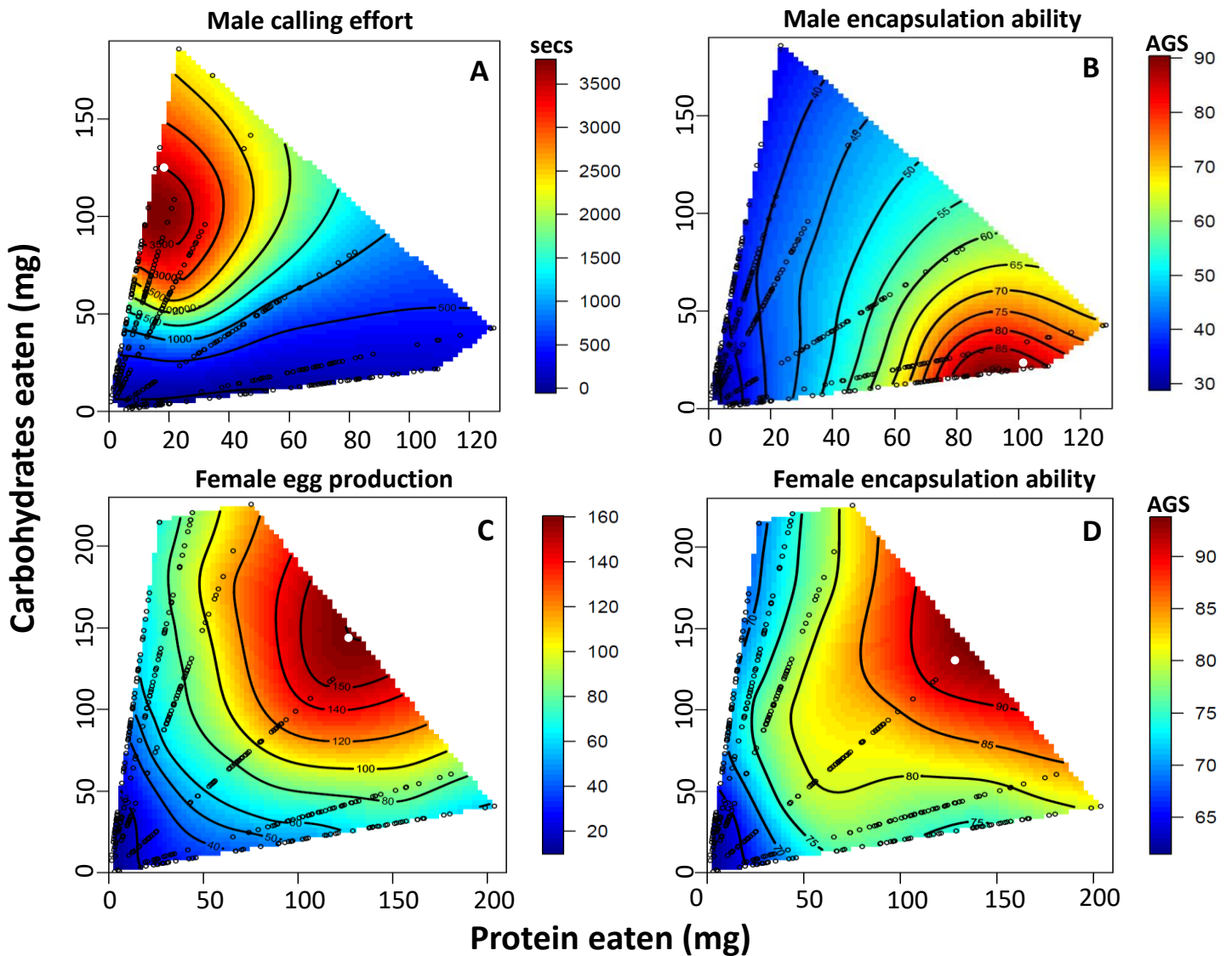


Figure 4.3. Nutritional landscapes illustrating the linear and nonlinear effects of protein and carbohydrate intake on (A) calling effort and (B) encapsulation ability in males and (C) egg production and (D) encapsulation ability in female *G. sigillatus*. On each landscape, high values of these traits are given in red and low values in blue. The open black circles represent the actual nutrient intake data for each cricket and the closed white circles represent the global maxima on each landscape.

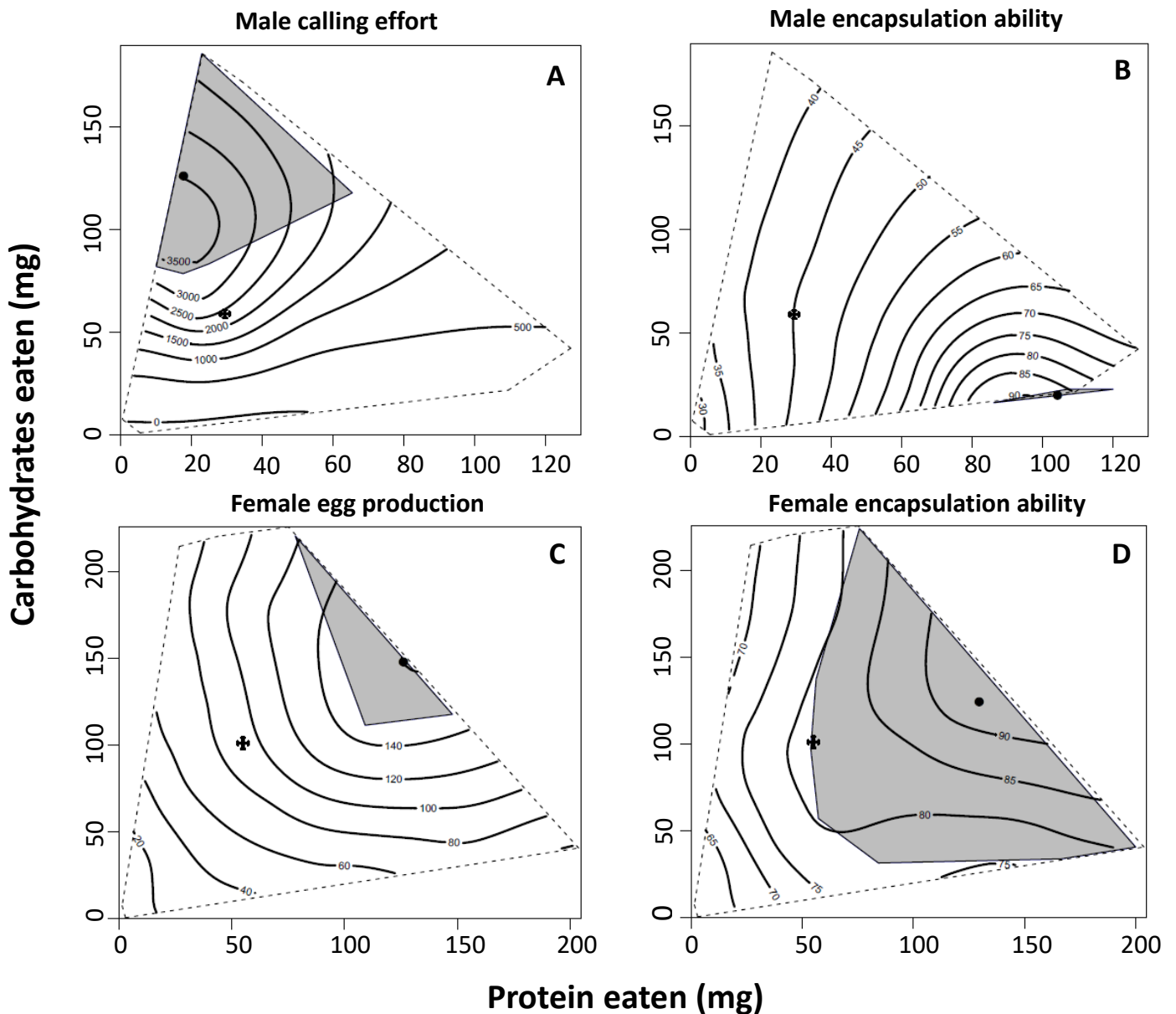


Figure 4.4. The 95% confidence region (solid grey fill) for the global maxima (closed black circle) on each landscape for (A) calling effort and (B) encapsulation ability in males and (C) egg production and (D) encapsulation ability in female *G. sigillatus*. On each landscape, the regulated intake point (\pm SE) is provided as a black cross and the dashed black line represents the boundary of the data. The regulation of nutrient intake under dietary choice is considered optimal for a given trait if the regulated intake point overlaps the 95% confidence region for the global maxima.

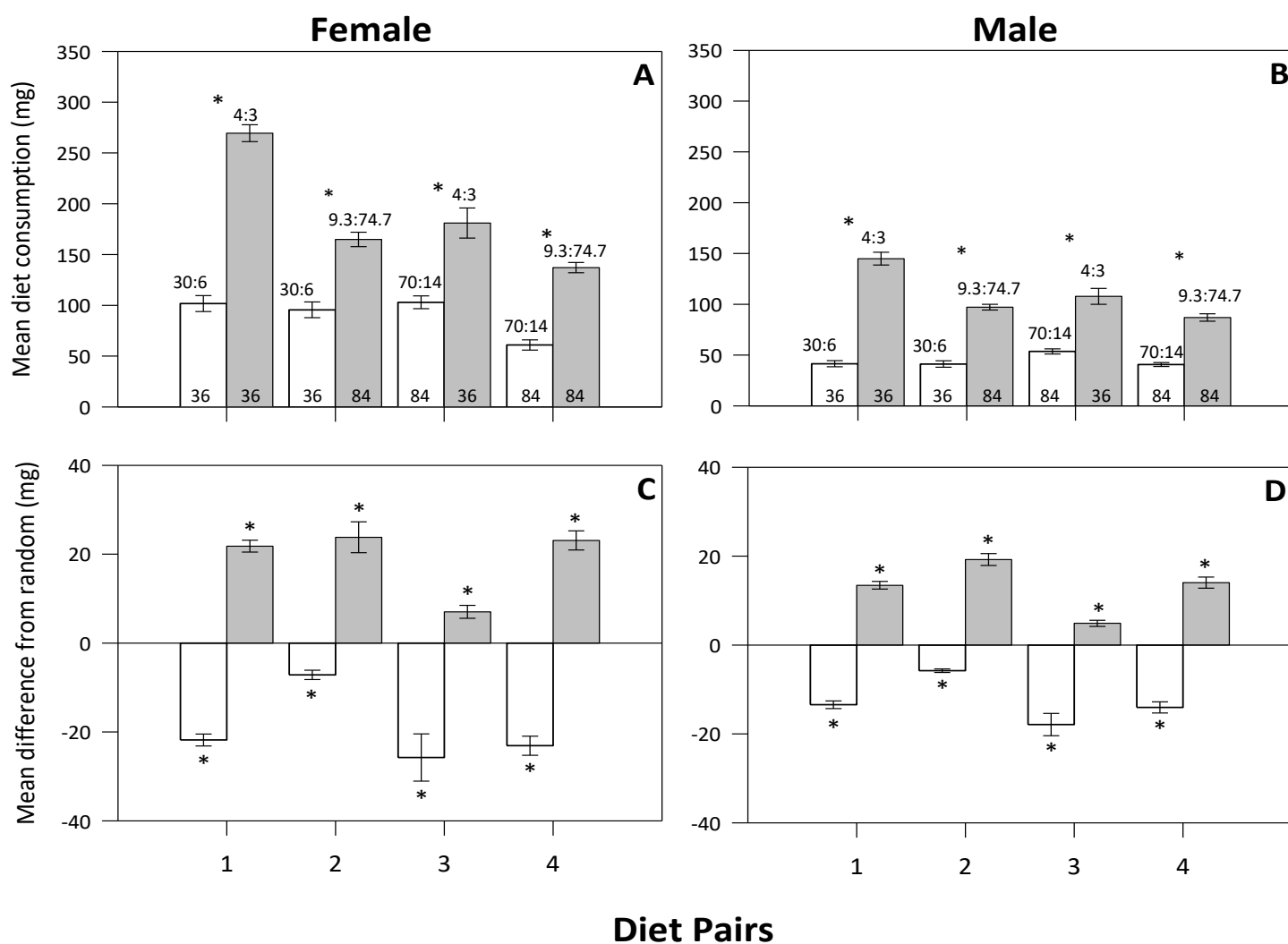


Figure 4.5. The mean (\pm SE) absolute consumption of each diet in the four diet pairs by (A) female and (B) male *G. sigillatus*. Grey bars represent the consumption of the high carbohydrate diet in the pair, whereas white bars represent the consumption of the high protein diet in the pair. The actual P:C ratio of alternate diets in each pair are provided above each bar and the total nutrient content of each diet are provided within the bar. The asterisks above each diet pair represents a significant difference (tested using a paired *t*-test) in the consumption of diets at $P < 0.05$. For each diet pair, males and females consumed significantly more of the high carbohydrate diet than the high protein diet. The difference in protein (white bars) and carbohydrate (grey bars) consumption from that expected if (C) females and (D) males fed at random from the diets in a pair. The asterisks above each bar represent a significant deviation from a mean of zero (tested using an unpaired *t*-test) which is expected under random feeding. For each diet pair, males and females consumed significantly more carbohydrates than expected by random feeding and less protein.

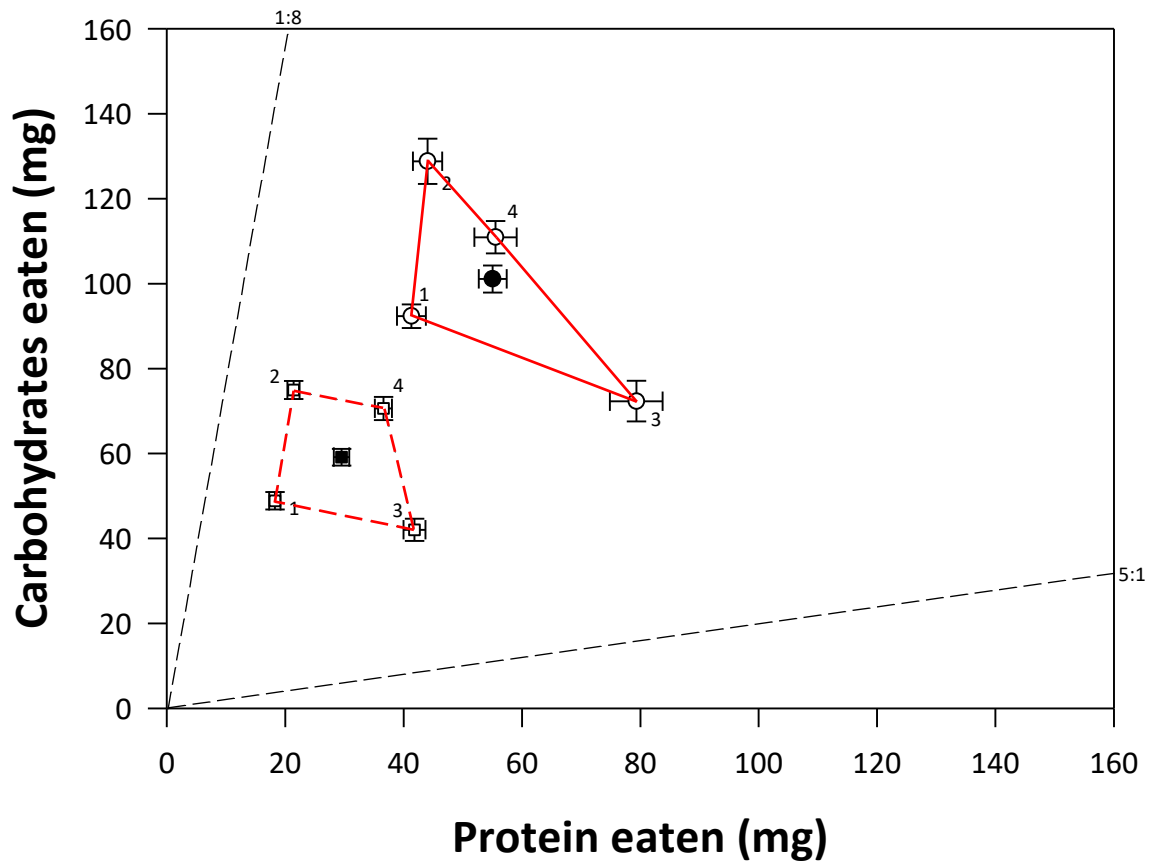


Figure 4.6. The mean (\pm SE) protein and carbohydrate intake of male (open squares) and female (open circles) *G. sigillatus* on each of the four diet pairs (labelled by number). The regulated intake point, calculated as the mean intake of nutrients across diet pairs is also presented for males (solid black square) and females (solid black circle) at a P:C ratio of 1:2.00 and 1:1.84, respectively. The red dashed lines and red solid lines represent the outer boundaries of my choice experimental design for males and females, respectively. The dashed black lines represent the expected intake of nutrients at a P:C ratio of 1:8, 1:3 and 1:1 (left to right of figure), respectively.

CHAPTER 5:

**LITTLE EVIDENCE FOR INTRALOCUS SEXUAL CONFLICT OVER THE
OPTIMAL INTAKE OF NUTRIENTS FOR LIFESPAN AND
REPRODUCTIVE EFFORT IN THE BLACK FIELD CRICKET
*TELEOGRYLLUS COMMODUS***

5.1. ABSTRACT

Life history traits that are expressed in both males and females are likely to have a shared genetic basis that prevents the independent evolution of these traits. If selection acting on these shared traits differs between the sexes then intralocus sexual conflict (ISC) will be generated and could prevent either sex from reaching their sex-specific phenotypic optima. Studies have shown significant sex differences in the effects of protein (P) and carbohydrate (C) intake on lifespan (LS) and reproductive effort (RE) with C intake important in maximising LS and male RE and P intake important for female RE. However, evidence suggests that the sexes cannot independently regulate their optimal nutrient intake, indicative of ISC. Here I used the cricket *Teleogryllus commodus* and the geometric framework of nutrition to examine two key parameters necessary for ISC to operate: sex-specific effects of P and C intake on LS and RE and strong positive genetic covariance between the sexes for the regulated intake of P and C. I found significant sex-specific effects of P and C intake on LS and RE and strong positive genetic covariance between the sexes over P and C intake under dietary choice. While this is often taken as strong evidence for ISC, I also show that the within sex, additive genetic variance-covariance matrix played more of a role in constraining the predicted response of nutrient regulation in the sexes. Furthermore, evidence of a dimorphism in nutrient regulation suggests that ISC over optimal nutrient intake is likely to be weak.

Key Words: Geometric Framework, Intralocus Sexual Conflict, Lifespan, Macronutrients, Reproduction

5.2. INTRODUCTION

There is incredible variation within and between animal species in the mean and maximal lifespan (LS) and this variation has puzzled researchers for decades, especially why the sexes often have different lifespans? (Massot *et al.*, 2002; Promislow, 2003; Mikolajewski *et al.*, 2004; Austad, 2006; Brodersen *et al.*, 2008; Perry & Rowe, 2010; Garratt & Brooks, 2012). In humans, women generally live longer than men (Promislow, 2003; Austad, 2006; Maklakov & Lummaa, 2013) and this pattern is apparent in a variety of taxonomic groups but is by no means a universal phenomenon (Garratt & Brooks, 2012). In most mammal species, females live longer than males (Promislow, 1992; Austad, 1997; Robinson *et al.*, 2006; Clutton-Brock & Isvaran, 2007), with the same pattern found in a number of insect species where females live longer than males (Promislow, 2003; Bonduriansky *et al.*, 2008; Morehouse, 2014). However, not all insect species follow this pattern (e.g. nematodes; McCulloch & Gems, 2003) and in birds, males are typically the longer lived sex (Triggs & Knell, 2012; Cayetano & Bonduriansky, 2015; Janicke & Chapuis, 2016), with the same pattern seen in some viviparous snake species (Sperry & Weatherhead, 2009).

Early biological explanations for this sexual dimorphism in LS focused on the role of sex chromosomes (“unguarded-X” hypothesis) and maternal mitochondrial inheritance (“mother’s curse” hypothesis) (Tower, 2006; Maklakov & Lummaa, 2013). The “unguarded-X” hypothesis posits that the heterogametic sex should be the shorter lived sex due to recessive deleterious mutations occurring on the X (or Z) chromosome which are not “guarded” by alleles on the second chromosome (Massot *et al.*, 2002; Tower, 2006; Maklakov & Lummaa, 2013). Support for this prediction generally comes from comparisons between mammals, where the male is the heterogametic sex and typically has a shorter LS than females, and birds where the female is the heterogametic sex and typically has a shorter LS than males (Promislow, 1992; Triggs & Knell, 2012). However, studies on polygynous birds where males engage in male-male competition similar to that seen in mammals, has shown an increase in male mortality despite being the homozygous sex (Promislow *et al.*, 1992). Furthermore, the “unguarded-X” hypothesis predicts that inbreeding, which increases homozygosity, should affect females more than males (Maklakov & Lummaa, 2013). However, two quantitative genetic studies on the seed beetle

Callosobruchus maculatus have found only limited support for this prediction. Both studies found that male LS was affected less by inbreeding than female LS but when kept in same-sex cohorts, sex differences in LS were essentially eliminated (Kolluru & Grether, 2005; Karell *et al.*, 2007). Similarly, the maternal inheritance of mitochondrial genetic material is predicted to increase the rate of male mortality by the accumulation of harmful mutations in male mitochondrial genomes, if they are only slightly deleterious, neutral or beneficial to females (Perry & Rowe, 2010; Maklakov & Lummaa, 2013; Clark *et al.*, 2015). A recent study on *Drosophila melanogaster* would seem to support the “mother’s curse” hypothesis with variance in mitochondrial haplotypes affecting LS and mortality rates in males but not females (Perry & Rowe, 2010), however, earlier studies found that variation in mitochondrial DNA did affect LS and aging in female *Drosophila* (Reznick, 1985; Zuk, 2009). Overall, it seems unlikely that the expression of damaging recessive alleles or the accumulations of male-damaging mutations are likely to adequately or completely explain the sex differences in LS. Instead, sex-specific selection on life history traits may select for different optimal expression of LS in males and females (Bonduriansky *et al.*, 2008; Maklakov & Lummaa, 2013).

Recently, the role that sexual selection plays in the evolution of sexual dimorphism in LS and rates of ageing has received considerable attention (Promislow, 2003; Bonduriansky *et al.*, 2008). Traditionally, sexual selection theory posits that in most sexually reproducing species, one sex (typically males) produce numerous, small gametes that compete for access to larger, less abundant gametes produced by the other sex (typically females). This dichotomy in reproductive investment means that males typically allocate more resources to mate competition than females (Trivers, 1972), which increases the variability in fitness and the opportunity for and intensity of sexual selection operating in males relative to females (Bateman, 1948; Trivers, 1972). This difference in the intensity of sexual selection across the sexes has implications for how males and females invest in reproductive effort over their LS (Bonduriansky *et al.*, 2008). Female fitness is limited by the amount of time available to amass the necessary resources for offspring production, therefore, natural selection is expected to promote a low-risk and low-cost strategy of reproductive effort that yields moderate rates of return over a long period (Bonduriansky *et al.*, 2008). Males, on the other hand, are expected to invest intensively in reproduction early in life by pursuing a high risk, “live fast, die young” strategy to achieve high fitness returns

over a shorter time scale (Hunt *et al.*, 2004; Bonduriansky *et al.*, 2008). Theory predicts that when adopting this high risk strategy, selection should favour the evolution of shorter LS and more rapid aging in males (Hunt *et al.*, 2004) than females which supports the pattern of increased male mortality seen in mammals (Promislow, 1992), insects (Promislow, 2003) and other taxa (Promislow, 2003; Austad, 2006; Garratt & Brooks, 2012). Given the divergent reproductive strategies of males and females and the differences over the timing of resource investment in reproduction, one would expect a divergence in the energetic requirements for reproduction in males and females (McCallum & Trauth, 2007; Bonduriansky *et al.*, 2008). By adopting a “live fast, die young” strategy, males should invest energy in sexual display and competition (Hunt *et al.*, 2004; Bonduriansky *et al.*, 2008) whereas females will be investing energy in expensive offspring/egg production and somatic maintenance to allow for reproduction over a longer time scale (Bonduriansky *et al.*, 2008). These investment strategies should result in a sex-specific allocation of resources to reproduction and LS (Stearns, 1992) and show a change in the allocation of resources to reproduction and LS in response to variation in the dietary environment.

Links between reproductive effort (RE), LS and diet have been shown in a number of studies, traditionally by restricting dietary intake. Modest dietary restriction (DR), through a reduction in food intake without malnutrition, has been shown to extend LS across a range of species (e.g. yeast (French *et al.*, 2007); *Drosophila* (Mair *et al.*, 2005); spiders (Mills *et al.*, 2010); mice (Weindruch *et al.*, 1986) and primates (Colman *et al.*, 2009)), with the effect found to be typically more pronounced in females than in males (Nakagawa *et al.*, 2012). The effects of DR on LS are traditionally explained through caloric restriction (CR) (Masoro, 2002; 2005; Partridge & Brand, 2005) with any sex differences explained by the divergent energetic costs of reproduction between males and females (McCallum & Trauth, 2007; Bonduriansky *et al.*, 2008). For instance, in females, the extension of LS through CR is explained by the associated reduction in fecundity (Chapman & Partridge, 1996) which frees up resources and allows for greater investment in somatic maintenance (Partridge & Brand, 2005). In contrast, the energetic demands of reproduction in males are considered to be lower than that of females and so the trade-off between LS and reproduction is less pronounced with less of a reallocation of resources to somatic maintenance when under CR (Bonduriansky *et al.*, 2008). However, recent studies have challenged this caloric centric viewpoint of LS extension through DR by showing that it is the intake of specific nutrients

and not calories *per se* that mediates the trade-off between reproduction and LS and is responsible for extending LS (Lee *et al.*, 2008; Maklakov *et al.*, 2008; Fanson *et al.*, 2009; Fanson & Taylor, 2012; Bruce *et al.*, 2013; Solon-Biet *et al.*, 2014). Distinguishing between caloric and nutrient specific effects on LS and reproduction is, therefore, important for understanding how diet influences sex-specific selection over investment in LS and reproduction (Simpson & Raubenheimer, 2009; Piper *et al.*, 2011; Tatar, 2011; Fanson & Taylor, 2012; Simpson & Raubenheimer, 2012; Tatar *et al.*, 2014), however, uncoupling the effects of calories from those of the nutrients from which they are derived, remains a major limitation of the DR method (Lee *et al.*, 2008; Archer *et al.*, 2009).

A solution to this problem is the use of chemically defined (holidic) diets within the Geometric Framework (GF) for nutrition (Simpson & Raubenheimer, 2012). The GF is a multidimensional nutritional approach within which, the effects of the intake of multiple nutrients (n) can be separated in n -dimensional nutritional space by restricting animals to an array of diets that differ in both nutrient composition and concentration (i.e. calories) (Simpson & Raubenheimer, 2012). When combined with precise measurements of diet consumption, thereby allowing nutrient intake to be calculated, the GF provides a powerful way of partitioning the effects of the intake of specific nutrients and calories on the expression of various life-history traits (Simpson & Raubenheimer, 2012). A number of studies have used the GF to show that the intake of the macronutrients: protein (P) and carbohydrate (C), have clear effects on LS and reproduction. For example, in *D.melanogaster* LS was shown to be maximized for both sexes at a high intake of nutrients at a protein:carbohydrate (P:C) ratio of approximately 1:16 (Lee *et al.*, 2008; Jensen *et al.*, 2015), with similar results seen in female Q-flies *Bactrocera tryoni* (Fanson *et al.*, 2009; Fanson & Taylor, 2012) and field crickets *Teleogryllus commodus* (Maklakov *et al.*, 2008), which also show increased LS on diets with a low P:C ratio. However, the exact P:C ratio was less C biased for female LS in *T.commodus* (P:C = 1:8, (Maklakov *et al.*, 2008) and more C biased in female *B.tryoni* with the magnitude depending on whether a yeast-based (P:C = 1:21, Fanson *et al.*, 2009) or holidic (P:C = 1:32, Fanson & Taylor, 2012) diet was used. Interestingly, Maklakov *et al.* (2008) also compared the effects of P and C intake on male LS and found that male and female crickets have different nutritional optima for LS, with male LS maximised at a P:C ratio of 1:5, which is contrary to the findings of Jensen *et al.* (2015). However, it should be noted that the difference in male and female nutritional optima for LS

resulted from LS declining at a high C intake in males but not females rather than a large shift in the P:C ratio maximising LS (Maklakov *et al.*, 2008). Overall, these studies support findings from DR studies which show that the effect of P intake (in relation to the intake of other macronutrients) on LS is more important than that of caloric intake (Nakagawa *et al.*, 2012). In contrast, GF studies have found a strong divergence in the effects of P and C on reproduction between the sexes. For example, in *D.melanogaster* male offspring production rate was maximised at a P:C ratio of 1:8, whereas female egg production was maximised at a high intake of diets with a P:C ratio of 1:2 (Lee *et al.*, 2008; Jensen *et al.*, 2015). Similarly, in *T.commodus* male reproductive effort was maximised at a P:C ratio of 1:8, whereas female egg production was maximised at a more balanced P:C ratio of 1:1 (Maklakov *et al.*, 2008). This difference in the nutritional optima for reproduction in males and females is most likely due to the differing nutritional requirements of the divergent reproductive strategies of the sexes (Bonduriansky *et al.*, 2008). With males competing for access to mates, they will require an abundant source of energy in the form of a high intake of C that can be accessed rapidly after digestion (Maklakov *et al.*, 2008; South *et al.*, 2011). On the other hand, with females typically investing in offspring production, access to P which plays an important role in stimulating oogenesis and regulating vitellogenesis (Wheeler, 1996) will be vital, while balancing P intake with C intake will be necessary to improve longevity in females (Bonduriansky *et al.*, 2008; Maklakov *et al.*, 2008).

There is clear evidence that optimal nutritional investment in LS and reproduction is highly divergent between the sexes (Lee *et al.*, 2008; Maklakov *et al.*, 2008; Jensen *et al.*, 2015). Optimal foraging theory predicts that animals will evolve foraging mechanisms that maximise their fitness (Stephens & Krebs, 1986) and so the divergence seen between the sexes over LS and reproduction suggests that fitness would be best maximised by the sexes regulating their intake of P and C independently. However, in choice feeding trials which compared the dietary choice of males and females, GF studies have failed to find a divergence in the feeding trajectories of the sexes with males and females both regulating their intake of nutrients at a P:C ratio of 1:4 in *D.melanogaster* (Jensen *et al.*, 2015) or a P:C ratio of 1:2.96 in *T.commodus* (Maklakov *et al.*, 2008).

When there are sex-specific optima for a trait that is expressed in both sexes, the shared genetic basis for this trait may prevent the sexes from evolving independently to their optima: a process referred to as intralocus sexual conflict (ISC) (Bonduriansky &

Chenoweth, 2009). In the case of nutrient optimization, previous GF studies argue that shared dietary choice is preventing the sexes from reaching their sex-specific nutrient optima for LS and reproduction (Maklakov *et al.*, 2008; Jensen *et al.*, 2015). However, to provide definite evidence for ISC over nutrient optimization would require showing that the sexes (i) have different nutritional optima for a shared trait and (ii) share a common genetic basis for their dietary preferences, which can be characterized by a strong and positive intersexual genetic correlation (Bonduriansky & Chenoweth, 2009). While many empirical examples of ISC exist (e.g. Lewis *et al.*, 2011; Mills *et al.*, 2012; Tarka, 2013; Berger *et al.*, 2014; Buzatto *et al.*, 2015), very little is known about the potential for ISC over nutrient intake to influence the evolution of key life-history traits. Indeed, to date only three studies have currently examined this topic, but each study is limited in their scope (Maklakov *et al.*, 2008; Reddiex *et al.*, 2013; Jensen *et al.*, 2015).

The two GF studies by Maklakov *et al.* (2008) and Jensen *et al.* (2015) have directly compared the nutritional optima for males and females over LS and reproduction and found that in *D.melanogaster* males and females had divergent nutritional optima for reproduction (Jensen *et al.*, 2015) while in *T.commodus* males and females had divergent nutritional optima for both LS and reproduction (Maklakov *et al.*, 2008). Furthermore, both studies reported that whilst there was evidence of active dietary regulation, there was little sexual dimorphism in nutrient regulation which is consistent with the presence of ISC (Maklakov *et al.*, 2008; Bonduriansky & Chenoweth, 2009; Jensen *et al.*, 2015). However, both studies have limitations that prevent them from definitively proving the presence of ISC. In Maklakov's *et al.* (2008) study, they compared nutritional landscapes for mated females and virgin males. This is problematic for two reasons; firstly, in crickets, the availability of mates has been shown to change the expression of a number of reproductive behaviours (Loher *et al.*, 1981; Souroukis *et al.*, 1992; Hill, 1998; Dowling & Simmons, 2012), which may all have particular nutritional requirements. Mating may, therefore, shift the nutritional optimum for fitness. Secondly, the prediction of optimal foraging theory (Stephens & Krebs, 1986) is that an individual of either sex will improve their fitness by optimising different life-history traits. In the absence of mates, virgins may maximise their LS until a mate becomes available and have different energetic demands compared to mated individuals. Indeed mating status has been shown to influence LS and age-dependent mortality in *T.commodus* with virgin females shown to live longer than mated females

(Zajitschek *et al.*, 2012). In contrast, mated males were shown to live longer than virgin males, possibly due to virgin males investing more in reproductive effort (amount of time spent calling) than mated males (Zajitschek *et al.*, 2012). Mating status, therefore, has the potential to change the optimal strategy of nutrient regulation across the sexes. In their study, Jensen *et al.* (2015) compared the nutritional landscapes between mated females and mated males, which prevents mating status from potentially confounding their results. Similarly to Maklakov *et al.* (2008), Jensen *et al.* (2015) found evidence for sex-specific nutritional optima for LS and reproduction in *D.melanogaster* and a shared trajectory for regulated nutrient intake. However, neither study investigated the underlying genetic architecture for dietary choice and so were unable to determine the nature of any genetic variation in dietary preference or get a definitive measure of any potential intersexual genetic correlation which would ultimately prove the presence of ISC over optimal nutrient intake.

Only the study by Reddiex *et al.* (2013) has used both key ISC parameters to examine the potential for ISC over the optimal intake of nutrients for reproduction in *D.melanogaster*. Despite showing sex-specific nutritional optima for reproduction and significant positive genetic (co)variance between the sexes for P and C intake, Reddiex *et al.* (2013) concluded that there was little scope for ISC over optimal nutrient intake in this species. However, there are a number of limitations to this study that perhaps make this an unsurprising result. Firstly, Reddiex *et al.* (2013) only measured nutrient intake over a very short time period (4 days) which is likely to explain the minor differences in the nutritional landscapes between the sexes in this study and an intersexual genetic correlation for P intake that did not differ statistically from zero (Reddiex *et al.*, 2013). Additionally, Reddiex *et al.* (2013) only examined the genetics of dietary choice for a single diet pair rather than using a number of pairs, that cover the extent of the nutritional landscape, to calculate the genetics of the regulated intake point (RIP). Limiting the genetic analysis of dietary choice to a single dietary pair restricts the measures of P and C intake to a small area of the nutritional landscape and does not represent the true regulated intake of nutrients, which overall limits the scope of Reddiex's *et al.* (2013) findings. Finally, Reddiex *et al.* (2013) do not measure LS or lifetime reproductive effort (LRE), instead only measuring the number of offspring produced in a single competitive mating event. We are, therefore, unable to compare the optimal nutrient investment between two key life-history traits and by

extension are unable to ascertain how investment in reproduction might trade-off with LS. Together, these studies show that there is the potential for ISC over optimal nutrient intake to influence the evolution of sex differences in LS and reproduction but clearly more research is needed to conclusively prove the presence of and effect of ISC over optimal intake.

In this study, I perform three experiments that build and improve upon previous GF studies, to document the existence of ISC over optimal nutrient intake in male and female *T.commodus* and how this affects the evolution of sex differences in LS, daily reproductive effort (DRE) and LRE. In Experiment 1, I determine the sex-specific effects of nutrient (P and C) intake and caloric intake on LS and reproductive effort by restricting males and females to one of 24 holidic diets. This allowed me to formally compare the nutritional optima for these traits between the sexes and identify sex-specific nutritional optima. In Experiment 2, utilizing a half-sib quantitative genetic breeding design, I conduct a choice feeding experiment using four pairs of diets that together encompass the full extent of the nutritional landscapes produced in Experiment 1. This allowed me to uncover the underlying genetics for dietary choice across the sexes as well as the strength and direction of any intersexual genetic correlations for P and C intake which may be constraining the evolution of nutrient optimization. Experiment 2, therefore, adds a genetic component (which is absent from the study by Maklakov *et al.* (2008)) to the comparison of sex-specific nutritional optima and studies the genetics of the RIP rather than a single dietary pair (as used by Reddix *et al.* (2013)). Finally in Experiment 3, I conduct a further choice feeding experiment on mated and virgin individuals to determine whether males and females regulate their nutrient intake differently and the effect that mating status has on this regulated intake of nutrients. By incorporating mating status into my experimental design I can specifically observe any differences over dietary choice caused by mating status, which was not possible in Maklakov's *et al.* (2008) study. I can then map the regulated intake points produced in Experiment 3 onto the nutritional landscapes from Experiment 1 to ascertain whether the dietary choice shown by the sexes is optimal for LS, DRE and LRE.

5.3. MATERIALS AND METHODS

5.3.1. Experimental animals

The *T. commodus* used in this study were collected from the wild in March 2009 from Smith's Lake, New South Wales, Australia and used to establish a panmictic lab population which has been maintained in large cultures of approximately 500 animals for 10 non-overlapping generations. Laboratory cultures are kept in 110L boxes at $28^{\circ}\text{C} \pm 1^{\circ}\text{C}$, under a 13:11 light:dark cycle, cleaned weekly and provided with cardboard egg carton for shelter, water *ad libitum* in 50mL test tubes plugged with cotton wool, egg pads consisting of damp cotton wool in a petri dish and a mixture of cat food (Purina Go Cat Senior[®]) and rat food (SDS Diets).

5.3.2. Artificial Diets and Feeding Protocol

I made 24 artificial, powdered diets that varied in P and C, as well as overall nutrition, based on the established protocol outlined by Simpson & Abisgold (1985). The composition of these diets can be found in Table 1.1 and the distribution of these diets in nutritional space can be seen in Figure 1.2. These represent the same 24 diets used throughout this thesis.

In each experiment, the same feeding protocols were used. At eclosion to adulthood, crickets were weighed using an electronic balance (Ohaus Explorer Professional model EP214C) and their pronotum width measured using an eyepiece graticule in a dissecting microscope (Leica model MZ5). Experimental animals were given either one (Experiment 1) or two (Experiments 2 and 3) dishes of diet of measured dry weight. Water was provided *ad libitum* in a 5ml test tube bunged with cotton wool. Food was provided in feeding platforms constructed by gluing the upturned lid of a vial (1.6 cm diameter, 1.6cm deep) onto the middle of a petri dish (5.5 cm diameter). Any diet spilled during feeding was collected in the petri dish and weighed. All diets were dried in an oven (Binder model FD115) at 30°C for 72 hrs before weighing. Feeding platforms were weighed before and after each feeding period using an electronic balance. Faeces were removed from the diet and feeding platform using forceps prior to re-weighing. Diet consumption was calculated as the difference in dry weight of diet before and after feeding. This amount of consumed diet was converted to a

weight of P and C ingested by multiplying by the proportion of these nutrients in the diet (South *et al.*, 2011; Bunning *et al.*, 2015).

5.3.3. Experiment 1: The effect of nutrient intake on lifespan and reproductive effort in the sexes

To determine the effects of P and C intake on male and female LS and reproductive effort, 10 males and 10 females were established at random on each of the 24 diets on their day of eclosion. However, some crickets escaped or died prematurely (due to accidental death) and were excluded from the final analysis (total males $n = 208$; females $n = 222$). Food of known dry mass was provided every three days and on the evening of day six post eclosion, the feeding platform was removed and a stock animal of the opposite sex was introduced to the container. This mate was removed on day seven when new food was provided and this weekly cycle was repeated throughout the experimental animal's lifetime. Animals were checked for mortality daily.

Reproductive effort of males and females was measured on the evening of day seven and once a week thereafter. To quantify female reproductive effort, females were provided with a small petri dish (5cm diameter) full of moist sand for oviposition for a seven day period, after which it was removed and frozen at -20°C for storage and replaced with a fresh dish of moist sand. To count eggs, the contents of each petri dish were emptied into a container of water and the eggs removed with fine forceps and counted. Male reproductive effort was measured as the amount of time spent calling each night (hereafter referred to as calling effort), which has been shown to be a good measure of mating success because females strongly prefer males that call more in both the laboratory (Hunt *et al.*, 2006) and the field (Bentsen *et al.*, 2006; Jacot *et al.*, 2008; Rodriguez-Munoz *et al.*, 2010). Furthermore, calling effort has been shown to be a good measure of reproductive effort because calling is metabolically expensive to produce in *T. commodus* (Kavanagh, 1987) and other field cricket species (e.g. *Gryllotalpa monanka* (Chapman & Partridge, 1996); *Gryllus lineaticeps* (Tatar, 2011); *Scapteriscus borellii* (Masoro, 2005); *Scapteriscus vicinus* (Masoro, 2005) and *Requena verticalis* (Bruce *et al.*, 2013)). Nightly calling effort was measured using a custom-built electronic monitoring device as described in full detail in (Archer *et al.*, 2012).

5.3.4. Experiment 2: The quantitative genetics of nutrient regulation

To estimate the quantitative genetics of nutrient regulation, I used a split-brood half-sib breeding design whereby sons and daughters from each full-sib family were split across four different diet pairs and their intake of nutrients measured under dietary choice for 21 days. The half-sib breeding design was established by mating each of 30 randomly chosen virgin sires with three randomly chosen virgin dams. A total of 50 offspring from each dam were collected and reared in a family group in an individual plastic container (10 x 10 x 5cm) for three weeks, with access to an *ad libitum* supply of ground cat food (Purina Go Cat Senior[®]) and water provided in a 5cm plastic tube plugged with cotton wool. After 3 weeks, 12 sons and 12 daughters per dam were isolated and established in individual plastic containers (5cm x 5cm x 5cm) and provided with *ad libitum* cat food pellets and water and checked daily for eclosion to adulthood. Containers were cleaned and fresh food and water were provided weekly. On the day of eclosion, I randomly allocated 3 sons and 3 daughters per dam to each of four diet pairs (total $n = 2160$). Fresh diet was provided every 3 days for a total of 21 days (i.e. a total of 7 feeding periods). Experimental animals were mated with a stock animal of the opposite sex on the evening of day 8 post-eclosion and removed on day nine with females provided with a petri dish of moist sand thereafter to measure reproductive effort. The four diet pairs used to examine nutrient regulation contained diets that differed in both the P:C ratio and total nutrition and provide a broad coverage in nutrient space (Figure 1.2). The diets chosen to form the four diet pairs were 2, 4, 22 and 24 and are the same diets as those used in the choice feeding experiment of Bunning *et al.* (2015) and Chapters 2 and 4 of this thesis.

5.3.5. Experiment 3: The effect of mating status on the regulated intake of nutrients by males and females

To examine the effect of mating status on the how male and female *T. commodus* regulate their intake of P and C under dietary choice, I conducted a further feeding choice experiment where I varied the mating status (i.e. virgin vs mated) of male and female crickets. Nymphs were collected on the day they hatched and raised in groups of 50 individuals for three weeks before being separated and raised individually until eclosion to adulthood, following the procedure outlined in Experiment 2. At eclosion, 60 crickets of each sex were randomly divided between the same four diet pairs used in Experiment 2.

Half the crickets on each diet pair were then further divided at random between two treatment groups: virgin and mated (total $n = 240$ males and 240 females). Individuals in the mated treatment group were provided with a stock virgin cricket of the opposite sex on the evening of day 8 post eclosion and this mating partner was removed the following morning. Experimental females were provided with a petri dish of moist sand immediately after the mating partner was removed. Fresh diet was provided to all crickets every two days for a total of 20 days (i.e. 10 feeding periods).

5.3.6 Statistical Analysis

Experiment 1: The effect of nutrient intake on lifespan and reproductive effort in the sexes

I quantified the linear and nonlinear (quadratic and correlational) effects of P and C intake on LS, DRE and LRE in Experiment 1 using multivariate response surface methodology (South *et al.*, 2011) implemented in the 'MCMCglmm' package of R (version 2.15.1, www.r-project.org)(Hadfield, 2010). I used the 'MCMCglmm' package (Hadfield, 2010) to calculate the linear and nonlinear (quadratic and correlational) effects of P and C intake on my traits because I used the posterior distribution of responses generated by 'MCMCglmm' in the later analysis of the predicted response to selection and genetic constraint in Experiment 2. Prior to analysis, P and C intake and all response variables were transformed to a mean of zero and standard deviation of one using a Z- transformation. Non-parametric thin-plate splines were used to visualize the nutritional landscape for each response variable and were constructed using the *Tps* function in the 'FIELDS' package of R (version 2.15.1, www.r-project.org).

I used a sequential model-building approach to determine whether the linear and nonlinear effects of P and C intake differed across the response variables (South *et al.*, 2011). Full details of this approach are outlined in Appendix 2. While the sequential approach provides a statistical test of the difference in magnitude of the linear and nonlinear gradients across response variables, it does not provide information on the direction of this difference in nutrient space. As such it is possible that response variables show differences in the magnitude of linear and nonlinear gradients, even though the optimal expression of these traits resides in a similar location in nutrient space. To account for this, I also calculated the angle (θ) and 95% confidence interval (CI) between linear

nutritional vectors for the two response variables being compared using the procedure outlined in Bunning *et al.* (2015) and the accompanying R code provided in Appendix 3.

Experiment 2: The quantitative genetics of nutrient regulation

I estimated the additive genetic variance-covariance matrix (**G**) and corresponding heritabilities (h^2) and genetic correlations (r_A) for P and C intake within and between the sexes using an multivariate animal model implemented in the ‘MCMCglmm’ package of R (version 2.15.1, www.r-project.org) using the following linear equation (Gilmour *et al.*, 2009; Hadfield, 2010; Wilson *et al.*, 2010):

$$y = \mu + a_i + \varepsilon_i \quad (1)$$

where μ is the multivariate mean, a_i is the breeding value (i.e. effects of i 's genotype relative to μ and ε_i is a residual term.

It is important to note that the above linear equation does not contain diet pair and therefore the resulting genetic parameters are taken across diet pairs. Consequently, these genetic parameters are for the RIP, defined as the point in nutrient space that individuals actively defend with given dietary choice, which is calculated as the mean intake of nutrients across diet pairs (Raubenheimer & Simpson 1993). I tested the significance of these genetic parameters using a one-sample Z-test, whereby the parameter of interest was divided by its associated standard error: if the resulting value exceeds 1.96 it is considered statistically significant at $P < 0.05$.

I estimated the predicted evolutionary response of the regulated intake of P and C in the sexes, as well as the 95% CIs for these responses, using the following equation (Lande, 1980):

$$\Delta\bar{z} = \frac{1}{2}\beta\mathbf{G} \quad (2)$$

where $\Delta\bar{z}$ represents the vector of predicted responses of the regulated intake of P and C in males ($\Delta\bar{z}_m$) and females ($\Delta\bar{z}_f$), respectively:

$$\Delta\bar{z} = \begin{pmatrix} \Delta\bar{z}_m \\ \Delta\bar{z}_f \end{pmatrix} \quad (3)$$

and $\boldsymbol{\beta}$ represents the vector of linear nutritional effects for males ($\boldsymbol{\beta}_m$) and females ($\boldsymbol{\beta}_f$), respectively (taken from Table 5.1):

$$\boldsymbol{\beta} = \begin{bmatrix} \boldsymbol{\beta}_f \\ \boldsymbol{\beta}_m \end{bmatrix} \quad (4)$$

\mathbf{G} represents the additive genetic variance-covariance matrix that can be partitioned into four sub-matrices, following Lande (1980):

$$\mathbf{G} = \begin{bmatrix} \mathbf{G}_m & \mathbf{B} \\ \mathbf{B}^T & \mathbf{G}_f \end{bmatrix} \quad (5)$$

where \mathbf{G}_m and \mathbf{G}_f are the within-sex additive genetic variance-covariance matrix for males and females respectively, while \mathbf{B} (and its transpose, \mathbf{B}^T) are the between-sex additive genetic covariance matrices that ultimately determines the extent to which the sexes are able to evolve independently. The constant $\frac{1}{2}$ appears because both parents are assumed to make equal autosomal contributions to the offspring of both sexes (Lande, 1980). As $\boldsymbol{\beta}_m$ and $\boldsymbol{\beta}_f$ measure the slope of the linear regression of P and C intake on a response variable, and I examined the effects of nutrients on three response variables (LS, DRE and LRE), there are three estimates of $\boldsymbol{\beta}$ and therefore $\Delta\bar{\mathbf{z}}$ for each sex. The 95% CIs for $\Delta\bar{\mathbf{z}}_m$ and $\Delta\bar{\mathbf{z}}_f$ were estimated by using the posterior distributions of $\boldsymbol{\beta}_m$, $\boldsymbol{\beta}_f$, \mathbf{G}_m , \mathbf{G}_f and \mathbf{B} in Equation 2. In short, a single data point was selected at random from the posterior distribution for each parameter and used in Equation 2 to calculate $\Delta\bar{\mathbf{z}}_m$ and $\Delta\bar{\mathbf{z}}_f$. This process was repeated for each data point in the posterior distribution ($n = 298$) using the ‘MCMCglmm’ package in R (version 2.15.1, www.r-project.org) and I used the posterior mean of these values as my parameter estimates of $\Delta\bar{\mathbf{z}}_m$ and $\Delta\bar{\mathbf{z}}_f$. To estimate the 95% CIs for $\Delta\bar{\mathbf{z}}_m$ and $\Delta\bar{\mathbf{z}}_f$, I used the highest posterior density (HPD) interval implemented in the ‘HPDinterval’ function of R. The associated R code for this procedure is provided in Appendix 4.

I examined the extent of genetic constraint on the evolution of the regulated intake of P and C within and between the sexes using a number of constraint metrics. Within the sexes, I calculated the angle (θ) and associated 95% CI between the vector of predicted responses ($\Delta\bar{\mathbf{z}}_m$ and $\Delta\bar{\mathbf{z}}_f$) and the vector of linear nutritional effects ($\boldsymbol{\beta}_m$ and $\boldsymbol{\beta}_f$) using the Bayesian approach outlined above for Experiment 1. This angle directly measures the degree to which \mathbf{G}_m and \mathbf{G}_f biases the predicted evolutionary response of the regulated

intake of P and C in males and females from the optimal direction favoured by β_m and β_f , respectively (Hansen & Houle, 2008; Blows & Walsh, 2009; Chenoweth *et al.*, 2010). An angle approaching 0° indicates an alignment of these vectors in the sexes and therefore a lack of genetic constraint to the evolution of nutrient regulation, whereas an angle approaching 180° suggests that G_m or G_f is constraining the predicted response of nutrient regulation in the sexes away from the direction favoured by β (Hansen & Houle, 2008; Blows & Walsh, 2009; Chenoweth *et al.*, 2010).

In addition, I used two constraint metrics to examine the extent to which \mathbf{B} influences the independent evolution of the regulated intake of nutrients in the sexes. First, I used a modified version of the rate of adaptation metric developed by Agrawal & Stinchcombe (2009). Although this metric was originally devised to examine the effects of genetic covariances on the response to selection within the sexes, it can easily be extended to quantify the effect that \mathbf{B} has on the predicted evolutionary response of nutrient regulation in the sexes by measuring the ratio:

$$\mathbf{R} = \frac{\Delta\bar{z}}{\Delta\bar{z}_{\mathbf{B}=0}} \quad (6)$$

where $\Delta\bar{z}$ is the predicted response of P and C intake in the sexes estimated from Equation (2) and $\Delta\bar{z}_{\mathbf{B}=0}$ are the same predicted responses when the genetic covariances in \mathbf{B} have been set to zero. Therefore \mathbf{R} measures the predicted response of P and C intake in the sexes taking into account the covariances in \mathbf{B} relative to response without these covariances (i.e. all traits are assumed to be genetically independent in the sexes). If $\mathbf{R}=0.5$, then the genetic covariance structure of \mathbf{B} causes the response of regulated nutrient intake to increase only 50% as quickly as expected if these traits were genetically independent in the sexes. In contrast, if $\mathbf{R}=2.0$ then the structure of \mathbf{B} accelerates the response of regulated nutrient intake in the sexes twice as much as expected under genetic independence. If $\mathbf{R}=1.0$, then the structure of \mathbf{B} has little effect on the response of regulated nutrient intake in the sexes (i.e. regulated nutrient intake is predicted to evolve as rapidly as if this trait was genetically independent in the sexes). To estimate the 95% CIs for \mathbf{R} , I used the approach outlined above for $\Delta\bar{z}_m$ and $\Delta\bar{z}_f$ and the associated R code is provided in Appendix 4.

Second, I used the framework of Hansen & Houle (2008) to measure the unconditional evolvability ($e(\boldsymbol{\beta})$) as:

$$e(\boldsymbol{\beta}) = \frac{[\boldsymbol{\beta}^T][\mathbf{G}][\boldsymbol{\beta}]}{[\boldsymbol{\beta}]^2} \quad (7)$$

where $|\boldsymbol{\beta}|$ is the normalized length of $\boldsymbol{\beta}$. $e(\boldsymbol{\beta})$ therefore describes the length of the vector of the predicted response ($\Delta\bar{\mathbf{z}}$) within the space of \mathbf{G} that has been projected onto the normalized vector of the optima nutritional response ($\boldsymbol{\beta}$). Consequently, it measures the amount of genetic variance available in the direction of the optimal nutrient regulation. As my response variables (LS, DRE and LRE) were measured on different scales, $e(\boldsymbol{\beta})$ was estimated using mean-standardized data to ease interpretability. As $e(\boldsymbol{\beta})$ represents a measure of evolvability in one direction in phenotypic space, it is therefore likely to be different with every choice of $\boldsymbol{\beta}$ (Hansen & Houle, 2008). Consequently, I also estimated the average evolvability (\bar{e}) of \mathbf{G} as:

$$\bar{e} = \frac{\sum_i \lambda_i}{k} \quad (8)$$

where λ_i are the eigenvalues of \mathbf{G} and k equals the total number of eigenvalues. \bar{e} therefore measures the average evolvability of the entire \mathbf{G} matrix taken across random nutritional effects ($\boldsymbol{\beta}$). Consequently, $e(\boldsymbol{\beta})$ can be compared to \bar{e} to determine how much genetic variation exists in the direction of optimal nutrient regulation relative to the entire \mathbf{G} matrix. To estimate the 95% CIs for $e(\boldsymbol{\beta})$ and \bar{e} , I used the approach outlined above for $\Delta\bar{\mathbf{z}}_m$ and $\Delta\bar{\mathbf{z}}_f$ and the associated R code is provided in Appendix 5.

Experiment 3: The effect of mating status on the regulated intake of nutrients by males and females under dietary choice

To examine the effect of mating status on the regulated intake of P and C by male and female, I used a multivariate analysis of variance (MANOVA) that included mating status, sex and diet pair as main effects, all possible interactions between these main effects, and P and C intake as the response variables. As the sex by diet pair interaction was significant in this overall model (Table 5.5), I ran a second MANOVA model within each sex that included mating status and diet pair as main effects, the interaction between these main effects and P and C intake as the response variables. I followed both MANOVA models

with univariate ANOVAs to determine which nutrient(s) contributed to any overall multivariate effects. As there were four diet pairs at each level of sex and mating status, I used Fisher's least significant difference (LSD) post-hoc analysis to determine which were significant at $P < 0.05$.

I calculated the regulated intake point (RIP), defined as the point in nutrient space that individuals actively defend when given dietary choice (Raubenheimer, 1993), as the mean intake of P and C across the four diet pairs in the two mating status treatments for each sex (i.e. virgin males, mated males, virgin females and mated females). To determine whether males and females optimally regulate their intake of nutrients under dietary choice, I mapped the RIP for mated crickets of each sex onto their respective nutritional landscapes: I focussed on the RIP of mated crickets as this is what the nutritional landscapes were derived from in Experiment 1. The RIP is considered optimal if it coincides with the peak on the nutritional landscape. To test for differences in the RIP, I used an analysis of covariance (ANCOVA). I was specifically interested in two contrasts: between virgin and mated crickets within each sex and between male and female crickets within each level of mating status. In the first contrast, I partitioned according to sex and ran an ANCOVA model that included mating status as a fixed effect, P intake as a covariate, the interaction between mating status and P intake and C intake as the response variable. In the second, I partitioned the data according to mating status and ran an ANCOVA model that included sex as a fixed effect, P intake as a covariate, the interaction between sex and P intake and C intake as the response variable. In both ANCOVA models, significance of the interaction term indicates that the RIP differs significantly across the fixed effect.

5.4. RESULTS

5.4.1. Experiment 1: The effect of nutrient intake on lifespan and reproductive effort in the sexes

The intake of both P and C had clear linear and nonlinear effects on LS in both sexes (Table 5.1). Female LS increased linearly with C intake (Table 5.1, Figure 5.1A). The significant negative quadratic term for C intake suggests a peak in LS with the intake of this nutrient and inspection of the nutritional landscape shows that this peak occurs at a high

intake of C and a low intake of P at an approximate P:C ratio of 1:8 (Table 5.1, Figure 5.1A). There was also a significant negative correlational term providing further evidence that female LS was maximised at a high intake of C and a low intake of P (Table 5.1). In contrast, male LS increased linearly with the intake of both P and C, although it was more responsive to the intake of C than P (Table 5.1, Figure 5.1B). The significant quadratic terms indicate a peak in male LS for both nutrients and inspection of the nutritional landscape shows that this peak occurs at a high intake of C and a low intake of P in an approximate P:C ratio of 1:2.5 (Figure 5.1B). There was also a significant negative correlational term providing further evidence that male LS is maximised at a high intake of C and low intake of P (Table 5.1). Formal statistical comparison of the nutritional landscapes using a sequential model approach showed that the linear and quadratic effects of P and C intake on LS in the sexes differed significantly but the correlational effects of these nutrients did not (Table 5.2). The sex difference in linear effects was due to the fact that LS increased with P intake in males but not in females and also because LS is more responsive to C intake in males than females (Table 5.2). The sex difference in quadratic effects is due to the fact that there is a peak in LS with P intake in females but not in males (Table 5.2). Importantly, the optima for LS in males and females occur in different regions on the nutritional landscapes (Figure 5.1A & B), as evidenced by the large angle between the two linear nutritional vectors (25.99° , Table 5.2).

DRE in females increased linearly with the intake of P and C, with both nutrients having an almost equal effect on this trait (Table 5.1). There was also a significant negative quadratic term for P intake indicating a peak in DRE with the intake of this nutrient and inspection of the nutritional landscape shows that this peak occurs at high intakes of P and C in an approximate P:C ratio of 1:1 (Figure 5.1C). The significant positive correlational term further demonstrates that DRE increases with the intake of both nutrients in females (Figure 5.2C, Table 5.1). In contrast, DRE in males significantly increased linearly with the intake of C and significantly decreased with the intake of P (Table 5.1). The significant negative quadratic term for C intake indicates a peak in DRE with the intake of this nutrient and inspection of the nutritional landscape shows that this peak occurs at a high intake of C and a low intake of P at a P:C ratio of approximately 1:8 (Figure 5.2D, Table 5.1). The significant negative correlational term further demonstrates that DRE in males is maximised at a high intake of C and low intake of P (Figure 5.2D, Table 5.1). Formal statistical comparison of the landscapes showed that the linear, quadratic and correlational effects of P and C intake on

DRE differed significantly in the sexes (Table 5.2). The sex differences in the linear effects is due to the fact that DRE increases with P intake in females but decreases with P intake in males and also because DRE is more responsive to the intake of C in males than females (Table 5.2). The sex differences in the quadratic effects is due to fact that DRE peaks with P intake in females but not in males, whereas the sex differences in the correlational effects occurs because the covariance between nutrients has a positive effect on DRE in females but a negative effect in males (Table 5.2). The large angle (55.19°) between the linear nutritional vectors indicates that the optima for DRE occur in different regions of the nutritional landscape for males and females (Table 5.2, Figure 5.1C & D).

The effects of P and C intake on LRE in the sexes show a similar pattern to that shown for DRE. LRE in females increased linearly with the intake of both P and C, although this trait was more responsive to the intake of C than P (Table 5.1). There were significant negative quadratic terms for both nutrients indicating a peak in LRE with both nutrients and inspection of the nutritional landscape shows that this peak occurs at a high intake of P and C in a P:C ratio of approximately 1:1.5 (Figure 5.2E, Table 5.1). In contrast, LRE in males increased linearly with C intake and decreased linearly with P intake (Table 5.1). The significant negative quadratic term for C intake indicates a peak in LRE with the intake of this nutrient and inspection of the nutritional landscape shows that this peak occurs at a high intake of C and low intake of P at a P:C ratio of approximately 1:8 (Figure 5.2F, Table 5.1). There was also a significant negative correlational gradient which further confirms LRE is optimized at a high intake of C and low intake of P (Figure 5.2F, Table 5.1). Formal statistical comparison of the landscapes showed that the linear, quadratic and correlational effects of P and C intake on LRE differed significantly in the sexes (Table 5.2). The sex differences in the linear effects are due to the fact that DRE increases with P intake in females but decreases with P intake in males (Table 5.2). The sex differences in the quadratic effects is due to the fact that LRE peaks with P intake in females but not in males, whereas the sex differences in the correlational effects occurs because the covariance between nutrients has a negative effect on LRE in males but not in females (Table 5.2). The optima for LRE in males and females occur in different regions on the nutritional landscapes (Figure 5.1E & F), as evidenced by the large angle between the two linear nutritional vectors (33.06° , Table 5.2).

The nutritional landscapes for LS, DRE and LRE also differed within each sex (Table 5.2). In females, the linear, quadratic and correlational effects of P and C intake on LS

differed significantly from the effects of these nutrients on DRE and LRE (Table 5.2). In both cases, the difference in linear effects was due to the positive effect of P on DRE and LRE but there was no effect on LS (Table 5.2). The difference in quadratic effects was due to the fact that DRE and LRE peaked with P intake but LS did not and because the peak in LS with C intake was more pronounced than the peaks for DRE and LRE (Table 5.2). The difference in correlational effects was due to the fact that the covariance between P and C intake had a negative effect on LS, a positive effect on DRE and no effect on LRE (Table 5.2). There was also a significant difference in the linear effects of P and C intake on DRE and LRE driven by the fact that DRE was more responsive than LRE to P intake, whereas the reverse pattern was true to C intake (Table 5.2). Collectively, this suggests that LS peaks in a different region on the nutritional landscape than DRE and LRE (Figure 5.1A, C & E), a finding that is supported by the large angles between the nutritional vectors these traits (51.18° and 32.50° , respectively)(Table 5.2). In contrast, DRE and LRE peak in similar regions (Figure 5.1C & E), as indicated by the much smaller angle between the nutritional vectors for these traits (18.66° , Table 5.2). As LS cannot be optimized at the same intake of nutrients that maximises DRE and LRE (and vice versa), this indicates a trade-off between these traits in females.

In contrast, only the linear and quadratic effects of P and C intake on LS differed significantly from the effects of these nutrients on DRE and LRE in males (Table 5.2). In both instances, the difference in linear effects was the result of P intake having a positive effect on LS but a negative effect on DRE and LRE (Table 5.2). Furthermore, the difference in quadratic effects was due to the fact that LS peaked with P intake but DRE and LRE did not and because the peak in LS with C intake was more pronounced than the peaks in DRE and LRE with the intake of this nutrient (Table 5.2). In contrast to females, the linear, quadratic and correlational effects of nutrient intake on DRE and LRE in males did not differ significantly indicating that these traits peak in the same region in nutrient space, as demonstrated by the small angle between the linear nutritional vectors for these traits (4.03° , Table 5.2). Collectively, this suggests that the nutritional optimum for LS in males occurs in a different region than the optima for DRE and LRE (Figure 5.1B, D & E). However, the angle between the nutritional linear vector for LS and DRE (30.48°) and LS and LRE (26.71°) is smaller than observed in females, suggesting that while these traits trade-off in males, the strength of this trade-off is weaker than in males.

5.4.2. Experiment 2: The quantitative genetics of nutrient regulation

The \mathbf{G} matrix for the regulated intake of P and C in male and female *T. commodus* is presented in Table 5.3. The regulated intake of P and C was significantly heritable and of a similar magnitude in the sexes (Table 5.3). In both sexes, the heritability estimates for the regulated intake of P were over twice as large as those for the regulated intake of C (Table 5.3). Furthermore, the genetic correlation (r_A) between the regulated intake of P and C was significant and positive for both sexes, although the estimate was higher for males than females (Table 5.3). Most importantly, I show strong and significant positive genetic correlations for the regulated intake of P and C between the sexes (Table 5.3). Together with the sex differences in the effects of P and C on LS, DRE and LRE, these strong positive genetic correlations between the sexes demonstrate the potential for ISC to constrain the evolution of nutrient regulation, LS and reproductive effort.

To examine the potential for genetic constraint, I first combined my estimates of the linear effects of nutrients on LS, DRE and LRE in each sex and the within-sex \mathbf{G} matrix (\mathbf{G}_m and \mathbf{G}_f) to estimate the degree of genetic constraint within each sex, as the angle (θ) between the vector of predicted evolutionary response of the regulated intake of P and C and the vector of linear nutritional effects of P and C intake on each response variable. Therefore, θ provides a direct measure of the degree to which \mathbf{G}_m and \mathbf{G}_f bias the evolution of the regulated intake of P and C in the sexes away from their phenotypic optima, whereby an θ of 0° represents a perfect alignment of these vectors and no genetic constraint and an angle of 90° indicates that these vectors are orthogonal and represents maximal genetic constraint. In males, θ was large for all traits, being the lowest for LS ($\theta = 33.41^\circ$, 95% CIs = 17.71° , 41.28°) and increasing for DRE ($\theta = 49.24^\circ$, 95% CIs = 39.32° , 55.37°) and LRE ($\theta = 54.18^\circ$, 95% CIs = 45.37° , 59.64°). In contrast, estimates of θ were, on average, smaller in females than males and this trait-specific pattern was reversed with θ being lowest for DRE ($\theta = 16.26^\circ$, 95% CIs = 3.30° , 32.79°), followed by LRE ($\theta = 32.19^\circ$, 95% CIs = 9.25° , 44.82°) and highest for LS ($\theta = 58.64^\circ$, 95% CIs = 45.01° , 72.45°). The impact of \mathbf{G}_m and \mathbf{G}_f on the predicted evolutionary response of the regulated intake of nutrients can be visualized in Figure 5.2 where the eigenstructure of \mathbf{G}_m and \mathbf{G}_f have been mapped onto the nutritional landscapes for each trait. The major axis of \mathbf{G}_f is well aligned with the peaks of the nutritional landscape for female DRE (Figure 5.2C) and LRE (Figure 5.2E) but less well

aligned with the peak for LS (Figure 5.2A). In contrast, the major axis of \mathbf{G}_m is well aligned with the peak of the nutritional landscape for male LS (Figure 5.2B) but less well aligned with the peak for DRE and LRE (Figure 5.2D & F). Collectively, this suggests that the structure of \mathbf{G}_f constrains female LS but poses less of a constraint to female DRE and LRE, whereas \mathbf{G}_m constrains male DRE and LRE but poses less of a constraint to male LS.

I also examined the magnitude of genetic constraint to the evolution of sexual dimorphism due to the across-sex genetic covariance (\mathbf{B}) in the regulated intake of P and C. First, I used the rate of adaptation metric (\mathbf{R}) of Agrawal & Stinchcombe (2009) that measures the ratio of the predicted evolutionary response of traits when \mathbf{B} is estimated directly from the quantitative genetic breeding design ($\Delta\bar{z}$) versus when \mathbf{B} is set to zero ($\Delta\bar{z}_{\mathbf{B}=0}$). Estimates of $\Delta\bar{z}$, $\Delta\bar{z}_{\mathbf{B}=0}$ and \mathbf{R} with 95% CIs for males and females are provided in Table 5.4. With the exception of the regulated intake of C for female DRE, all other traits in the sexes showed higher predicted responses for $\Delta\bar{z}$ than $\Delta\bar{z}_{\mathbf{B}=0}$ (Table 5.4). In males, \mathbf{R} values for the regulated intake of P and C for DRE and the regulated intake of C for LRE were significantly greater than 1.0 (95% CIs did not overlap 1.0), whereas the remaining traits did not differ significantly from 1.0 (Table 5.4). In females, the \mathbf{R} value for the regulated intake of P for LS was significantly greater than 1.0, however, all other traits did not differ significantly from 1.0 (Table 5.4). Collectively, this suggests that for most traits in the sexes, the genetic covariance contained in \mathbf{B} did little to constrain the predicted response of the regulated intake of nutrients and in those few instances where it did, \mathbf{B} appeared to accelerate the predicted response rather than constrain it.

Second, I used the unconditional evolvability ($e(\boldsymbol{\beta})$) of Hansen & Houle (2008) to measure the availability of genetic variance in the direction of the optimal regulated intake of P and C. The mean standardized estimates of $e(\boldsymbol{\beta})$ were surprisingly similar for LS (0.12, 95% CIs = 0.09, 0.16), DRE (0.13, 95% CIs = 0.11, 0.16) and LRE (0.12, 95% CIs = 0.09, 0.15). The average evolvability for the entire \mathbf{G} matrix is over 3 times lower than any individual estimates $e(\boldsymbol{\beta})$ (\bar{e} = 0.034, 95% CIs = 0.032, 0.036). This suggests that there is ample genetic variation available in the direction of the optimal regulated intake of nutrients for each trait.

5.4.3. Experiment 3: The effect of mating status on the regulated intake of nutrients by males and females under dietary choice

A MANOVA revealed a significant multivariate effect of mating status, sex and diet pair, as well as a significant interaction between sex and diet pair on the intake of nutrients (Table 5.5). Univariate ANOVAs revealed that P intake contributed to the observed difference between the sexes, the intake of both nutrients contributed to the observed differences across mating status and diet pair, whereas it was the intake of C that contributed to the observed interaction between sex and diet pair (Table 5.5). Given the significant sex by diet pair interaction, I examined the effect of mating status and diet pair (and their interaction) within each sex in separate MANOVA models (Table 5.6). In females, there was a significant multivariate effect of mating status and diet pair on the intake of nutrients but no significant interaction between mating status and diet pair (Table 5.6). The significant multivariate effect of mating status is driven by differences in the intake of both P and C (Table 5.6), with mated females on average having a higher intake of both nutrients than virgin females (Figure 5.4). The significant multivariate effect of diet pair was also driven by differences in the intake of both nutrients (Table 5.6). In mated females, the pattern of P intake was diet pair $2 < 1 < 4 < 3$ and C intake was diet pair $3 < 1 = 4 = 2$ (Figure 5.3A), whereas in virgin females the pattern of P intake was diet pair $2 = 1 < 4 < 3$ and C intake was diet pair $3 < 4 = 1 = 2$ (Figure 5.3C). In males, there was a significant multivariate effect of diet pair on the intake of nutrients but no significant effect of mating status or the interaction between mating status and diet pair (Table 5.6, Figure 5.3B & D). The significant multivariate effect of diet pair on nutrient intake was driven by the intake of both P and C (Table 5.6). In mated males, the pattern of P intake was diet pair $2 = 1 < 4 < 3$ and C intake was diet pair $3 < 1 < 2 = 4$ (Figure 5.3B), whereas in virgin males the pattern of P intake was diet pair $1 = 2 < 4 < 3$ and C intake was diet pair $3 = 1 < 2 = 4$ (Figure 5.3D).

The regulated intake point (RIP) was at a mean P intake of $262.69 \pm 7.33\text{mg}$ and C intake of $577.10 \pm 11.54\text{mg}$ for mated females (P:C ratio = 1:2.20), a mean P intake of $238.28 \pm 5.02\text{mg}$ and C intake of $538.68 \pm 10.22\text{mg}$ for virgin females (P:C ratio = 1:2.26), a mean P intake of $179.22 \pm 5.02\text{mg}$ and C intake of $578.57 \pm 12.51\text{mg}$ for mated males (P:C ratio = 1:3.23) and a mean P intake of $176.11 \pm 4.80\text{mg}$ and C intake of $561.78 \pm 13.60\text{mg}$ for virgin males (P:C ratio = 1:3.19). The RIP for these groupings can be visualized in Figure 5.4. ANCOVA revealed that the RIP was more P biased for mated than virgin females (mating

status: $F_{1,236} = 2.47$, $P = 0.12$, P intake: $F_{1,236} = 17.17$, $P = 0.0001$, mating status x P intake: $F_{1,236} = 4.80$, $P = 0.029$) but mating status had little effect on the RIP of males (mating status: $F_{1,236} = 0.08$, $P = 0.78$, P intake: $F_{1,236} = 4.56$, $P = 0.034$, mating status x P intake: $F_{1,236} = 0.32$, $P = 0.58$)(Figure 5.4). The largest difference in RIP, however, was between the sexes with females choosing to consume a more P biased diet than males, both when the sexes were mated (sex: $F_{1,236} = 4.08$, $P = 0.04$, P intake: $F_{1,236} = 13.96$, $P = 0.0001$, mating status x P intake: $F_{1,236} = 4.96$, $P = 0.027$) and when they were virgin (sex: $F_{1,236} = 3.97$, $P = 0.05$, P intake: $F_{1,236} = 12.38$, $P = 0.0001$, mating status x P intake: $F_{1,236} = 4.18$, $P = 0.04$)(Figure 5.4). Mapping the RIP for mated crickets onto the nutritional landscapes presented in Figure 5.1 showed that the RIP did not coincide exactly on the peaks in LS, DRE or LRE suggesting that neither males nor females are optimally regulating their intake of P and C when given dietary choice.

5.5. DISCUSSION

Phenotypic traits that are expressed in both males and females are likely to have a shared genetic basis that prevents the independent evolution of these traits. If selection acting on these shared traits differs between the sexes then ISC will be generated and could prevent either sex from reaching their sex specific phenotypic optima (Lande, 1980). In this study I have empirically shown that the sexes in *T. commodus* show significant differences in the effects of P and C intake on LS, DRE and LRE and that there is a strong positive genetic covariance between the sexes for the regulated intake of these nutrients when given dietary choice. Together, this provides the fundamental requirements necessary for ISC to operate over the optimal intake of nutrients for LS, DRE and LRE in *T. commodus* (Bonduriansky & Chenoweth, 2009). My work, however, cautions against using these requirements as definitive evidence that ISC exists and plays an important role in the evolution of sex differences in shared phenotypic traits. Indeed, despite possessing the characteristic hallmarks of ISC, I show that **B** had very little effect on the predicted response of nutrient regulation in the sexes, with this shared trait in males and females being more constrained by the structure of \mathbf{G}_m and \mathbf{G}_f , respectively. Furthermore, although neither sex optimally regulated their intake of P and C when given dietary choice, males and females exhibit a clear dimorphism in nutrient regulation irrespective of whether they are virgin or mated.

Therefore, contrary to the earlier work of Maklakov *et al.* (2008) supporting a key role for ISC over the optimal intake of nutrients for LS, DRE and LRE in *T. commodus*, my work suggests that this process is likely to be weak.

My study found that P and C intake had a clear effect on LS, DRE and LRE in both males and females. For males, LS was maximised at a high intake of nutrients with a P:C ratio of approximately 1:2.5 (Figure 5.2B), while for females the same high intake of nutrients was found but on a P:C ratio of approximately 1:8 (Figure 5.2A). These findings strongly resemble earlier work using the GF on *T. commodus* by Maklakov *et al.* (2008) who found that male LS was maximised at a P:C ratio of 1:5 while female lifespan was maximised at a more C biased P:C ratio of 1:8. Indeed, my finding of high C biased diets maximising LS is consistent with a number of DR studies on *D.melanogaster* which found that LS extension under CR was due to a restricted intake of P relative to C and not calories *per se* (Mair *et al.*, 2005; Skorupa, 2008; Tatar, 2011; Nakagawa *et al.*, 2012; Bruce *et al.*, 2013) and a number of GF studies across a range of species all reporting C biased diets increasing LS. Two GF studies on *D.melanogaster* found that LS was maximised for both sexes at a high intake of nutrients at a P:C ratio of approximately 1:16 (Lee *et al.*, 2008; Jensen *et al.*, 2015) while GF studies on female Queensland fruit flies also showed that LS was maximised on a C biased diet with a P:C ratio of 1:21 or 1:32 depending on whether a yeast-based or holidic diet (respectively) were used (Fanson *et al.*, 2009; Fanson & Taylor, 2012). In mice, median LS was reported to increase with low-P, high-C diets, with LS maximised at a P:C ratio of approximately 1:13 (Solon-Biet *et al.*, 2014). In female mealworm beetles *Tenebrio molitor*, the GF revealed that female LS was maximised on a P:C ratio of 1:5 although there was not a significant difference in mean female LS on a balanced P:C ratio of 1:1 and male LS was also found to be maximised on a balanced P:C ratio of 1:1 (Rho & Lee, 2016). While this study does not seem to conform to the findings of other GF studies, Rho & Lee (2016) were unable to measure male reproductive effort and so we cannot be sure how LS is trading-off with other life-history traits. Interestingly, when given a choice male *T.molitor* were actively regulating their intake to a slightly C-biased diet with a P:C ratio of approximately 1:1.54-1:1.64 (Rho & Lee, 2016)

In contrast to LS, there was a much stronger divergence in the effects of P and C intake on both DRE and LRE between the sexes. For males, DRE and LRE were both maximised on a P:C ratio of 1:8 (Figure 5.2D & F), whereas in females, DRE was maximised

on a P:C ratio of 1:1 (Figure 5.2C); and LRE on 1:1.5 (Figure 5.2E). These differences in optimal reproductive nutritional requirements are reflective of the divergent reproductive strategies of the sexes. In most species, males are under more intense sexual selection than females because males typically contribute far less to each offspring than females (Bonduriansky *et al.*, 2008). Consequently, males often face intense competition for access to mates and those with the most elaborate sexual trait or behaviour will be most successful (Bonduriansky *et al.*, 2008). In *T. commodus* a key determinant in male mating success is an advertisement call (Bentsen *et al.*, 2006; Hunt *et al.*, 2006; Jacot *et al.*, 2008; Rodriguez-Munoz *et al.*, 2010) which has been shown to be metabolically costly to produce (Kavanagh, 1987). To fuel this energetically expensive trait, males require a high intake of C to provide an abundant source of energy that can be easily and rapidly accessed after digestion. In contrast, females typically do not have to compete for matings, their reproductive success being determined by the number of eggs they produce rather than the number of matings they achieve (Bonduriansky *et al.*, 2008). In many insect species, egg production is closely linked to nutrition with P playing an important role in stimulating oogenesis and regulating vitellogenesis (Wheeler, 1996). It is therefore unsurprising that females in a range of species require a higher intake of P than males to maximise their reproductive success (Lee *et al.*, 2008; Maklakov *et al.*, 2008; Fanson *et al.*, 2009; Fanson & Taylor, 2012; Reddiex *et al.*, 2013; Jensen *et al.*, 2015).

This clear divergence in the effect of P and C on LS and RE in the sexes suggests that fitness would be maximised by the sexes regulating their intake of P and C independently. A number of studies using the GF have found similar sexual divergence in nutritional optima but failed to find any divergence in the feeding trajectories of the sexes under dietary choice (Lee *et al.*, 2008; Maklakov *et al.*, 2008; Fanson *et al.*, 2009; Jensen *et al.*, 2015). Maklakov *et al.* (2008) and Jensen *et al.* (2015) argued that this common pattern of dietary choice could be preventing the sexes from reaching their sex-specific nutritional optima and could signal the presence of ISC. However, a major limitation to both of these studies is the absence of any genetic parameters, without which it is impossible to definitively determine the nature of any genetic variation over dietary preference or get a definitive measure of any potential intersexual genetic correlation to prove the presence of ISC over optimal nutrient intake (Bonduriansky & Chenoweth, 2009). Only one study has so far used the GF to look at the key genetic parameters for ISC and despite showing sex-specific nutritional optima for

reproduction in *D.melanogaster* and significant genetic (co)variance between the sexes for P and C intake, concluded that there was little evidence for the presence of ISC over nutrient optimization for reproduction (Reddiex *et al.*, 2013). However, there are a number of problems with this study which perhaps make this an unsurprising result. Firstly, nutrient intake was only measured over a short time period (4 days) which is likely to explain the minor differences in the nutritional landscapes between the sexes in this study and an intersexual genetic correlation for P intake that did not differ statistically from zero (Reddiex *et al.*, 2013). Reddiex *et al.* (2013) also use inbred lines (DGRP *Drosophila* lines) in their study which could distort the findings of their genetic analysis and additionally, only investigate the genetics of dietary choice using one diet pair. The use of a single dietary pair in a choice feeding experiment within the context of the GF, does not represent the true RIP of the complete nutritional landscape, therefore, the use of one diet pair limits the findings of any investigation into the genetics of optimal dietary regulation.

In contrast to the study by Reddiex *et al.* (2013), my study has a number of differences, firstly my feeding experiments were conducted over a much longer time-frame (20 days/21 days vs 4 days). I used a genetically diverse outbred population of crickets in a split-brood half-sib breeding design rather than using inbred lines and I used four diet pairs to measure the genetics of the RIP rather than one diet pair. A result of these differences is that my results contrast sharply with those reported by Reddiex *et al.* (2013), for example, I found an intersexual genetic correlation of 0.87 ± 0.16 for male vs female C intake compared to 0.95 in Reddiex *et al.* (2013) and an intersexual genetic correlation of 0.99 ± 0.07 for male vs female P intake compared to 0.28 in Reddiex *et al.* (2013). While my measure of intersexual genetic correlation for C-intake is comparable to that found by Reddiex *et al.* (2013) my measure of genetic correlation for P-intake is much higher, with both measures suggesting that a change in the regulated intake of nutrients in one sex should result in an almost equal response in the other sex. Interestingly, while both P and C intake are heritable in the sexes, my heritability estimates for P intake (males: 0.31 ± 0.06 ; females: 0.28 ± 0.06) were greater than for C intake (males: 0.14 ± 0.04 ; females: 0.11 ± 0.04) which suggests that the intake of P is under stronger genetic control in *T. commodus*. This stronger genetic control may reflect the fact that above a certain intake, P has a detrimental effect on LS and reproduction in both sexes: an effect which has been termed the lethal P hypothesis (Simpson & Raubenheimer, 2009; Fanson & Taylor, 2012). The most

likely causes of this detrimental effect of high P intake are the elevated production of toxic nitrogenous wastes (Singer, 2003) or the increase of mitochondrial reactive oxygen species which have been shown to increase with P consumption (Sanz *et al.*, 2004; Ayala *et al.*, 2007). Furthermore, a recent study on mice has suggested that the reduction in LS and cardio-metabolic health observed in mice consuming diets with a high P:C ratio results from a high expression of mTOR (Solon-Biet *et al.*, 2014). The nutritional landscapes for *T. commodus* (Figure 5.1) clearly highlights that LS and reproduction declines rapidly with increasing P intake in both sexes, however, more work is needed to understand the proximate reason(s) for this decline.

By combining my estimates of the effects of P and C intake on LS, DRE and LRE in the sexes and my genetic estimates of the sign and strength of the covariance across the sexes (**B**) in the regulated intake of these nutrients, I was able to predict the evolutionary response of nutrient intake in males and females and characterise the magnitude of the ISC over the optimal intake of nutrients. Specifically, I compared the predicted response of the regulated intake of nutrients ($\Delta\bar{z}$) when **B** was estimated directly from the breeding design to when **B** was set to zero ($\Delta\bar{z}_{\mathbf{B}=0}$) to reflect a scenario where there is no genetic constraint and the sexes are free to evolve independently. The ratio of $\Delta\bar{z}$ to $\Delta\bar{z}_{\mathbf{B}=0}$ (defined as **R**, (Agrawal & Stinchcombe, 2009) therefore provides a measure of the degree to which **B** is predicted to constrain the evolution of sexual dimorphism through ISC. I show that most values of **R** for LS, DRE and LRE in the sexes did not differ significantly from a value of 1.0 meaning that **B** did little to constrain the evolution on nutrient regulation in the sexes. Notable exceptions to this were the regulated intake of P and C for DRE and the regulated intake of C for LRE in males and the regulated intake of P for LS in females (Table 5.4) that had **R** values exceeding 2.0 (and deviating significantly from **R** = 1.0) indicating that the predicted response of nutrient intake due to **B** will be over twice as fast as expected under genetic independence. Interestingly, the unconditional evolvability ($e(\beta)$) was over 3 times higher for each trait than the average evolvability (\bar{e}) of the entire **G** matrix. This demonstrates that there is ample genetic variance available in the direction of the optimal regulated intake of P and C for nutrient regulation to evolve to the optima for each trait, which Figure 5.1 shows is clearly not occurring. A similar result was also found by Reddiex *et al.* (2013) over the optimal intake of P and C for reproduction in *D.melanogaster* and raises the obvious question: if the covariance structure of **B** is not constraining the independent

evolution of sexes and there is ample genetic variance in the direction of optimal nutrient regulation, why is ISC not stronger? In the case of *T. commodus*, it is clear that the extent of divergence in the nutritional optima of the sexes is a major limit to the strength of ISC. Despite showing significant divergence in the nutritional optima, it is important to note that the maximum angle (θ) between linear nutritional vectors in the sexes was only 55° for DRE and that θ was less than half this for LS (25.99°) and LRE (33.06°) (Table 5.2). By comparison, Lewis *et al.* (2011) showed strong ISC over life-history traits (development time, body size, and LS) in the moth *Plodia interpunctella* that was driven by a much larger θ of 127.91° between the linear selection gradients in the sexes. This illustrates that although strong positive genetic correlations between shared traits in the sexes provides the stage for ISC, selection (or in the case of my study, nutritional effects) must be sufficiently divergent for this ISC to operate.

In comparison to the relatively negligible effects of \mathbf{B} on ISC, constraints imposed by the structure of \mathbf{G}_f and \mathbf{G}_m appeared to play a more important role on the predicted evolution of nutrient regulation. With the exception of DRE in females, where the vector of linear nutritional effects and the vector of predicted response of nutrient regulation were well aligned (characterised by a small θ), all other traits showed poor alignment between these vectors indicative of a genetic constraint. The role of \mathbf{G}_f and \mathbf{G}_m in this process can be visualized in Figure 5.2 which shows that the major eigenvector of these matrices do not align well with linear effects of P and C in the nutritional landscapes (again with the exception of DRE in females). This poor alignment between the vector of linear nutritional effects and the predicted response of nutrient regulation should not only prevent the sexes from reaching their nutritional optima but should also hinder the evolution of sexual divergence in nutrient regulation strategies. In agreement with this first prediction, Figure 5.1 shows that neither sex optimally regulates its intake of P and C for LS, DRE or LRE when given dietary choice. However, the sexes have clearly evolved divergence in their RIP and this sexual divergence was irrespective of mating status. This raises the obvious question: just how effective are genetic constraints at biasing phenotypic? In a seminal paper, Schluter (1996) showed that morphological evolutionary divergence across vertebrate species was biased in the direction of the major eigenvector of \mathbf{G} (known as \mathbf{g}_{\max}). However, studies in both animals (e.g. Colman *et al.*, 2009) and plants (e.g. Souroukis *et al.*, 1992)

have shown that phenotypic evolution across the direction of genetic constraint can be readily achieved in a few generation of artificial selection. Thus, the debate about the relative importance of genetic constraints to phenotypic evolution is still very much ongoing.

Despite the strong genetic correlations for the regulated intake of P and C across the sexes, I show clear evidence that males and females regulate their P and C intake independently and had clearly divergent RIPs. This is in contrast to previous GF work on *T. commodus* (Maklakov *et al.*, 2008), two *Drosophila* species (Reddiex *et al.*, 2013; Jensen *et al.*, 2015) and the cockroach *Nauphoeta cinerea* (Bunning *et al.*, 2016) that also failed to find divergence in the nutrient regulation of the sexes. These previous studies argue that this shared dietary choice is evidence that ISC is preventing the sexes from reaching their sex-specific nutritional optima. My finding that current ISC is weak and the sexes show divergent nutrient regulation, therefore, suggests that ISC may be resolved in *T. commodus* (or at least in the initial stages of resolution). Given sufficient evolutionary time, selection is expected to resolve ISC (Bonduriansky & Chenoweth, 2009) and various mechanisms are known to facilitate this process including sex-specific expression of autosomal loci via sex linked modifiers (Long *et al.*, 1995; Foley *et al.*, 2007), alternative splicing mechanisms (McIntyre *et al.*, 2006), gene duplication (Partridge & Hurst, 1998; Rice & Chippindale, 2001; Rice & Chippindale, 2002), sex biased gene expression (Ellegren & Parsch, 2007) and sex linkage (Rice, 1984; Rice & Chippindale, 2002). Genomic imprinting (Day & Bonduriansky, 2004), condition dependence (Bonduriansky *et al.*, 2005; Bonduriansky, 2007) and maternal effects (Foerster *et al.*, 2007) may also contribute to a resolution of ISC but empirical support for these mechanisms is still largely lacking (Bonduriansky & Chenoweth, 2009; Lewis *et al.*, 2011). Whether ISC has indeed been resolved, and the proximate mechanisms that may be responsible, require further study in *T. commodus* and it is likely that genomic investigation will prove most profitable.

I speculated that one possible reason for the lack of sex difference in the intake of P and C shown in Maklakov *et al.* (2008) for *T. commodus* was because virgin crickets were used. I found little evidence to support this hypothesis, with clear sex differences in the RIP of the sexes independent of mating status (Figure 5.4). I did find, however, that mating status significantly influenced the RIP for P and C in *T. commodus* but this effect was sex-specific. In females, mated crickets consumed more P and C in total and also had a more P

biased RIP relative to virgin females, whereas mated and virgin males showed little differences in both the total intake of nutrients or the P:C ratio of the RIP (Figure 5.4). It is likely that these sex differences in the effects of mating status on nutrient regulation reflect the divergence in the nutritional demands of reproduction. Mated female *T. commodus* have been shown to lay, on average, 6 to 8 more eggs per unit time than virgin females (Loher & Edson, 1973) a pattern that has also been demonstrated in other field cricket species (e.g. *Acheta domestica* (Murtaugh & Denlinger, 1985; Clifford & Woodring, 1986); *Teleogryllus emma* (Zhao & Zhu, 2011)). Furthermore, males transfer prostaglandin synthesising complex (Avila *et al.*, 2011) in their ejaculate to females at mating which has been shown to increase oviposition rate (Loher *et al.*, 1981). This higher production of eggs with mating is likely to increase the demand for energy (i.e. higher intake of both nutrients) and P in females relative to males (Mair *et al.*, 2005). In contrast, even though male calling effort is metabolically costly in *T. commodus* (Kavanagh, 1987) and has been shown to be an important determinant of male mating success in this species (Bentsen *et al.*, 2006; Hunt *et al.*, 2006; Jacot *et al.*, 2008; Rodriguez-Munoz *et al.*, 2010), there is conflicting evidence which suggests that virgin males call for longer than mated males. In their study on *T.commodus*, Zajitschek *et al.* (2012) found that virgin males had a shorter LS than mated males because virgins males invested more in their reproductive effort (time spent calling). However, while mated males did have a slightly higher nutrient intake (mean P intake of $179.22 \pm 5.02\text{mg}$ and C intake of $578.57 \pm 12.51\text{mg}$) than virgin males (mean P intake of $176.11 \pm 4.80\text{mg}$ and C intake of $561.78 \pm 13.60\text{mg}$), there was no significant difference between these nutrient intakes suggesting that the disparity in calling effort between mated and virgin males is not expected to be nutritionally severe and mated males may be investing in other reproductive traits for example, sperm production, as seen in *N.cinerea* (Bunning *et al.*, 2015). A study on sagebrush crickets (*Cyphoderris strepitans*) also found that recently mated males called less than virgin males (Sakaluk & Snedden, 1990). In this species, however, females feed on the fleshy hind wings of the male during mating and this is likely to deplete energy reserves that could be used for calling (Sakaluk & Snedden, 1990). Given that male *T. commodus* do not use nuptial feeding or present females with a nuptial gift during copulation, the disparity in calling effort between mated and virgin males is again not expected to be as severe as shown in *C. strepitans*.

In conclusion, my study has provided evidence that males and females have divergent nutritional optima for LS, DRE and LRE and strong positive additive genetic correlations for the regulated intake of P and C across the sexes. This alone is often taken as strong evidence for ISC over the optimal intake of nutrients. I show, however, that the strong positive genetic covariance between the sexes (defined by \mathbf{B}) has little effect on the predicted response of nutrient regulation in the sexes: if anything, the structure of \mathbf{B} accelerated rather than constrained the predicted response of nutrient regulation in the sexes. The structure of additive genetic variance-covariance matrix for the regulated intake of nutrients within males and females (\mathbf{G}_m and \mathbf{G}_f , respectively) appeared to play a more important role in constraining the predicted response of nutrient regulation in the sexes. With the exception of DRE in females, the direction of nutritional effects and the predicted response of nutrient regulation were poorly aligned and this was characterised by large θ between these vectors. Finally, even though the sexes have evolved independent patterns of nutrient regulation and this divergence was unaffected by mating status, the RIP of neither sex coincided with the peak of the nutritional optima for LS, DRE or LRE suggesting that nutrient regulation was not optimal. The finding of weak ISC over the optimal intake of nutrients combined with divergent nutrient regulation in the sexes suggests that ISC may be resolved (or in the initial steps of resolution) in *T. commodus*, but more work is needed to test this fully.

Table 5.1. Linear and nonlinear effects of protein (P) and carbohydrates (C) on lifespan (LS), daily reproductive effort (DRE) and lifetime reproductive effort (LRE) in the sexes. DRE and LRE were measured as calling effort and egg production in males and females, respectively.

| Response variables | Linear effects | | Nonlinear effects | | |
|--------------------|----------------|-------------|-------------------|--------------|--------------|
| | P | C | P x P | C x C | P x C |
| (A) Females | | | | | |
| LS | | | | | |
| Coefficient ± SE | -0.06 ± 0.06 | 0.60 ± 0.06 | -0.08 ± 0.05 | -0.32 ± 0.05 | -0.33 ± 0.08 |
| <i>t</i> | 1.10 | 10.46 | 1.53 | 5.99 | 4.13 |
| <i>df</i> | 219 | 219 | 216 | 216 | 216 |
| <i>P</i> | 0.27 | 0.0001 | 0.13 | 0.0001 | 0.0001 |
| DRE | | | | | |
| Coefficient ± SE | 0.52 ± 0.06 | 0.53 ± 0.06 | -0.35 ± 0.05 | -0.09 ± 0.05 | 0.21 ± 0.08 |
| <i>t</i> | 8.58 | 8.59 | 6.48 | 1.67 | 2.58 |
| <i>df</i> | 219 | 219 | 216 | 216 | 216 |
| <i>P</i> | 0.0001 | 0.0001 | 0.0001 | 0.09 | 0.011 |
| LRE | | | | | |
| Coefficient ± SE | 0.35 ± 0.06 | 0.70 ± 0.06 | -0.33 ± 0.05 | -0.18 ± 0.05 | 0.06 ± 0.08 |
| <i>t</i> | 6.09 | 12.29 | 6.41 | 3.49 | 0.75 |
| <i>df</i> | 219 | 219 | 216 | 216 | 216 |
| <i>P</i> | 0.0001 | 0.0001 | 0.0001 | 0.001 | 0.45 |
| (B) Males | | | | | |
| LS | | | | | |
| Coefficient ± SE | 0.28 ± 0.06 | 0.76 ± 0.06 | -0.25 ± 0.03 | -0.22 ± 0.04 | -0.19 ± 0.06 |
| <i>t</i> | 4.89 | 13.35 | 7.44 | 5.37 | 3.03 |
| <i>df</i> | 205 | 205 | 202 | 202 | 202 |
| <i>P</i> | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.003 |
| DRE | | | | | |
| Coefficient ± SE | -0.15 ± 0.04 | 0.80 ± 0.04 | -0.01 ± 0.02 | -0.11 ± 0.03 | -0.25 ± 0.05 |
| <i>t</i> | 3.80 | 20.84 | 0.31 | 3.78 | 4.60 |
| <i>df</i> | 205 | 205 | 202 | 202 | 202 |
| <i>P</i> | 0.0001 | 0.0001 | 0.76 | 0.0001 | 0.0001 |
| LRE | | | | | |
| Coefficient ± SE | -0.09 ± 0.04 | 0.79 ± 0.04 | -0.04 ± 0.03 | -0.10 ± 0.04 | -0.22 ± 0.05 |
| <i>t</i> | 2.17 | 18.42 | 1.33 | 2.93 | 4.32 |
| <i>df</i> | 205 | 205 | 202 | 202 | 202 |
| <i>P</i> | 0.03 | 0.0001 | 0.18 | 0.004 | 0.001 |

Table 5.2. Sequential model results for differences between the linear and nonlinear effects of protein (P) and carbohydrate (C) ingestion LS, DRE and LRE between and within the sexes and the angle (θ) with 95% CI between the linear vectors for the variable being compared.

| | SS_R | SS_C | DF_1 | DF_2 | F | P | θ | 95% CI |
|--------------------------|--------|--------|--------|--------|--------|---------------------|----------|----------------|
| Females vs. Males | | | | | | | | |
| <i>LS</i> | | | | | | | | |
| Linear | 253.60 | 243.44 | 2 | 424 | 8.85 | 0.0002 ^A | 25.99° | 13.11°, 39.39° |
| Quadratic | 208.83 | 201.79 | 2 | 420 | 7.33 | 0.0007 ^B | | |
| Correlational | 190.38 | 189.46 | 1 | 418 | 2.03 | 0.15 | | |
| <i>DRE</i> | | | | | | | | |
| Linear | 272.22 | 199.81 | 2 | 424 | 76.83 | 0.0001 ^C | 55.19° | 45.93°, 64.40° |
| Quadratic | 187.97 | 168.05 | 2 | 420 | 24.89 | 0.0001 ^D | | |
| Correlational | 167.64 | 158.03 | 1 | 418 | 25.41 | 0.0001 | | |
| <i>LRE</i> | | | | | | | | |
| Linear | 218.14 | 192.99 | 2 | 424 | 27.63 | 0.0001 ^E | 33.06° | 24.05°, 42.42° |
| Quadratic | 177.74 | 165.68 | 2 | 420 | 15.29 | 0.0001 ^F | | |
| Correlational | 163.86 | 160.27 | 1 | 418 | 9.35 | 0.002 | | |
| Females | | | | | | | | |
| <i>LS vs. DRE</i> | | | | | | | | |
| Linear | 325.66 | 282.74 | 2 | 438 | 118.45 | 0.0001 ^G | 51.18° | 38.14°, 64.93° |
| Quadratic | 256.64 | 239.89 | 2 | 434 | 15.15 | 0.0001 ^H | | |
| Correlational | 239.33 | 227.54 | 1 | 432 | 22.37 | 0.0001 | | |
| <i>LS vs. LRE</i> | | | | | | | | |
| Linear | 279.34 | 263.34 | 2 | 438 | 13.31 | 0.0001 ^I | 32.50° | 19.70°, 45.66° |
| Quadratic | 235.88 | 224.64 | 2 | 434 | 20.52 | 0.0001 ^J | | |
| Correlational | 221.67 | 215.61 | 1 | 432 | 12.15 | 0.0005 | | |
| <i>DRE vs. LRE</i> | | | | | | | | |
| Linear | 289.78 | 280.07 | 2 | 438 | 7.59 | 0.0006 ^K | 18.66° | 9.40°, 28.65° |
| Quadratic | 225.82 | 225.09 | 2 | 434 | 0.70 | 0.50 | | |
| Correlational | 222.17 | 221.23 | 1 | 432 | 1.84 | 0.18 | | |
| Males | | | | | | | | |
| <i>LS vs. DRE</i> | | | | | | | | |
| Linear | 180.69 | 160.51 | 2 | 410 | 25.77 | 0.0001 ^L | 30.48° | 21.65°, 39.40° |
| Quadratic | 142.37 | 129.95 | 2 | 406 | 19.40 | 0.0001 ^M | | |
| Correlational | 120.15 | 119.94 | 1 | 404 | 0.71 | 0.40 | | |
| <i>LS vs. LRE</i> | | | | | | | | |
| Linear | 188.49 | 173.09 | 2 | 410 | 18.24 | 0.0001 ^N | 26.71° | 17.39°, 35.97° |
| Quadratic | 151.81 | 142.83 | 2 | 406 | 12.76 | 0.0001 ^O | | |
| Correlational | 134.19 | 134.12 | 1 | 404 | 0.21 | 0.65 | | |
| <i>DRE vs. LRE</i> | | | | | | | | |
| Linear | 113.06 | 112.73 | 2 | 410 | 0.60 | 0.55 | 4.03° | 0.00°, 10.69° |
| Quadratic | 109.02 | 108.63 | 2 | 406 | 0.72 | 0.48 | | |
| Correlational | 97.11 | 97.07 | 1 | 404 | 0.17 | 0.68 | | |

Univariate test: ^A P: $F_{1,424} = 17.68$, $P = 0.0001$, C: $F_{1,424} = 3.98$, $P = 0.047$; ^B P x P: $F_{1,420} = 12.39$, $P = 0.0005$, C x C: $F_{1,420} = 0.87$, $P = 0.35$; ^C P: $F_{1,424} = 83.05$, $P = 0.0001$, C: $F_{1,424} = 13.73$, $P = 0.0002$; ^D P x P: $F_{1,420} = 49.58$, $P = 0.0001$, C x C: $F_{1,420} = 0.43$, $P = 0.51$; ^E P: $F_{1,424} = 37.17$, $P = 0.0001$, C: $F_{1,424} = 1.53$, $P = 0.22$; ^F P x P: $F_{1,420} = 29.59$,

$P = 0.0001$, C x C: $F_{1,420} = 3.37$, $P = 0.07$; ^G P: $F_{1,438} = 48.92$, $P = 0.0001$, C: $F_{1,438} = 0.85$, $P = 0.36$; ^H P x P: $F_{1,434} = 22.66$, $P = 0.0001$, C x C: $F_{1,434} = 3.99$, $P = 0.046$; ^I P: $F_{1,438} = 25.67$, $P = 0.0001$, C: $F_{1,438} = 1.48$, $P = 0.22$; ^J P x P: $F_{1,434} = 18.26$, $P = 0.0001$, C x C: $F_{1,434} = 0.89$, $P = 0.35$; ^K P: $F_{1,438} = 4.47$, $P = 0.035$, C: $F_{1,438} = 4.43$, $P = 0.036$; ^L P: $F_{1,410} = 38.16$, $P = 0.0001$, C: $F_{1,410} = 0.33$, $P = 0.57$; ^M P x P: $F_{1,406} = 36.17$, $P = 0.0001$, C x C: $F_{1,406} = 4.62$, $P = 0.03$; ^N P: $F_{1,410} = 27.15$, $P = 0.0001$, C: $F_{1,410} = 0.21$, $P = 0.65$; ^O P x P: $F_{1,406} = 22.28$, $P = 0.0001$, C x C: $F_{1,406} = 4.91$, $P = 0.03$.

Table 5.3. Additive genetic variance-covariance (**G**) for protein (P) and carbohydrate (C) intake in male and female *T. commodus*. The subscripts *m* and *f* refer to males and females, respectively. h^2 refers to heritability estimates and standard errors in brackets (SE). The additive genetic (co)variance within males and females is along the diagonal and the additive genetic covariance between the sexes is on the lower off-diagonal. Genetic correlations (r_A) are provided in bold above off-diagonal, with the SE in brackets. Estimates of h^2 and r_A in italics are statistically significant at $P < 0.05$.

| | h^2 | P_m | C_m | P_f | C_f |
|-------|--------------------|-------|---------------------------|---------------------------|---------------------------|
| P_m | <i>0.31 (0.06)</i> | 9.92 | <i>0.94 (0.20)</i> | <i>0.99 (0.07)</i> | <i>0.59 (0.16)</i> |
| C_m | <i>0.14 (0.04)</i> | 5.62 | 8.18 | <i>0.95 (0.14)</i> | <i>0.87 (0.16)</i> |
| P_f | <i>0.28 (0.06)</i> | 8.95 | 8.06 | 9.08 | <i>0.68 (0.19)</i> |
| C_f | <i>0.11 (0.04)</i> | 4.65 | 6.38 | 3.84 | 6.28 |

Table 5.4. The predicted response of protein (P) and carbohydrate (C) intake in the sexes when the additive genetic covariance matrix between the sexes (**B**) is estimated directly from the breeding design ($\Delta\bar{z}$) versus when it is set to zero ($\Delta\bar{z}_{B=0}$) and the corresponding **R** constraint metric of Agrawal & Stinchcombe (2009). The 95% CIs for $\Delta\bar{z}$, $\Delta\bar{z}_{B=0}$ and **R** are provided in brackets beneath the estimates.

| | Males | | | Females | | |
|------------|-----------------------|-----------------------|----------------------|----------------------|-----------------------|----------------------|
| | $\Delta\bar{z}$ | $\Delta\bar{z}_{B=0}$ | R | $\Delta\bar{z}$ | $\Delta\bar{z}_{B=0}$ | R |
| LS | | | | | | |
| P | 6.50 (3.84, 8.78) | 5.75 (4.15, 8.15) | 1.10 (0.59, 1.68) | 6.33 (4.22, 8.50) | 2.71 (1.15, 0.84) | 2.31 (1.01, 4.58) |
| C | 6.46 (4.45, 9.36) | 4.54 (2.97, 6.35) | 1.41 (0.78, 2.30) | 4.59 (2.02, 6.56) | 2.14 (1.59, 2.37) | 2.10 (0.72, 4.36) |
| DRE | | | | | | |
| P | 6.98 (4.60, 8.93) | 3.11 (2.13, 4.55) | 2.19 (1.15, 3.42) | 7.06 (5.18, 9.19) | 6.85 (4.61, 8.74) | 1.05 (0.61, 1.55) |
| C | 7.07 (5.08, 9.68) | 2.50 (1.49, 3.55) | 2.84 (1.52, 4.45) | 5.05 (3.20, 7.45) | 6.25 (4.10, 8.07) | 0.81 (0.42, 1.35) |
| LRE | | | | | | |
| P | 6.82 (4.29, 9.07) | 3.51 (2.03, 4.78) | 1.95 (0.97, 3.09) | 7.03 (4.94, 9.51) | 6.40 (4.42, 8.57) | 1.11 (0.59, 1.74) |
| C | 6.99 (5.03, 10.21) | 2.79 (1.69, 4.04) | 2.53 (1.29, 4.27) | 5.27 (2.89, 8.07) | 4.79 (3.26, 6.96) | 1.08 (0.51, 1.97) |

Table 5.5. Multivariate Analysis of Variance (MANOVA) examining the effects of sex, mating status (virgin or mated) and diet pair on the total intake of protein (P) and carbohydrates (C) in *Teleogryllus commodus*. This overall multivariate model was followed by a series of ANOVAs to determine which nutrients contributed to any overall multivariate effects.

| | MANOVA | | | |
|--------------------|--------------------------|-----------|----------|----------|
| Model terms | Pillai's Trace | <i>df</i> | <i>F</i> | <i>P</i> |
| Sex (A) | 0.42 | 2,463 | 164.12 | 0.0001 |
| Mating status (B) | 0.02 | 2,463 | 5.207 | 0.006 |
| Diet pair (C) | 0.60 | 6,928 | 66.01 | 0.0001 |
| A x B | 0.01 | 2,463 | 2.63 | 0.073 |
| A x C | 0.05 | 6,928 | 3.66 | 0.001 |
| B x C | 0.00 | 6,928 | 0.25 | 0.96 |
| A x B x C | 0.01 | 6,928 | 0.55 | 0.77 |
| | Univariate ANOVAs | | | |
| Model terms | Nutrient | <i>df</i> | <i>F</i> | <i>P</i> |
| Sex (A) | P | 1,464 | 246.32 | 0.0001 |
| | C | 1,464 | 1.21 | 0.27 |
| Mating status (B) | P | 1,464 | 8.79 | 0.003 |
| | C | 1,464 | 6.12 | 0.014 |
| Diet pair (C) | P | 3,464 | 103.88 | 0.0001 |
| | C | 3,464 | 22.19 | 0.0001 |
| A x B | P | 1,464 | 5.27 | 0.02 |
| | C | 1,464 | 0.94 | 0.33 |
| A x C | P | 3,464 | 0.95 | 0.42 |
| | C | 3,464 | 7.34 | 0.0001 |
| B x C | P | 3,464 | 0.42 | 0.74 |
| | C | 3,464 | 0.01 | 0.99 |
| A x B x C | P | 3,464 | 0.34 | 0.80 |
| | C | 3,464 | 0.29 | 0.83 |

Table 5.6. The effects of mating status and diet pair on the total intake of protein (P) and carbohydrates (C) in female and male *T. commodus*. As in Table 5.5, each multivariate model was followed by a series of ANOVAs to determine which nutrients contributed to any overall multivariate effects.

| | MANOVA | | | |
|-------------------------|----------------|-----------|----------|----------|
| Model terms | Pillai's Trace | <i>df</i> | <i>F</i> | <i>P</i> |
| Females | | | | |
| Mating status (A) | 0.04 | 2,231 | 5.20 | 0.006 |
| Diet pair (B) | 0.47 | 6,464 | 23.84 | 0.0001 |
| A x B | 0.01 | 6,464 | 0.37 | 0.90 |
| Univariate ANOVA | | | | |
| Model terms | Nutrient | <i>df</i> | <i>F</i> | <i>P</i> |
| Mating status (A) | P | 1,232 | 9.07 | 0.003 |
| | C | 1,232 | 6.41 | 0.012 |
| Diet pair (B) | P | 3,232 | 34.65 | 0.0001 |
| | C | 3,232 | 4.41 | 0.005 |
| A x B | P | 3,232 | 0.31 | 0.82 |
| | C | 3,232 | 0.15 | 0.93 |
| Males | | | | |
| Mating status (A) | 0.01 | 2,231 | 0.57 | 0.57 |
| Diet pair (B) | 0.85 | 6,464 | 57.54 | 0.0001 |
| A x B | 0.01 | 6,464 | 0.54 | 0.78 |
| Univariate ANOVA | | | | |
| Model terms | Nutrient | <i>df</i> | <i>F</i> | <i>P</i> |
| Mating status (A) | P | 1,232 | 0.47 | 0.49 |
| | C | 1,232 | 1.05 | 0.31 |
| Diet pair (B) | P | 3,232 | 109.66 | 0.0001 |
| | C | 3,232 | 23.66 | 0.0001 |
| A x B | P | 3,232 | 0.60 | 0.62 |
| | C | 3,232 | 0.14 | 0.93 |

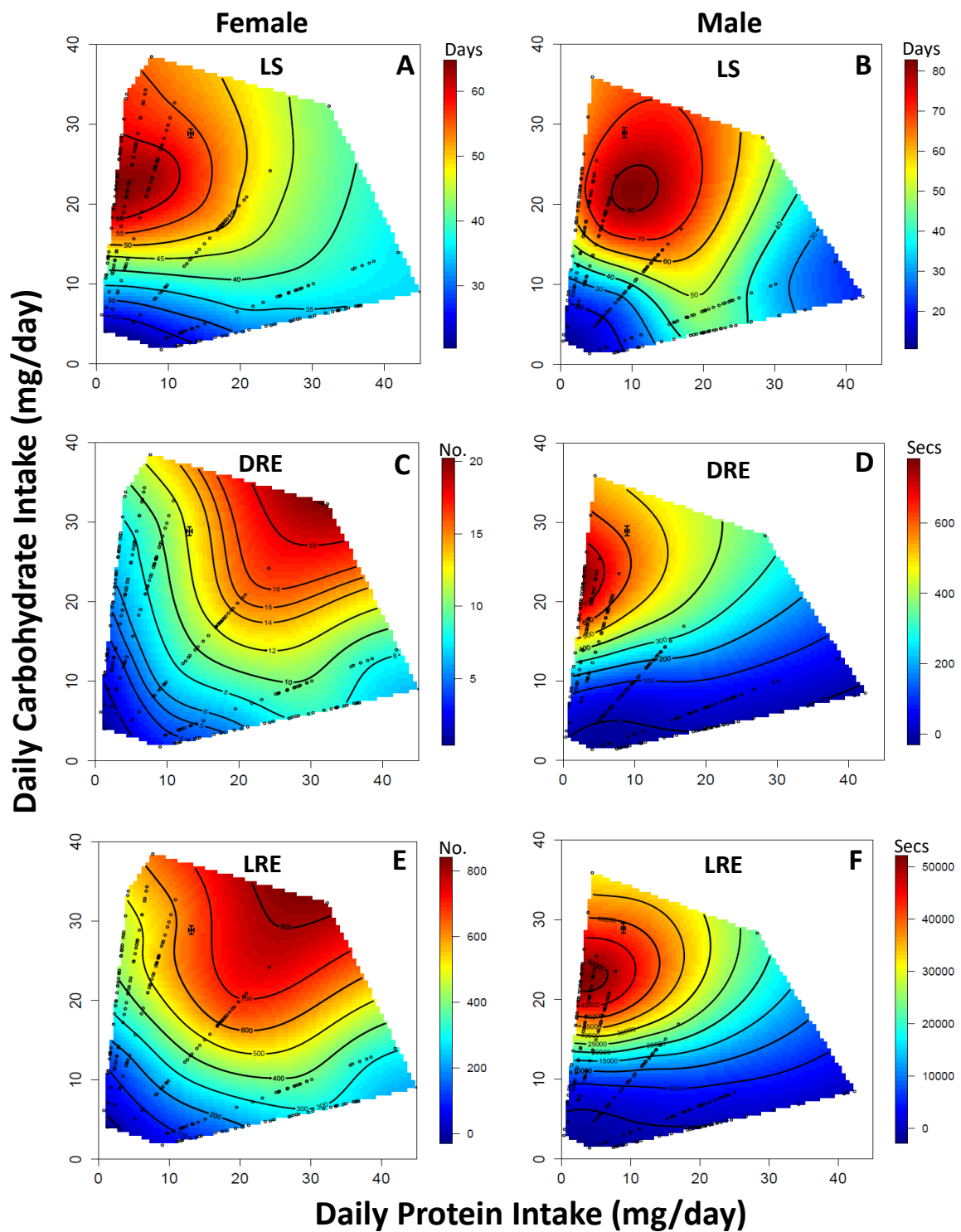


Figure 5.1. The nutritional landscapes for female and male LS (A & B, respectively), female DRE and male DRE (C & D, respectively) and female and male LRE (E & F, respectively). In each landscape, the red regions represent higher values for the response variable, whereas blue regions represent lower values. The black cross represents the regulated intake point (and 95% CIs) estimated in Experiment 3. The small black circles represent the actual feeding data for individual crickets.

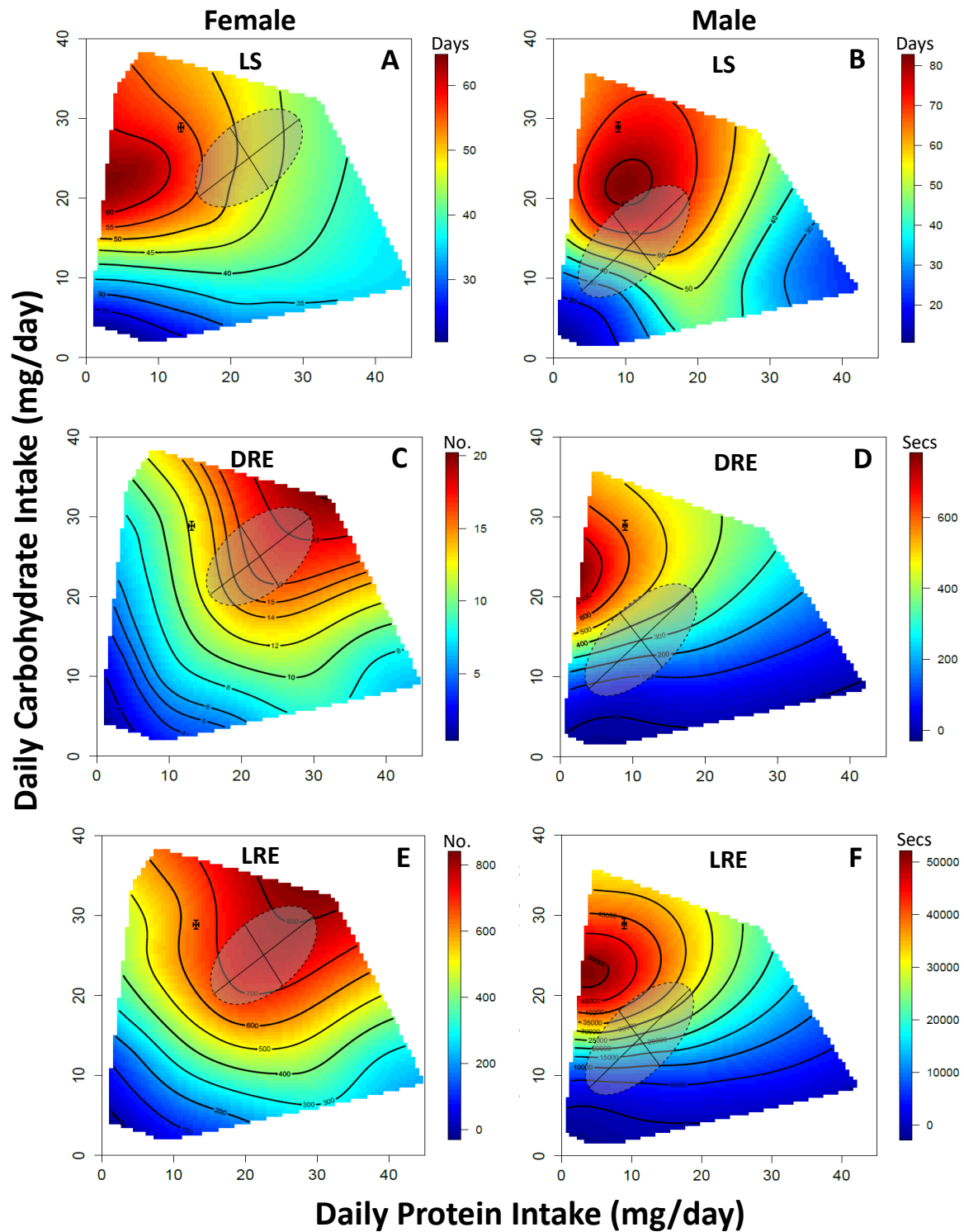


Figure 5.2. The nutritional landscapes female and male LS (A & B, respectively), female DRE and male DRE (C & D, respectively) and female and male LRE (E & F, respectively) with the two major eigenvectors (and 95% CIs) of G_f and G_m overlaid (grey ellipsoids). The black cross represents the regulated intake point (and 95% CIs) estimated in Experiment 3.

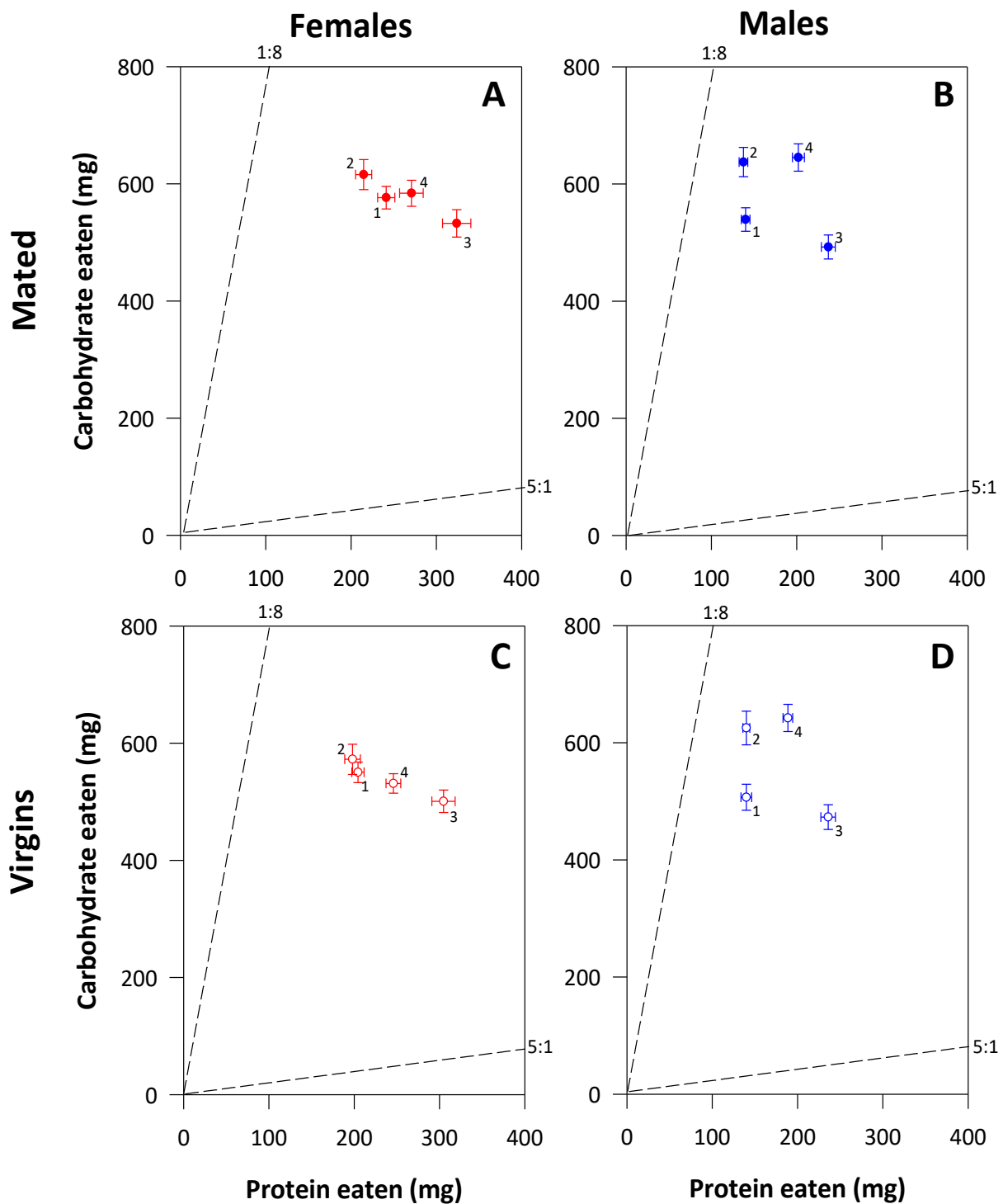


Figure 5.3. The mean (and 95% CIs) protein (P) and carbohydrate (C) intake of mated female and male (A & B, respectively; closed symbols) and virgin female and male (C & D, respectively; open symbols) *T. commodus* on the four different diet pairs in Experiment 3. Diet pairs are labelled by their number. The black dashed lines (at P:C ratios of 5:1 and 1:8) represent the outer nutritional rails for the individual diets contained in the diet pairs. Consequently, any dietary choice will occur within these outer margins.

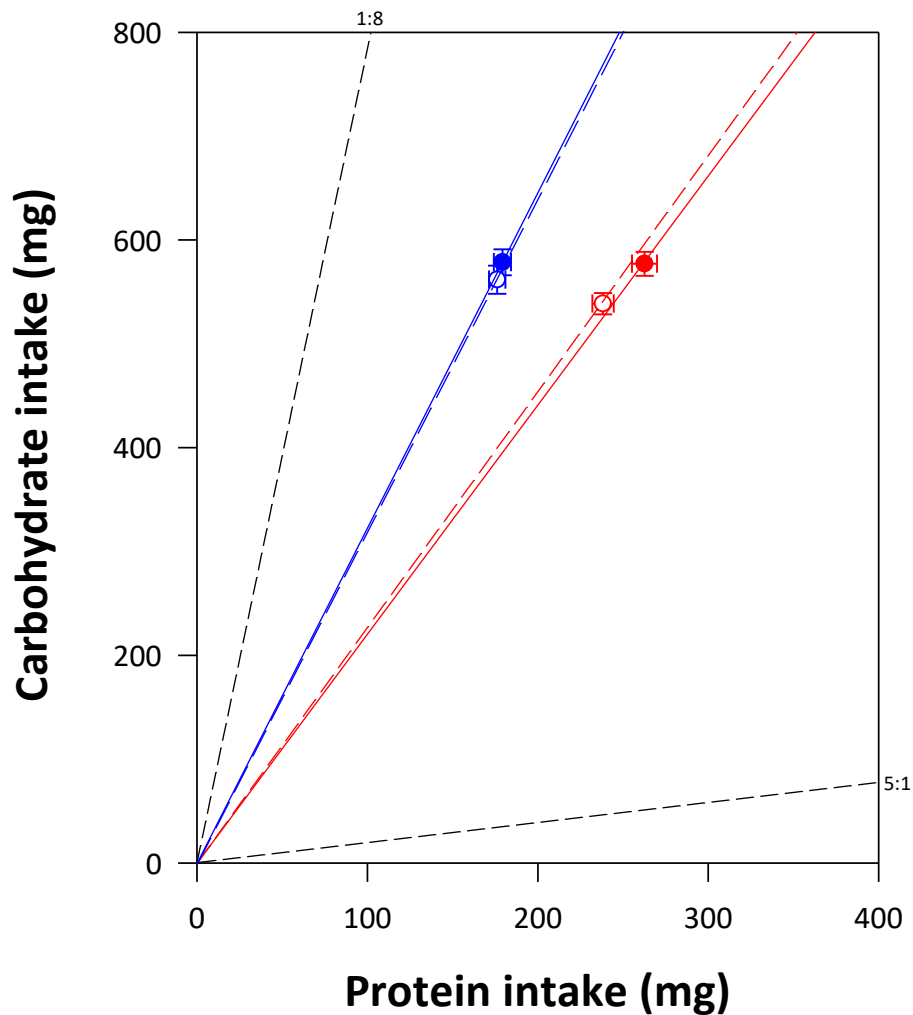


Figure 5.4. The regulated intake point (RIP), calculated as the mean intake of protein (P) and carbohydrate (C) across diet pairs for mated and virgin males (blue symbols, solid and open symbols, respectively) and mated and virgin females (red symbols, solid and open symbols, respectively). The black dashed lines (at P:C ratios of 5:1 and 1:8) represent the outer nutritional rails for diets. The solid and dashed red lines represent the RIP for mated and virgin females, respectively. The solid and dashed blue lines represent the RIP for mated and virgin males, respectively.

CHAPTER 6:
COMPLEX GENOTYPE-BY-ENVIRONMENT INTERACTIONS FOR
DIETARY CONSUMPTION, NUTRIENT PREFERENCE AND LIPID
DEPOSITION IN NUTRITIONALLY IMBALANCED ENVIRONMENTS IN
FIELD CRICKETS

6.1. ABSTRACT

Obesity has recently been described as a worldwide pandemic, although its effects are not just limited to humans, with excess weight gain found in a variety of animal species ranging from invertebrates to vertebrates. When in nutritionally imbalanced environments, animals have been shown to exhibit a range of compensatory mechanisms in order to maintain a specific nutrient intake. However, this often comes at the cost of over-ingesting nutrients and causing detrimental effects such as obesity. Although genes are known to play an important role in regulating dietary intake, it is still largely unknown how the interaction between genes and the dietary environment determines nutrient intake and weight gain and whether this relationship differs across the sexes. Using a half-sib breeding design I presented male and female black field crickets (*Teleogryllus commodus*) with one of four diet pairs (DP) of nutritionally imbalanced artificial diets differing in both the ratio of protein (P) to carbohydrate (C) and total nutrition, and measured the total amount of diet eaten (TE), total nutritional preference (TP, total P intake/total C intake) and lipid mass (LM). I found evidence for significant genotype-by-diet pair interactions and genotype-by-sex-by-diet pair interactions for each trait, as well as significant genotype-by-sex interactions for TE and LM but not TP. Furthermore, I demonstrate abundant additive genetic variance in TE, TP and LM in both sexes and all DPs, as well as substantial additive genetic covariance between these traits. The extent of these genetic correlations between traits, however, were more pronounced in males than females, especially the observed positive genetic correlation between TE and TP and the negative genetic correlation between TP and LM. Collectively, my work shows that complex interactions between genotype, sex and the nutritional environment play a central role in how the sexes in *T. commodus* regulate their feeding behaviour and nutrient intake in response to a nutritionally imbalanced environment and this has important implications for lipid deposition.

Key Words: Genotype-by-Environment Interactions, Lipids, Nutrient Regulation, Obesity, Sexual Selection, *Teleogryllus commodus*

6.2. INTRODUCTION

The unparalleled rise in the worldwide rates of obesity along with accompanying health problems (e.g. diabetes and cardiovascular disease) (Martens *et al.*, 2013) has recently led to obesity being considered a pandemic in many human populations (Raubenheimer *et al.*, 2015). The associated health problems of obesity are, however, not limited to humans and have been found in a variety of animal species ranging from vertebrates (cats (Scarlett *et al.*, 1994); dogs (German, 2006; Zoran, 2010); horses (Wyse *et al.*, 2008); and mice (Sorensen *et al.*, 2008; Huang *et al.*, 2013)) to invertebrates (caterpillars (Simpson *et al.*, 2004; Warbrick-Smith *et al.*, 2006); dragonflies (Schilder & Marden, 2006); and *Drosophila* (Skorupa, 2008; Birse *et al.*, 2010)). It has been argued that this rise in obesity in humans is occurring due to an imbalance between energy intake and energy expenditure (Mathes *et al.*, 2011), driven by the over ingestion of energy dense foods and lower activity levels of modern day humans.

This over ingestion of energy dense foods is perplexing because optimal foraging theory predicts that animals will evolve foraging mechanisms that maximise their fitness (Stephens & Krebs, 1986). Traditional optimal foraging theory assumes that optimizing fitness occurred by maximising energy intake (Stephens & Krebs, 1986). However, the more recent development of the multidimensional geometric framework of nutrition (GF) has shown that animals typically regulate both their energy intake and the specific balance of nutrients to maximise fitness (Simpson & Raubenheimer, 2012). This is because many fitness-related traits have been shown to be maximised in individuals that consume certain amounts and specific combinations of nutrients (Simpson & Raubenheimer, 2012), although different traits often have divergent dietary optima. For example, in female *Drosophila melanogaster* fecundity is maximised on nutrient rich diets containing protein (P) and carbohydrate (C) in a 1:2 P:C ratio but lifespan is maximised on diets with a P:C ratio of 1:16 (Lee *et al.*, 2008; Jensen *et al.*, 2015). Having trait-specific dietary optima therefore means that the diet that maximises fitness will depend on which trait(s) an individual must invest in to increase fitness, for instance somatic maintenance or reproduction, which is likely to vary with age, condition and the nutritional environment (Lihoreau *et al.*, 2015).

In most sexually reproducing species, one sex (typically males) produce numerous, small gametes that compete for access to larger, less abundant gametes produced by the

other sex (typically females). This divergence in reproductive investment means that females typically allocate more resources to offspring production, whereas males typically allocate more resources to mate competition (Trivers, 1972). As these divergent reproductive strategies often require different nutritional demands, sexual selection can drive the evolution of sex-specific nutritional optima for reproduction (Maklakov *et al.*, 2008; Simpson & Raubenheimer, 2012; Jensen *et al.*, 2015). For example, in *D. melanogaster*, males maximise the number of offspring they sire in a competitive environment when fed carbohydrate rich diets on a P:C ratio of 1:16, contrasting with maximum female fecundity at a P:C ratio of 1:2 (Jensen *et al.*, 2015). Similarly, in the field cricket *Teleogryllus commodus*, male calling effort (an important determinant of male mating success (Bentsen *et al.*, 2006)) is maximised on carbohydrate rich diets with a P:C ratio of 1:8, while female fecundity is maximised on a 1:1 P:C ratio (Maklakov *et al.*, 2008). Furthermore, sexual selection can also promote sexual divergence in the strength and direction of nutritional trade-offs between life history traits (Chapter 4). For example, male and female *D. melanogaster* live longest on a diet with a P:C ratio of 1:16 and this enables males to maximise both reproduction and lifespan (Jensen *et al.*, 2015). In contrast, females are unable to maximise both lifespan and fecundity on the same diet and therefore experience a nutritional trade-off between these traits (Lee *et al.*, 2008; Jensen *et al.*, 2015). A similar pattern has been reported in *T. commodus*, whereby male lifespan and calling effort are maximised on diets with similar P:C ratios (P:C = 1:5 and 1:8, respectively), whereas female lifespan and fecundity are maximised on more divergent P:C ratio diets (P:C = 1:8 and 1:1, respectively)(Maklakov *et al.*, 2008). How males and females regulate their intake of nutrients is therefore important in determining the optimal expression of multiple fitness determining traits (Chapters 4 & 5; Simpson & Raubenheimer, 2012).

Foragers can regulate their nutrient intake in a number of ways (Simpson & Raubenheimer, 2012). If restricted to a single diet that is deficient in an important nutrient, an animal will consume a larger volume of that diet in order to maintain the intake of the specific limiting nutrient; a process known as compensatory feeding (Simpson & Raubenheimer, 1993). In doing so an animal must decide how much diet to consume given the costs of over- and under- ingesting all nutrients contained in that diet (Simpson & Raubenheimer, 2012). Alternatively, an animal can regulate the total intake and the balance of nutrients by foraging from different nutritionally imbalanced foods to maintain a constant

intake of an optimal nutrient ratio (Simpson & Raubenheimer, 2012). It is possible to identify this optimal nutrient ratio, known as the regulated intake point (RIP) (Simpson & Raubenheimer, 2012), by providing an animal with a pair of nutritionally imbalanced diets and measuring the total intake of nutrients. If the animal eats non-randomly from each food in the pair then this suggests active nutrient regulation (Simpson & Raubenheimer, 2012). For example, male and female *D. melanogaster* feed non-randomly from different pairs of diet varying in P and C content and converge on a RIP that has a P:C ratio of 1:4 (Lee *et al.*, 2008; Jensen *et al.*, 2015). Similarly, male and female *T. commodus* have been shown to converge on a RIP with a P:C ratio of 1:2.96 when given dietary choice (Maklakov *et al.*, 2008).

It is important to note, however, that even though active nutrient regulation is well documented in animals (Maklakov *et al.*, 2008; Jensen *et al.*, 2012; Bunning *et al.*, 2015; Jensen *et al.*, 2015; Bunning *et al.*, 2016), it is not always optimal for trait expression or fitness (Chapters 3-5; South *et al.*, 2011; Harrison *et al.*, 2014; Bunning *et al.*, 2015; Jensen *et al.*, 2015; Bunning *et al.*, 2016). For example, the RIP observed in both in *T. commodus* (P:C = 1:2.96) and *D. melanogaster* (P:C = 1:4) is sub-optimal for lifespan and reproduction in both sexes (Maklakov *et al.*, 2008; Jensen *et al.*, 2015). Sub-optimal nutrient regulation may reflect an active compromise, whereby individuals regulate their intake of nutrients to balance the expression of multiple traits. For example, the RIP of male cockroaches (*Nauphoeta cinerea*) occurs at a P:C ratio of 1:4.95, which is midway between the P:C ratio that maximises sperm production (P:C = 1:2, Bunning *et al.*, 2015) and the P:C ratio that maximises pheromone production and pre-copulatory attractiveness (P:C = 1:8, South *et al.*, 2011; Bunning *et al.*, 2016). Alternatively, the existence of sub-optimal nutrient regulation may indicate that individuals are in some way constrained from feeding to their nutritional optima. This constraint may be genetic in origin if nutrient intake has divergent effects on males and females but the genes that govern the regulated intake of nutrients are shared in the sexes. This shared genetic basis for nutrient regulation can, in theory, prevent the sexes from independently evolving to their nutritional optima: a process known as intralocus sexual conflict (ISC) (Bonduriansky & Chenoweth, 2009). There is, however, currently little support for this process (Chapter 5; Reddiex *et al.*, 2013). It is also possible that physiological constraints on feeding behaviour prevent individuals from reaching their nutritional optima. Dietary assimilation, digestion, absorption and utilization can all

constrain feeding behaviour in animals (Henson & Hallam, 1995) and the efficiency of these processes is known to be intimately linked to gut morphology (e.g. Penry & Jumars, 1990; McWhorter & del Rio, 2000). Therefore, if the morphology of the gut limits the efficiency of one or more of these processes, it may prevent individuals from reaching their nutritional optima. While it is widely appreciated that animals do not always optimally regulate their intake of nutrients, more work is clearly needed to understand why this occurs.

Most studies examining the link between nutrition and obesity have focussed on the importance of lipid (L) and C intake and have largely ignored the role of P intake because of its comparatively minor role in the total energy budget of most animals (Simpson & Raubenheimer, 2005). However, recent insights from nutritional ecology have demonstrated that animals ranging from insects to mammals have separate appetite systems for the intake of P, C and L (Raubenheimer, 1997) and that when restricted to a diet of fixed macronutrient intake, animals regulate their intake of P more strongly than C and L (Raubenheimer, 1997; Sorensen *et al.*, 2008; Gosby *et al.*, 2014). This dominant mechanism for regulating P intake appears widespread in animals (Raubenheimer *et al.*, 2015) and has led to the recent development of the Protein Leverage Hypothesis (PLH) (Simpson & Raubenheimer, 2005). The PLH postulates that when the proportion of P contained in a diet is reduced, the powerful P appetite stimulates an increased consumption of diet (and therefore the intake of energy) in an attempt to gain more of the limited supply of P. Accordingly, any dietary shift towards foods that are higher in C and/or L will dilute the availability of P and this will increase consumption and the overall intake of energy (Gosby *et al.*, 2014). It has been argued that this may predispose individuals to increased lipid deposition and obesity (Simpson & Raubenheimer, 2005). Although a number of studies have provided support for the PLH and how it effects lipid deposition (Warbrick-Smith *et al.*, 2006; Skorupa, 2008; Sorensen *et al.*, 2008; Huang *et al.*, 2013), no study has yet attempted a complete genetic dissection of this relationship. In particular, how an individual regulates its intake of nutrients and the effect this has on lipid deposition will not only depend on the independent effects of genotype and the nutritional environment, but also on how these factors interact. The differential expression of genes in alternate environments, referred to as genotype-by-environment interactions, are therefore expected to be important and their presence would indicate that certain individuals are genetically pre-disposed to regulate their nutrient intake or deposit lipids in a specific way depending on variation in the

nutritional environment. Furthermore, as the sexes often have divergent nutritional requirements (Chapters 4 and 5; Maklakov *et al.*, 2008; Harrison *et al.*, 2014; Jensen *et al.*, 2015) and sexual dimorphism in the RIP has evolved in at least some species (Chapter 5; Harrison *et al.*, 2014), the effect of genotype and the nutritional environment on nutrient regulation and lipid deposition may also be different in males and females. This will result in a significant genotype-by-sex-by-nutritional environment interaction and indicate that the sexes are genetically pre-disposed to regulate their nutrient intake or deposit lipids in different ways that is contingent on the nutritional environment. It is important to note, however, that even if the sexes respond to the nutritional environment in the same manner, males and females may still be genetically pre-disposed to regulate their nutrient intake or deposit lipids in different ways if the underlying physiological processes that regulate these traits are sex-specific. This will result in a significant genotype-by-sex interaction and this interaction has been shown to be important to the expression of a range of physiological processes implicated in obesity (e.g. Towne *et al.*, 1997; North *et al.*, 2007; Santos *et al.*, 2014).

Here I combine the GF with quantitative genetics to determine how male and female black field crickets (*Teleogryllus commodus*) of known genetic relatedness responded when placed into four different nutritionally imbalanced environments. Each nutritionally imbalanced environment consisted of two diets that varied in their P:C ratio, as well as total nutritional content (P+C). For each cricket, I measured the total amount of diet eaten, the nutritional preference (calculated as total protein intake divided by total carbohydrate intake) and total lipid mass (as a measure of lipid deposition) to quantify both the independent effects of genotype and the nutritional environment on each of these traits, as well as the importance of the interaction between these main effects to trait expression (i.e. genotype-by-diet pair interaction). Furthermore, by measuring these traits in both sons and daughters in my pedigree I also determined whether the genetic contribution to these traits differed across the sexes (i.e. genotype-by-sex interaction) and across the sexes with regard to diet pair (i.e. genotype-by-sex-by-diet pair interaction). If individuals are actively regulating their intake of nutrients, I predict that there will be differences in the total amount of diet eaten and total nutrient preference across diet pairs and this will influence lipid deposition. Moreover, if males and females differentially regulate their intake of nutrients (see Chapter 5), I predict that the total amount of diet eaten and total nutrient

preference will differ across the sexes, as will the relationship between these traits and lipid deposition. Finally, if nutrient regulation is under genetic control I predict that there will be significant additive genetic variance in and additive genetic covariance between these traits in both sexes, as well as complex interactions between genotype, diet pair and sex (i.e. genotype-by-diet pair, genotype-by-sex and genotype-by-sex-by-diet pair interactions).

6.3. MATERIALS AND METHODS

6.3.1. Experimental animals

A total of 200 mated female *T. commodus* were collected from Smith's Lake, New South Wales in eastern Australia in March 2009 and used to establish a large panmictic lab population, maintained in 10 large culture containers (100L) of approximately 500 animals per culture for 10 non-overlapping generations prior to this experiment. Lab populations are kept at $28^{\circ}\text{C} \pm 1^{\circ}\text{C}$, under a 13:11 light:dark cycle, cleaned weekly and provided with cardboard for shelter, water *ab libitum*, egg pads consisting of damp cotton wool and a mixture of cat food (Purina Go Cat Senior[®]) and rat food (SDS Diets). Nymphs were moved at random between culture containers each generation to ensure gene flow.

6.3.2. Artificial Diets

Using the established protocol outlined by Simpson & Abisgold (1985) I made four artificial, dry diets that differed in both P:C ratio and total nutrition (Diets 2, 4, 22 and 24 in Table 1.1). I paired these diets to make four diet pairs (Pair 1: D2 and D22; Pair 2: D2 and D24; Pair 3: D4 and D22; Pair 4: D4 and D24), which have been used previously in choice feeding experiments (Chapters 3-5; South *et al.*, 2011; Bunning *et al.*, 2015) and were selected from a possible 24 diets because they provide a complete coverage of potential nutrient space (see Figure 1.2 for the distribution of these diets in nutritional space).

6.3.3. Quantitative Genetic Breeding Design

To estimate the quantitative genetics of total diet eaten, nutritional preference and lipid mass, I used a split-brood half-sib breeding design whereby sons and daughters from each full-sib family were split across four different diet pairs and their intake of nutrients measured under dietary choice for 21 days. The half-sib breeding design was established by

mating each of 30 randomly chosen virgin sires with 3 randomly chosen dams. A total of 50 offspring from each dam were collected and reared in a family group in an individual plastic container (10 x 10 x 5cm) for three weeks, with access to an *ad libitum* supply of ground cat food (Purina Go Cat Senior[®]) and water provided in a 5cm plastic tube plugged with cotton wool. After 3 weeks, 12 sons and 12 daughters per dam were isolated and established at random in individual plastic containers (5cm x 5cm x 5cm) and provided with *ad libitum* cat food pellets and water and checked daily for eclosion to adulthood. Containers were cleaned and fresh food and water were provided weekly. On the day of eclosion, I randomly allocated 3 sons and 3 daughters per dam to each of four diet pairs (total $n = 1080$ sons and 1080 daughters). Fresh diet was provided every 3 days for a total of 21 days (i.e. a total of 7 feeding periods). Experimental animals were mated with a stock animal of the opposite sex on the evening of day 8 post-eclosion and removed on day nine with females provided with a petri dish of moist sand thereafter for oviposition.

6.3.4. Feeding Regime

Experimental feeding followed established protocols used previously (Chapters 2-5; South *et al.*, 2011; Bunning *et al.*, 2015). In brief, two dishes of diet of measured dry weight were provided to each cricket according to assigned diet pair. Food was provided in feeding platforms constructed by gluing the upturned lid of a vial (1.6 cm diameter, 1.6cm deep) onto the middle of a petri dish (5.5 cm diameter) and water was provided *ad libitum* in a 5ml test tube plugged with cotton wool. Any diet spilled during feeding was collected in the petri dish and weighed. All diets were dried in an oven (Binder model FD115) at 30°C for 72 hrs before weighing. Feeding platforms were weighed before and after each feeding period using an electronic balance (Ohaus Explorer Professional model EP214C). Faeces were removed from the diet and feeding platform using forceps prior to re-weighing. Diet consumption was calculated as the difference in dry weight of diet before and after feeding. This amount of consumed diet was converted to a weight of P and C ingested by multiplying by the proportion of these nutrients in the diet (South *et al.*, 2011; Bunning *et al.*, 2015).

6.3.5. Measuring Lipid Mass

On day 21, crickets were frozen at -20°C and stored until lipid analysis could be performed. Lipid extraction was performed using the protocol outlined in South *et al.* (2011)

and Folch *et al.* (1957). In brief, each cricket was defrosted to room temperature and a slit was made along the abdomen using dissecting scissors. The cricket was then dried at 60°C for 24 hours and weighed using an electronic balance. Each cricket was then placed in 10ml of a 2:1 (v/v) solution of dichloromethane:methanol and agitated for 48 hrs to extract lipids. Crickets were then removed from this solution and dried for a further 24 hours at 60°C and then weighed. The difference between the pre- and post-extraction weights of each cricket was taken as the lipid mass.

6.3.6. Statistical Analysis

Quantitative genetic analyses were performed using animal models and restricted maximum likelihood approach in ASReml v.3.0 (Gilmour *et al.*, 2009; Wilson *et al.*, 2010). I examined three phenotypic traits: the total amount of diet eaten (TE), total nutritional preference (TP) (calculated as total protein intake divided by total carbohydrate intake) and lipid mass (LM). Prior to analysis each trait was standardized to a mean of zero and standard error of one using a Z-transformation. Model comparisons were made using a log-likelihood ratio test (LRT) where each of two competing models (the null model and the alternate model) is separately fitted to the data and the log-likelihood (LogL) value recorded. The test statistic (D) is twice the difference in the log-likelihoods and the probability distribution of D approximates a chi-squared distribution with degrees of freedom (df) equal to the number of additional parameters estimated in the alternate model (Pinheiro & Bates, 2000). Due to limitations in the ASReml software (which is common to all available software for analysing pedigrees with the animal model), I was unable to run a multivariate (multi-trait) model which included each trait split by sex and diet pair treatments. I therefore, ran separate univariate models for each trait. Body size (BS) was included as a fixed effect in all models to control for any size effects on TE, TP or LM.

Initially, I examined the effects of Diet Pair and Sex on TE, TP and LM. To examine the effect of Diet Pair, I ran a univariate model for each trait that included BS and Sex (S) as fixed effects (Model A, Table 6.1). The resulting LogL values from these models were compared to those derived from a second univariate model for each trait that also included Diet Pair (DP) as a fixed effect (Model B, Table 6.1). To examine the effect of sex, I ran a univariate model for each trait that included BS and DP as fixed effects (Model C, Table 6.1) and compared the LogL values from this model to those from a further univariate model for each trait that

included S as a fixed effect (Model D, Table 6.1). Given the differences in TE, TP and LM across DPs and the sexes (see Results), I also explored the effects of P and C intake on LM and whether this differed across the sexes. I used a response surface approach to characterize the linear and nonlinear (quadratic and correlational) effects of nutrients on LM in each sex (South *et al.*, 2011). Prior to this analysis, the intake of nutrients and LM were standardized to a mean of zero and standard deviation of one using a Z transformation to facilitate direct comparison across the sexes. I visualized the effects of P and C intake on LM in each sex using thin-plate splines constructed using the *Tps* function in the 'FIELDS' package of R (version 2.15.1, www.r-project.org). I statistically compared the linear (P and C), quadratic (P x P and C x C) and correlational (P x C) effects of nutrient intake across the sexes using a sequential model-building approach (South *et al.*, 2011).

I then examined the presence of significant additive genetic variance in TE, TP and LM by running a univariate model for each trait that included BS and a Sex x Diet Pair (S:DP) interaction term (Model E, Table 6.1) and compared the resultant LogL values to those obtained from a further univariate model which also included the breeding value (G) as a random effect in the model (Model F, Table 6.1). A Sex x Diet Pair term was included in this model to ensure that a separate mean was fitted for males and females in each of the four diet pairs.

Next I tested specifically for a significant G x Diet Pair interaction on TE, TP and LM by running a univariate model on each trait that controlled for BS and Sex and included G (Model G, Table 6.1). To run this model in ASREML requires splitting each trait across the four diet pairs (T_1 - T_4 , Model G, Table 6.1). For each trait, Model G was compared to the same model with a G x DP (G:DP) term included (Model H, Table 6.1). I also performed a secondary analysis to further explore sex differences in the effects of G x DP interactions on trait expression by re-running the G x Diet Pair models (Models G and H) but restricting the data to one sex at a time, for each trait, the Sex (S) term was therefore excluded from the model. It is important to note that even though these models include a conventional multivariate structure (i.e. there are multiple traits, T_1 - T_4 , included in Model G and H), these are the same trait measured in the different diet pairs (i.e. uni-trait model) rather than 4 different traits (i.e. multi-trait model). Consequently, I refer to these as univariate models not multivariate models. This same logic also applies to models I to L below.

I then tested for a G x Sex interaction on TE, TP and LM by running a univariate model on each trait that controlled for BS and DP and contained G (Model I, Table 6.1). To run this model in ASREML requires splitting each trait across each sex (T_M and T_F , Model I, Table 6.1). Model I for each trait was compared to the same model that included a G x Sex (G:S) term (Model J, Table 6.1). I also performed a secondary analysis to further explore whether G x Sex interactions differ across diet pairs. To do so, I re-ran the G x Sex models (Models I and J) but using data restricted to one diet pair at a time, for each trait, the Diet Pair (DP) term was therefore excluded from the models.

Finally, I performed an overall test for a G x Sex x Diet Pair interaction in TE, TM and LM by running a univariate model on each trait that controlled for BS and included G (Model K, Table 6.1). To run this model in ASREML requires splitting each trait across each diet pair for males ($T_{M1} - T_{M4}$) and females ($T_{F1} - T_{F4}$)(Model K, Table 6.1). Model K for each trait was compared to the same model that included a G x Sex x Diet Pair (G:S:DP) term (Model L, Table 6.1).

I also used ASREML to extract the additive genetic variances in and additive genetic co-variances between TE, TP and LM, as well as the corresponding standardized estimates of heritability (h^2) and genetic correlations (r_A). Due to the significant G x Sex x Diet Pair interactions for each trait (see Results), the above genetic parameters were estimated separately for males and females within each diet pair. To test the significance of my h^2 estimates, separate univariate models were run for each trait: one that controlled for BS and one that controlled for BS but also included a breeding value (G) term. To test the significance of my r_A estimates, two bivariate (i.e. two-trait) models were run for each estimate: one where the genetic covariance between the two traits was constrained to zero and one where the covariance structure was unconstrained (i.e. allowed to vary with the structure of the data). For both h^2 and r_A estimates, LogL values from each contrasting model were compared using the LRT procedure outlined above.

6.4. RESULTS

There was a significant effect of DP on TE, TP and LM, as demonstrated by the fact that univariate models including BS and S as fixed effects (Model A, Table 6.1) were significantly improved by the addition of a DP term (Model B, Table 6.1). Likewise, there was

a significant effect of Sex on TE, TP and LM as a univariate model including BS and DP as fixed effects (Model C, Table 6.1) were significantly improved by the addition of a S term (Model D, Table 6.1). The effects of DP and S on TE, TP and LM can be visualized in Figure 6.1. For both sexes, TE was highest on DP1, followed by DP3, DP2 and was lowest of DP4 (Figure 6.1A). This pattern of dietary consumption demonstrates compensatory feeding in the sexes, with males and females increasing their consumption of diet by 58% and 72% respectively, when feeding on the lowest (DP1, 36% nutrition) versus the highest (DP4, 84% nutrition) nutrient diet pair. Females consumed more diet than males on each DP and their consumption of diets was, on average, 20% higher than males across all DPs (Figure 6.1A). For both sexes, TP values were highest for DP3, followed by DP1, DP4 and DP2 (Figure 6.1B). Furthermore, TP values were greater for females than males on each DP (Figure 6.1B). The effects of DP and S on the TP for nutrients can be further visualized in Figure 6.2, which shows the mean P and C intake of the sexes on each DP, as well as the regulated intake point (RIP) for each sex which is calculated as the mean intake of these nutrients across diet pairs and represents the point in nutrient space that individuals actively defend when given dietary choice. With the exception of crickets of both sexes on DP3 that showed an equal intake of nutrients, crickets on all other DPs showed a preference to consume relatively more C than P (Figure 6.2). This C biased preference, however, was more prominent in males than females, as indicated by a RIP at a P:C ratio of 1:2.02 in males and 1:1.71 in females (Figure 6.2). Finally, for both sexes LM was highest on DP4, followed by DP2, DP3 and DP1 (Figure 6.1C). Despite the higher consumption of diets by females, LM was actually higher in males than females (Figure 6.1C). Response surface analysis showed that LM increased linearly with the intake of C in both sexes and decreased linearly with P intake in males but not in females (Table 6.2). There were, significant positive quadratic effects of P intake on LM in both sexes but no significant quadratic effects of C intake (Table 6.2). There was a significant negative correlational effect of nutrient intake on LM in males but not females (Table 6.2). The effect of nutrient intake on LM in the sexes is presented as thin-plate splines in Figure 6.3 and they confirm that LM is maximised at a high intake of C and low intake of P in both sexes. Indeed, a sequential model-building approach revealed that linear ($F_{2,2068} = 1.16, P = 0.31$), quadratic ($F_{2,2064} = 2.33, P = 0.10$) and correlational ($F_{1,2062} = 2.80, P = 0.10$) effects of P and C intake on LM did not differ significantly between the sexes.

The fit of a univariate model controlling for BS and containing a Sex x Diet Pair interaction (S:DP) term as a fixed effect (to fit separate trait means for each sex and diet pair, Model E) was significantly improved by the addition of a breeding value term (G, Model F) for each trait examined (Table 6.1). This indicates significant additive genetic variance for TE, TP and LM in each sex across the four diet pairs tested. This finding is verified by the abundance of significant h^2 estimates in Table 6.3, the only exception being for the TP of females in DP4.

There was evidence for a significant G x DP interaction for each trait (Table 6.1). That is, separate univariate models for TE, TP and LM that controlled for BS and Sex and included G and G x DP interaction terms (Model H) provided a significantly better fit to the data than corresponding models that did not include the G x DP terms (Model G)(Table 6.1). Further exploration within each sex showed that this interaction term was significant for all three traits in both males and females, being especially pronounced for TP (Table 6.4). The reaction norms for this interaction in TE, TP and LM are presented in Figures 6.4 to 6.6, respectively, for each sex. It is readily apparent from these reactions norms for males and females that there is both substantial additive genetic variation for each trait in each of the DPs and substantial crossing over of reactions norms across DPs resulting in significant G x DP interactions.

There was also evidence for a significant G x Sex interaction for TE and LM but not for TP (Table 6.1). That is, separate univariate models for TE and LM that controlled for BS and DP and included G as a random effect (Model I, Table 6.1) was significantly improved by adding a G x Sex interaction term (Model J, Table 6.1). Further exploration within each DP showed that this interaction was significant in all four DPs for TE and LM but was only significant in DPs 1, 2 and 3 for TP (Table 6.4). The reaction norms illustrating the G x Sex interaction in TE, TP and LM are presented in Figures 6.7 to 6.9, respectively, for each diet pair. The reactions norms for the G x Sex interaction show that there is ample additive genetic variance in the sexes for each trait on the different DPs and substantial crossing over of the reaction norms. The extent of this crossing over, however, is visibly greater for TE and LM than TP, especially for DP4 (Figure 6.7).

Finally, there was evidence for a significant G x Sex x DP interaction for TE, TP and LM as the fit of univariate models including BS and G as fixed effects (Model K, Table 6.1) was significantly improved by the addition of this interaction term (Model L, Table 6.1). This

finding suggests that complex interaction between genes, sex and the nutritional environment are key to the intake of nutrients and lipid deposition in *T. commodus*. More specifically, it indicates that individuals are genetically pre-disposed to regulate their nutrient intake or deposit lipids but this depends on variation in the nutritional environment and their sex.

The significance of the G x Sex x DP interaction for TE, TP and LM also suggests that the additive genetic variance-covariance structure for these traits vary with Sex and DP. In Table 6.3, I provide estimates of the additive genetic variances in and covariance between these traits for each sex in the four different DPs. With the only exception of TP for females in DP4, all other h^2 estimates for the sexes in each DP were significantly greater than zero. There was, however, substantial variability in h^2 estimates, ranging from 0.25 to 0.94, and there was no clear pattern with regards to DP or sex. In contrast, estimates of genetic correlations (r_A) between traits showed a number of clear differences across the sexes and DPs. First, estimates of r_A were more pronounced in males than females, with 9 estimates being significantly greater than zero in males, compared to only two in females (Table 6.3). Second, there is a significant positive r_A between TE and TP for all DPs in males, whereas this genetic correlation is only significant for DP2 in females (Table 6.3). Third, there is a significant negative r_A between TE and LM for DP1 in males but a significant positive r_A between these traits in DP3 (Table 6.3). In contrast, there is no significant covariance between TE and LM in females (Table 6.3). Finally, there is a significant negative r_A between TP and LM for DP1, DP2 and DP4 in males, but a negative r_A between these traits is only significant for DP1 in females (Table 6.3).

6.5. DISCUSSION

In this study, I combined the GF with a half-sib quantitative genetic breeding design to examine the interactions between genes and the dietary environment in order to understand how male and female black field crickets (*T. commodus*) regulate their feeding behaviour and intake of nutrients when encountering a nutritionally imbalanced environment and the consequences this has for lipid deposition. I predicted that if *T. commodus* actively regulate their feeding behaviour and nutrient intake, there would be differences in TE and TP across diet pairs and this would have important implications for LM.

Moreover, due to the divergence in the nutritional requirements of the sexes, I further predicted that any differences in TE and TP across diet pairs would be sex-specific, as would the relationship between TE, TP and LM. In agreement with these predictions, I found that male and female *T. commodus* showed considerable differences in TE and TP across DPs that is consistent with active nutrient regulation. There were, however, clear sex differences with females consuming more diet and showing a stronger preference for the intake of P relative to C than males on each DP. Interestingly, despite their higher dietary consumption, females exhibited lower LM on each DP compared to males. I further predicted that if nutrient regulation is under genetic control, there will be significant additive genetic variance in and additive genetic covariance between these traits in both sexes, as well as complex interactions between genotype, diet pair and sex. Consistent with this prediction, I show that there is ample additive genetic variance in TE, TP and LM in both sexes and across all DPs (the only exception being for TP in females in DP4), as well as substantial additive genetic covariance between these traits. This covariance between traits was more pronounced in males than females, most notable being the consistent positive genetic correlation between TE and TP and the negative genetic correlation between TP and LM across DPs. Most importantly, I provide evidence for significant genotype-by-diet pair interactions (G x DP) and genotype-by-sex-by-diet pair interactions (G x Sex x DP) for each trait, as well as significant genotype-by-sex interactions (G x Sex) for TE and LM but not TP. Together, my findings demonstrate that complex interactions between genotype, sex and the nutritional environment play a central role in how *T. commodus* regulate their feeding behaviour and nutrient intake in response to nutritionally imbalanced environments and that this has important implications for lipid deposition in the sexes.

Optimal foraging theory (Stephens & Krebs, 1986) predicts that when in a nutritionally imbalanced environment, an animal may increase its consumption when feeding on diets with diluted nutrient levels (known as compensatory feeding) or eat non-randomly from multiple food sources to actively regulate their intake of nutrients (Simpson & Raubenheimer, 2012). My finding that there is considerable variation in both TE and TP across DPs and the sexes suggests that both processes are operating in male and female *T. commodus* but to differing degrees. I found that both sexes increased the total amount of diet they consumed on the lowest nutrition pair (DP1, 36% nutrition) compared to highest nutrition pair (DP4, 85%) but this increase was larger in females (72%) than males (52%).

While compensatory feeding has been demonstrated in a wide variety of animal taxa ranging from invertebrates to mammals (e.g. Cruz-Rivera & Hay, 2000; Fanson *et al.*, 2012; Jensen *et al.*, 2014), fewer studies have reported sex differences in this behaviour and both existing studies show that the magnitude of compensatory feeding is higher in males than in females (Barreto *et al.*, 2003; Butzen *et al.*, 2013). I also show that females have consistently higher TP values than males on each DP and although both sexes show an overall preference for C intake over P intake, the RIP was relatively more P biased in females (P:C ratio = 1:1.71) than males (P:C ratio = 1:2.02). This contrasts earlier work in *T. commodus* (Maklakov *et al.*, 2008), as well as studies on *D. melanogaster* (Reddiex *et al.*, 2013; Jensen *et al.*, 2015) and the cockroach *N. cinerea* (Bunning *et al.*, 2016), that show no sex-difference in the regulated intake of P and C. The differences I observe in *T. commodus*, however, can be explained by the divergent reproductive strategies of the sexes. Male *T. commodus* produce an advertisement call that is used to attract females and while metabolically demanding to produce (Kavanagh, 1987), the amount of time spent calling is a major determinant of male mating success (Bentsen *et al.*, 2006). To fuel this signalling behaviour, males therefore require a high intake of C which provides an abundant source of energy that is available rapidly after digestion and calling effort is subsequently maximised at a P:C ratio of 1:8 (Chapter 4; Maklakov *et al.*, 2008). Reproductive success in females, however, is largely determined by the number of eggs they can produce and P intake is known to play an important role in stimulating oogenesis and regulating vitellogenesis in insects (Wheeler, 1996). Females therefore require a higher intake of P relative to males to maximise egg production and the RIP of female *T. commodus* has been shown to be more P biased than in males (P:C = 1:1, (Maklakov *et al.*, 2008); P:C = 1:2.02, (Chapter 5)). It is important to note, however, that despite this sexual divergence, neither sex optimally regulate their relative intake of P and C to maximise their reproductive success, although females do appear closer to regulate closer the optimal P:C ratio than males (Chapter 5).

Current theories on the link between diet and obesity have highlighted the over ingestion of energy dense foods as a primary factor in weight gain (Mathes *et al.*, 2011; Raubenheimer *et al.*, 2015). In agreement with this view, I show that lipid deposition in male and female *T. commodus* was significantly greater on the DP with the highest total nutrition (DP4, 84% nutrition) and lowest on the DP containing lowest total nutrition (DP1, 36% nutrition). Contrary to this general prediction, however, I show that lipid deposition is not

only contingent on the energy (caloric) content of the diet but also the relative intake of nutrients. This is illustrated by the difference in lipid deposition of both sexes when feeding from DP2 and DP3; both DPs contain the same total energy content, but the highest nutrient diet in DP2 is C biased (P:C = 1:8, 84% total nutrition) whereas it is P biased on DP3 (P:C = 5:1, 84% total nutrition). Consequently, the significantly higher lipid deposition of males and females feeding from DP2 than DP3 suggests that the intake of C is more important to lipid deposition than P intake. This claim is further substantiated by my response surface analysis which showed that LM was maximised in both sexes at a high intake of C and low intake of P. This finding therefore supports the well-established link between increased C intake and lipid deposition reported in a range of animal taxa (e.g. invertebrates (Simpson, 2002); fish (Dias *et al.*, 2004); birds (Zhang *et al.*, 2011); mammals (Sorensen *et al.*, 2008), including humans (e.g. Horton *et al.*, 1995). It also explains the lower LM of females than males on each of the DPs, despite their higher overall consumption of diets: by consuming relatively more P to C than males, females deposit lower levels of lipids. However, other mechanisms that may explain this reduced LM in females cannot be ruled out, including a more evolved “resistance” to effects of diet on lipid deposition in females than males (Warbrick-Smith *et al.*, 2006) or physiological differences in the way the sexes use and store lipids. For example, egg production causes a substantial mobilization of lipid reserves from the fat body to the ovaries in insects (Ziegler & Ibrahim, 2001; Lorenz & Anand, 2004; Ziegler & Van Antwerpen, 2006). It is therefore possible that females are utilizing more of their lipid stores to provision eggs, whereas males are using relatively less C for calling and storing the remainder as lipids. Measuring the LM of virgin females, with reduced egg production, on each of the DPs is likely to be a useful way to test this hypothesis (Nestel *et al.*, 2005).

Classic molecular genetic studies using mice (Marie *et al.*, 2000) and human models (Sørensen, 1988), as well as more recent genomic approaches (Scuteri *et al.*, 2007), have revealed the highly complex, polygenic contribution of genes to the physiological systems that underlie obesity (Mathes *et al.*, 2011). Despite the progress of such studies, pinpointing the exact gene(s) responsible for lipid deposition and obesity have proved difficult (Qi & Cho, 2008; Mathes *et al.*, 2011). This is perhaps not surprising given that lipid deposition and obesity are complex, multidimensional traits (or disorders) that are influenced by the interaction between many variables, such as behaviour, diet, environment, social structures and genetics (Qi & Cho, 2008; Mathes *et al.*, 2011).

Furthermore, lipid deposition and obesity are often linked to other important life-history traits due to competition for a common pool of resources and/or because they share a common metabolic pathway (Hansen *et al.*, 2013). For example, gonadectomy or germline ablation increases lipid mass in various species (Crane, 1991; Salmeri *et al.*, 1991; Fettman *et al.*, 1997; Hansen *et al.*, 2013) but also extends lifespan (Partridge *et al.*, 2005; Flatt *et al.*, 2008; Flatt & Heyland, 2011; Judd *et al.*, 2011; Hansen *et al.*, 2013) and the Insulin/IGF-1 signalling pathway (e.g. Perez & Van Gilst, 2008; Wolf, 2010; Post & Tatar, 2016), TOR nutrient sensing pathway (e.g. Kapahi *et al.*, 2004; Kapahi *et al.*, 2010) and NHR receptors (e.g. Goudeau *et al.*, 2011) are all known to play an important role in regulating reproduction, lipid metabolism and lifespan. My results are in broad agreement with the general view that lipid deposition is a complex trait that is influenced by the interaction between many variables. I show that LM in *T. commodus* is influenced by a complex interaction between genotype, the nutritional environment and sex. Furthermore, there is considerable additive genetic covariance between LM, TE and TP and that these latter two feeding behaviours are also subject to complex G x DP x Sex interactions. Collectively, these findings demonstrate that to understand lipid deposition in *T. commodus* it is not simply enough to characterize the independent contributions of the genotype, nutritional environment and sex to this trait: context is important. That is, these complex interactions in *T. commodus* mean that whether an individual is predisposed to increased lipid deposition cannot be predicted with complete accuracy from any one of these variables in isolation. Consequently, before any specific measures for obesity prevention that are tailored to an individual's personalized genetic make-up will be effective (Qi & Cho, 2008), a better understanding of how these complex interactions regulate LM is essential.

My finding that the additive genes for feeding behaviour (TE and TP) are linked to those for lipid deposition suggests that these traits share a common genetic pathway and are, therefore, not free to evolve independently (Lande, 1980). Despite the large number of additive genetic covariances estimated in my study, a number of consistent patterns exist. First, the magnitude of significant genetic correlations between TE, TP and LM was greater in males than females (9 versus 2, respectively). The fact that h^2 estimates were large for all traits and there were no systematic differences in these estimates across the sexes indicates that this pattern is not due to a simple lack of additive genetic variance for these traits in females (with the notable exception of TP in DP4). It does suggest that either the genetic

pathway regulating feeding behaviour and LM is different in the sexes or it is the same but more tightly regulated in males than females. Second, there were consistent positive genetic correlations between TE and TP across all DPs in males and also in DP2 for females. In my study, TP was measured as the total intake of P divided by the total intake of C. Higher values of TP, therefore, mean a preference for more P relative to C, even when there is an absolute preference for C ($TP < 1.0$, as shown for DP1, 2 and 3 in Figure 6.1). Consequently, this positive genetic correlation indicates that in males and in some nutritional environments for females, the genes that govern the preference for P relative to C are positively associated with the genes for dietary consumption. Finally, there were negative genetic correlations between TP and LM on DPs 1, 3 and 4 in males and DP1 in females. This indicates that the genes for LM are negatively associated with those governing the preference for P relative to C. Taken collectively, both of these patterns of additive genetic covariance between traits provide partial support for the Protein Leverage Hypothesis (PLH) at the genetic level. The PLH predicts that in a nutritionally imbalanced environment where P is limited, the powerful P appetite will stimulate individuals to increase their dietary consumption in an attempt to gain more P (Simpson & Raubenheimer, 2005; Sorensen *et al.*, 2008; Gosby *et al.*, 2014), a pattern that is supported by the positive genetic correlation between TP and TE. Furthermore, the PLH predicts that a side effect of the attempt to consume a limited supply of P is the over-ingestion of more abundant nutrients (such as C) that cause increased lipid deposition and predispose an individual to obesity (Simpson & Raubenheimer, 2005; Sorensen *et al.*, 2008; Gosby *et al.*, 2014). The observed negative genetic correlations between TP and LM agree with this prediction, although it also supports the alternate view that the genes for C preference are directly linked to those for LM. Further support for this prediction would have come from positive genetic correlations between TE and LM, however, this relationship was inconsistent in males being negative in DP1 and positive in DP3.

In conclusion, while my work is in general agreement with the commonly held view that the consumption of energy rich diets is a major contributor to the increased rates of obesity in most developed societies, it also clearly demonstrates that the causes of increased lipid deposition are far more complex than this in *T. commodus*. Complex interactions between genotype, the nutritional environment and sex for feeding behaviour (TE and TP) and LM, as well as additive genetic covariance between these traits, means that

focussing on any one of these variables in isolation will provide an incomplete understanding on whether an individual is predisposed to lipid deposition (or obesity) or not. The obvious question that remains from my work is what are the consequences of high lipid deposition in male and female *T. commodus*? In humans, as well as a range of mammalian models, there is clear evidence that excessive lipid deposition and obesity are responsible for a number of different metabolic and cardiovascular disorders (Kahn *et al.*, 2006; Corona *et al.*, 2009; Szendroedi & Roden, 2009; Raubenheimer *et al.*, 2015) which negatively impact health. There is some evidence that similar disorders exist in insects (e.g. *Drosophila* (Musselman *et al.*, 2011); dragonflies (Schilder & Marden, 2006)) but no study has yet fully dissected the fitness costs of obesity in an insect. One notable exception is a study showing that caterpillars of the diamond back moth (*Plutella xylostella*) maintained in populations fed a C rich diet for 8 generations evolved the ability to consume this excess C without storing it as lipids (Warbrick-Smith *et al.*, 2006). This suggests that *P. xylostella* has evolved resistance to obesity, which would only occur if the excessive deposition of lipids was costly in this species. Understanding the genetic mechanisms behind this adaptation to the over-consumption of energy rich and C biased diets would clearly be a useful avenue for future obesity research.

Table 6.1. Model rationales and ASREML model summaries incorporating Trait (T), Body Size (BS), Sex (S), Diet Pair (DP) and Breeding Value (G). Models testing for the effect of Diet Pair (DP), Sex (S), additive genetic variance (V_A), G x DP (G:S) interactions, G x S (G:S) interactions G x S x DP (G:S:DP) interactions. Log-Likelihoods (LogL) are reported for each model and are compared using LRTs to determine significance. Significant differences between models are highlighted in bold.

| Model | Model Rationale | Model Explanation | ASREML Model Summary | TE LogL | TP LogL | LM LogL |
|-------|-----------------|--|---|-----------------|----------------|-----------------|
| A | Test for DP | Trait controlling for BS and S | T BS S | -7427.65 | 1281.81 | -7834.22 |
| B | | Include DP | T BS S DP | -6834.38 | 2408.05 | -7524.41 |
| C | Test for S | Trait controlling for BS and DP | T BS DP | -7020.77 | 2352.60 | -7652.59 |
| D | | Include S | T BS DP S | -6834.38 | 2408.05 | -7524.41 |
| E | Test for V_A | Trait controlling for BS, S and DP | T BS S:DP | -350.04 | 63.90 | -612.96 |
| F | | Include G | T BS S:DP G | -187.42 | 260.67 | -533.95 |
| G | Test for G:DP | Trait split by DP, controlling for BS and S, including G | $T_1 T_2 T_3 T_4$ BS S G | -98.67 | 703.33 | -438.248 |
| H | | Include G:DP interaction | $T_1 T_2 T_3 T_4$ BS S G G:DP | -72.85 | 919.67 | -417.69 |
| I | Test for G:S | Trait split by S, controlling for BS and DP, including G | $T_M T_F$ BS DP G | -185.42 | 260.98 | -456.18 |
| J | | Include G:S interaction | $T_M T_F$ BS DP G G:S | -139.20 | 261.86 | -443.62 |
| K | Test for G:S:DP | Trait split by S and DP, controlling for BS, including G | $T_{M1} T_{M2} T_{M3} T_{M4} T_{F1} T_{F2} T_{F3} T_{F4}$ BS G | -103.13 | 702.57 | -324.94 |
| L | | Include G:S:DP interaction | $T_{M1} T_{M2} T_{M3} T_{M4} T_{F1} T_{F2} T_{F3} T_{F4}$ BS G G:S:DP | 0.99 | 940.22 | -275.14 |

Table 6.2. Response surface analysis quantifying the linear and nonlinear effects of protein (P) and carbohydrate (C) intake on lipid deposition in male and female *Teleogryllus commodus*.

| Sex | Linear effects | | Nonlinear effects | | |
|----------------|----------------|-------------|-------------------|--------------|--------------|
| | P | C | P x P | C x C | P x C |
| Males | | | | | |
| Gradient ± SE | -0.08 ± 0.03 | 0.52 ± 0.03 | 0.09 ± 0.02 | -0.00 ± 0.02 | -0.10 ± 0.03 |
| t_{1029} | 3.14 | 19.38 | 4.23 | 0.06 | 2.87 |
| <i>P</i> | 0.002 | 0.0001 | 0.0001 | 0.95 | 0.004 |
| Females | | | | | |
| Gradient ± SE | -0.03 ± 0.03 | 0.49 ± 0.03 | 0.04 ± 0.02 | 0.02 ± 0.03 | -0.00 ± 0.03 |
| t_{1041} | 1.09 | 18.12 | 2.00 | 0.81 | 0.07 |
| <i>P</i> | 0.27 | 0.0001 | 0.04 | 0.42 | 0.95 |

Table 6.3. Additive genetic variance-covariance matrices (**G**) for total diet eaten (TE), total nutrient preference (TP) and lipid mass (LM) in males and females across the four diet pairs tested. Genetic correlations (r_A , in italics) are above the diagonal, additive genetic variances are along the diagonal and the additive genetic covariance between the traits are provided below the diagonal, with SEs for these parameters being provided in brackets. Heritability (h^2) estimates for each trait are provided in a separate column (with SEs provided in brackets). Significant estimates of r_A and h^2 are in bold where * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

| Males | | | | | Females | | | | |
|--------------------|---------------|-----------------------|------------------------|-----------------------|---------|---------------|-----------------------|------------------------|-----------------------|
| | TE | TP | LM | h^2 | | TE | TP | LM | h^2 |
| Diet Pair 1 | | | | | | | | | |
| TE | 0.38 (3.64) | 0.79 (0.09)*** | -0.56 (0.23)** | 0.56 (0.13)** | TE | 0.16 (1.91) | -0.24 (0.26) | 0.06 (0.29) | 0.25 (0.12)* |
| TP | 0.25 (4.19) | 0.25 (5.28) | -0.64 (0.15)*** | 0.94 (0.12)*** | TP | -0.41 (-1.06) | 0.18 (5.09) | -0.54 (0.15)*** | 0.80 (0.12)*** |
| LM | -0.15 (-2.49) | -0.13 (-3.33) | 0.18 (2.71) | 0.57 (0.14)*** | LM | 0.08 (0.22) | -0.74 (-3.01) | 0.10 (3.27) | 0.49 (0.13)*** |
| Diet Pair 2 | | | | | | | | | |
| TE | 0.27 (3.92) | 0.93 (0.16)*** | -0.23 (0.23) | 0.67 (0.13)*** | TE | 0.36 (4.30) | 0.47 (0.15)*** | 0.06 (0.22) | 0.81 (0.13)*** |
| TP | 0.49 (3.45) | 0.01 (2.26) | -0.55 (0.24)* | 0.31 (0.13)* | TP | 0.48 (2.43) | 0.29 (3.56) | 0.11 (0.25) | 0.69 (0.15)*** |
| LM | -0.86 (-1.07) | -0.40 (-1.79) | 0.51 (2.67) | 0.59 (0.14)*** | LM | 0.15 (0.24) | 0.08 (0.46) | 0.19 (2.28) | 0.68 (0.13)*** |
| Diet Pair 3 | | | | | | | | | |
| TE | 0.30 (3.45) | 0.68 (0.18)*** | 0.34 (0.20)* | 0.53 (0.13)*** | TE | 0.31 (3.51) | -0.02 (0.17) | 0.09 (0.23) | 0.65 (0.14)*** |
| TP | 0.25 (3.25) | 0.45 (3.67) | -0.01 (0.20) | 0.60 (0.13)*** | TP | -0.07 (-0.09) | 0.66 (4.77) | -0.21 (0.19) | 0.93 (0.13)*** |
| LM | 0.11 (1.61) | -0.02 (-0.03) | 0.38 (3.35) | 0.39 (0.14)** | LM | 0.16 (0.40) | -0.56 (-1.11) | 0.11 (2.83) | 0.56 (0.13)*** |
| Diet Pair 4 | | | | | | | | | |
| TE | 0.24 (4.07) | 0.47 (0.22)* | -0.31 (0.22) | 0.72 (0.13)*** | TE | 0.26 (4.12) | 0.84 (0.91) | 0.00 (0.27) | 0.77 (0.13)*** |
| TP | 0.53 (1.90) | 0.52 (2.15) | -0.57 (0.26)* | 0.27 (0.12)* | TP | 0.40 (1.72) | 0.09 (0.48) | -0.52 (0.85) | 0.06 (0.12) |
| LM | -0.11 (-1.44) | -0.97 (-1.83) | 0.56 (2.66) | 0.59 (0.14)*** | LM | -0.00 (-0.00) | -0.17 (-0.65) | 0.12 (1.61) | 0.79 (0.12)*** |

Table 6.4. Models testing for G x Diet Pair interactions within each sex were re-run using data split between the sexes and models for the G x Sex interaction were re-run using data split between the diet pairs. Log-likelihoods were reported for each model and compared using LRT to determine statistical significance (at $P < 0.05$). Significant differences between models are highlighted in bold.

| Data | Model Re-Run | TE LogL | TP LogL | LM LogL |
|-------------|--------------|----------------|----------------|----------------|
| Female | C | -75.05 | 362.35 | 40.16 |
| Female | D | -25.67 | 471.82 | 56.14 |
| Male | C | -13.09 | 339.67 | -384.99 |
| Male | D | -7.68 | 428.78 | -369.34 |
| Diet Pair 1 | E | -172.29 | 172.91 | 73.05 |
| Diet Pair 1 | F | -146.90 | 176.23 | 75.77 |
| Diet Pair 2 | E | 30.87 | 626.840 | -184.17 |
| Diet Pair 2 | F | 34.64 | 634.61 | -177.60 |
| Diet Pair 3 | E | -82.94 | -135.16 | -5.29 |
| Diet Pair 3 | F | -69.26 | -132.17 | -0.39 |
| Diet Pair 4 | E | 92.99 | 214.44 | -230.89 |
| Diet Pair 4 | F | 96.27 | 215.19 | -227.08 |

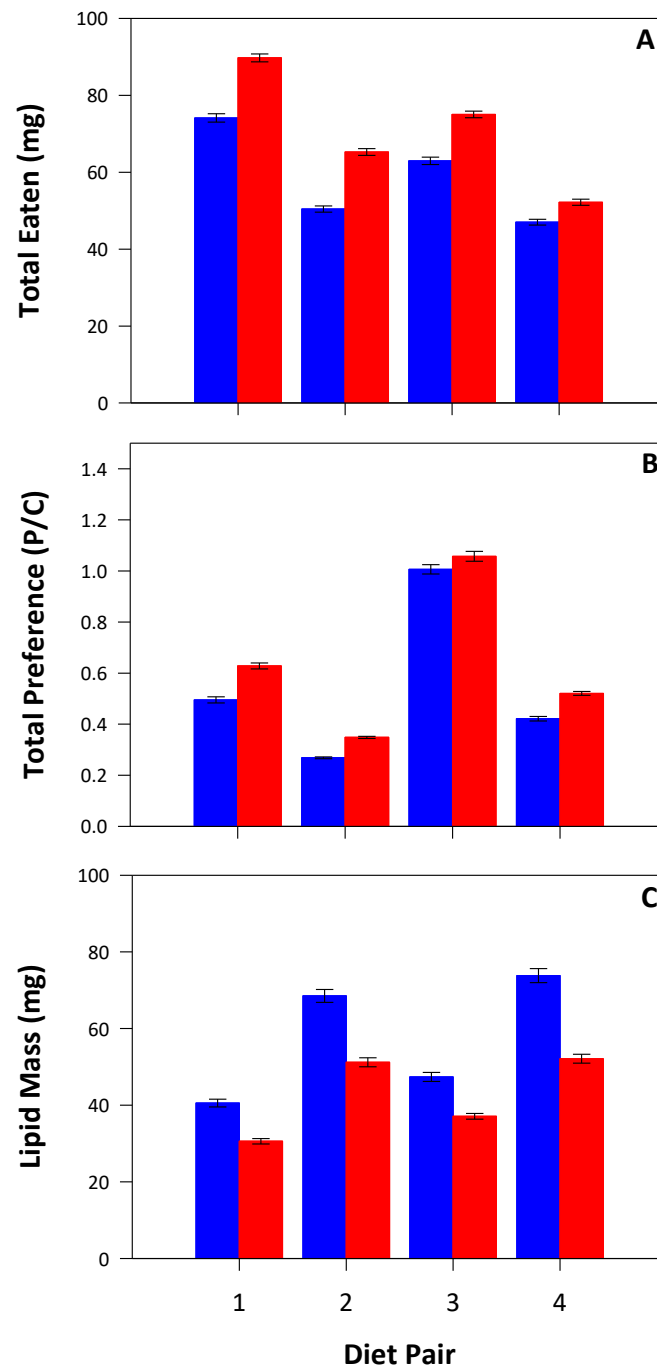


Figure 6.1. The mean (\pm SE) (A) total amount of diet eaten, (B) total nutrient preference (P intake/C intake) and (C) lipid mass for male (blue bars) and female (red bars) *T. commodus* on each of the four experimental diet pairs.

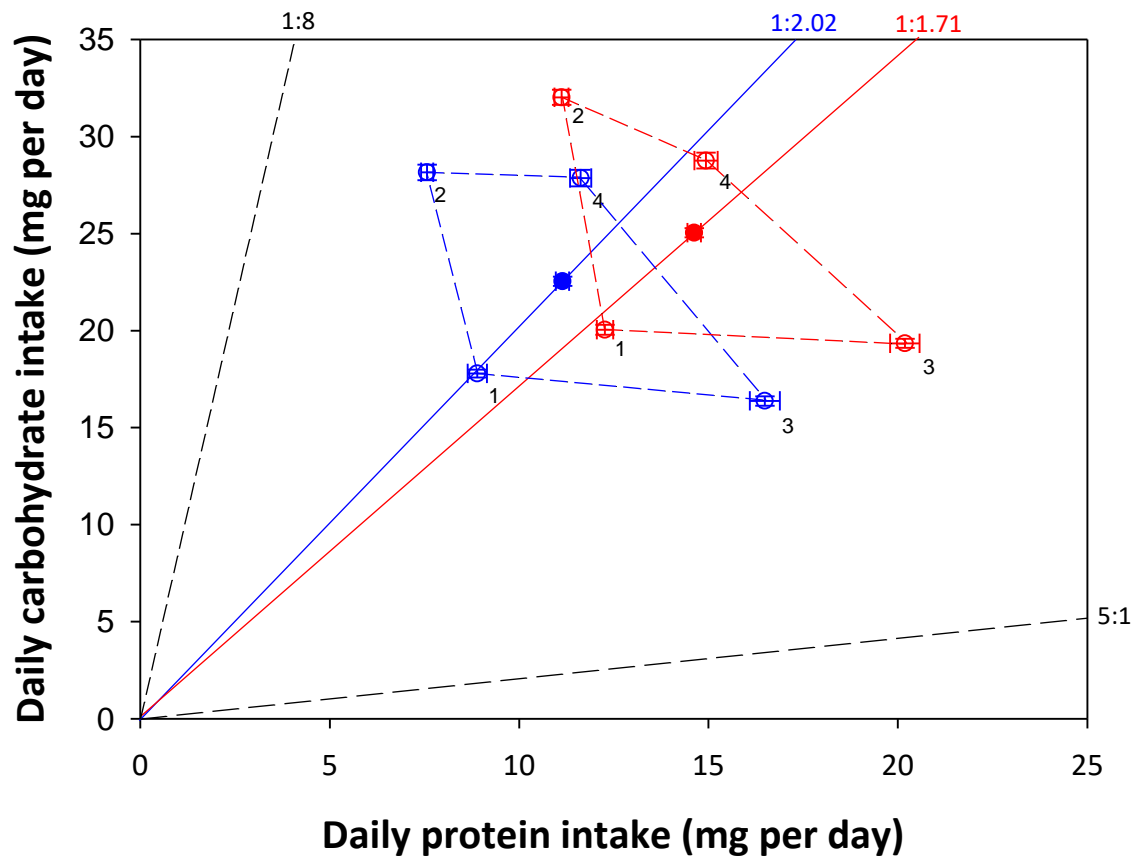


Figure 6.2. The mean (\pm SE) intake of P and C by male (blue symbols) and female (red symbols) *T. commodus*. The open symbols represent the mean intake of nutrients in each of the four diet pairs (denoted by pair number), whereas the solid symbols represent the regulated intake point (RIP), calculated as the mean of the four diet pairs. The solid blue and red lines represent the nutritional rails (lines in nutrient space that represents a fixed intake of nutrients) that passes through the RIP for males (P:C ratio of 1:2.02) and females (P:C ratio of 1:1.71). The black dashed lines (P:C ratios of 5:1 and 1:8) represent the outer nutritional rails of the nutritional landscape.

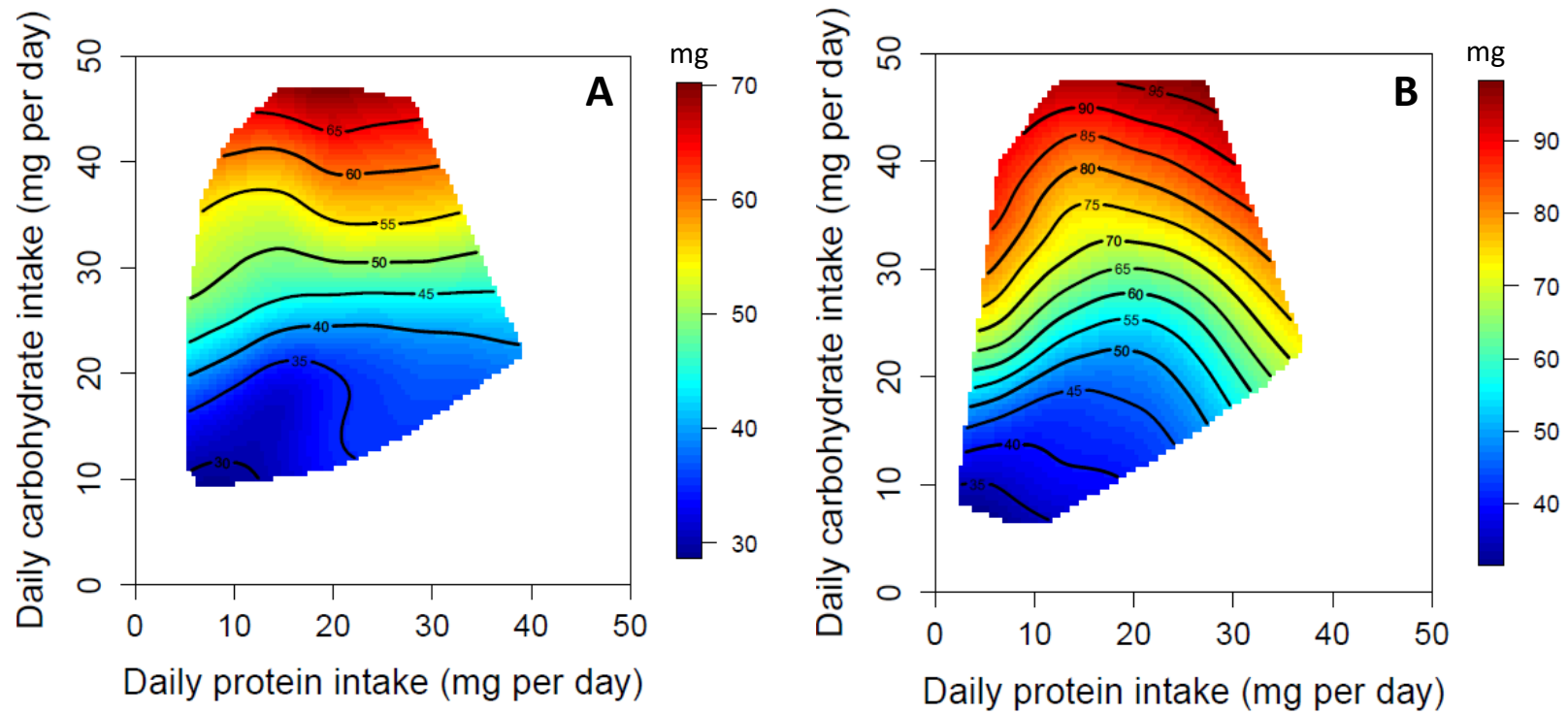


Figure 6.3. Thin-plate spline (contour view) visualizations of the effects of protein (P) and carbohydrate (C) intake on lipid mass in (A) female and (B) male *Teleogryllus commodus*. In each spline, the red regions represent higher values for the measured trait, whereas blue regions represent lower values.

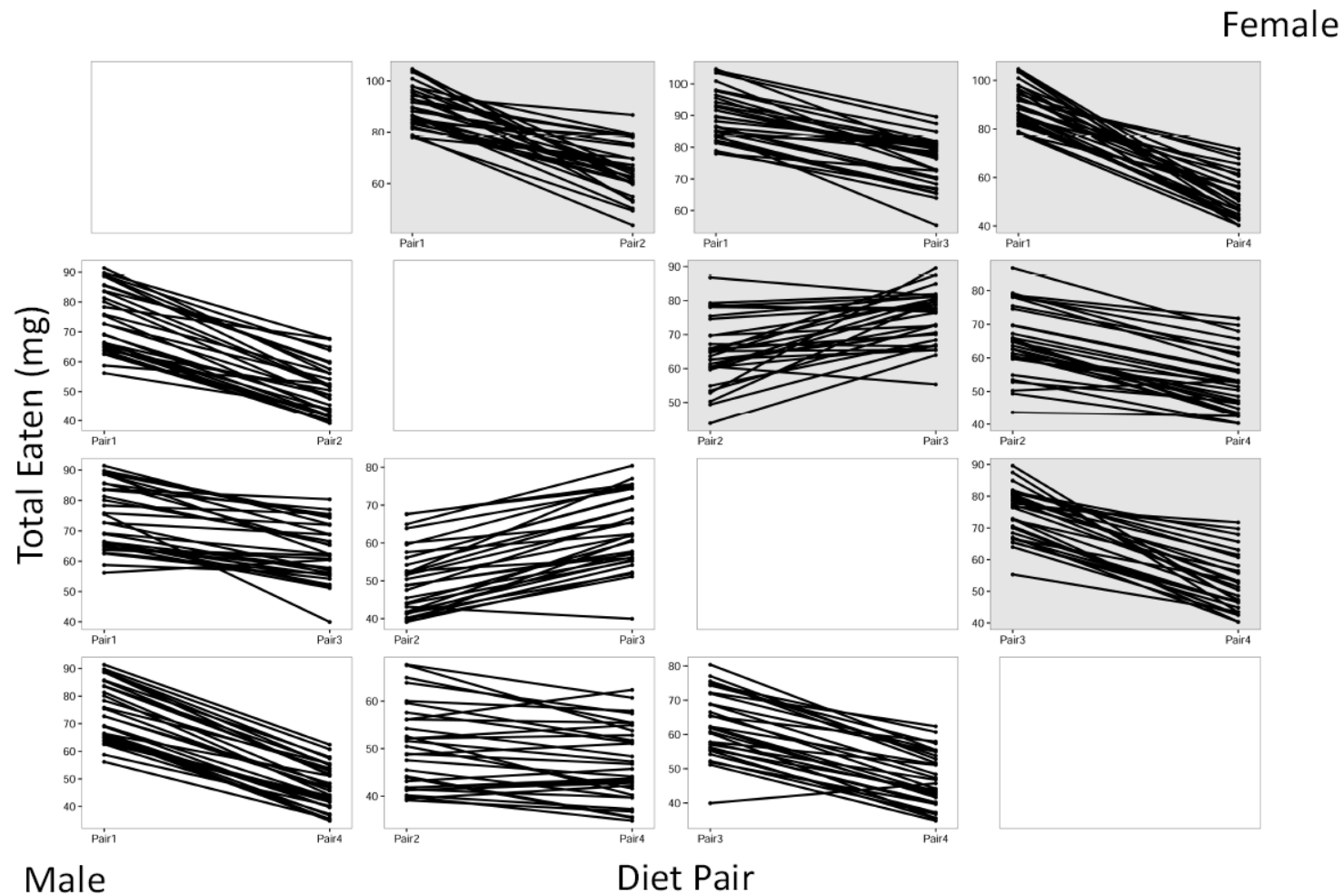


Figure 6.4. Reactions norms illustrating the genotype-by-diet pair interaction (G:DP) for the total amount of diet eaten (TE) in male and female *T. commodus*. Females are presented above the diagonal (grey background) and males beneath the diagonal (white background). In each panel, lines represent the response of a given genotype across two diet pairs.

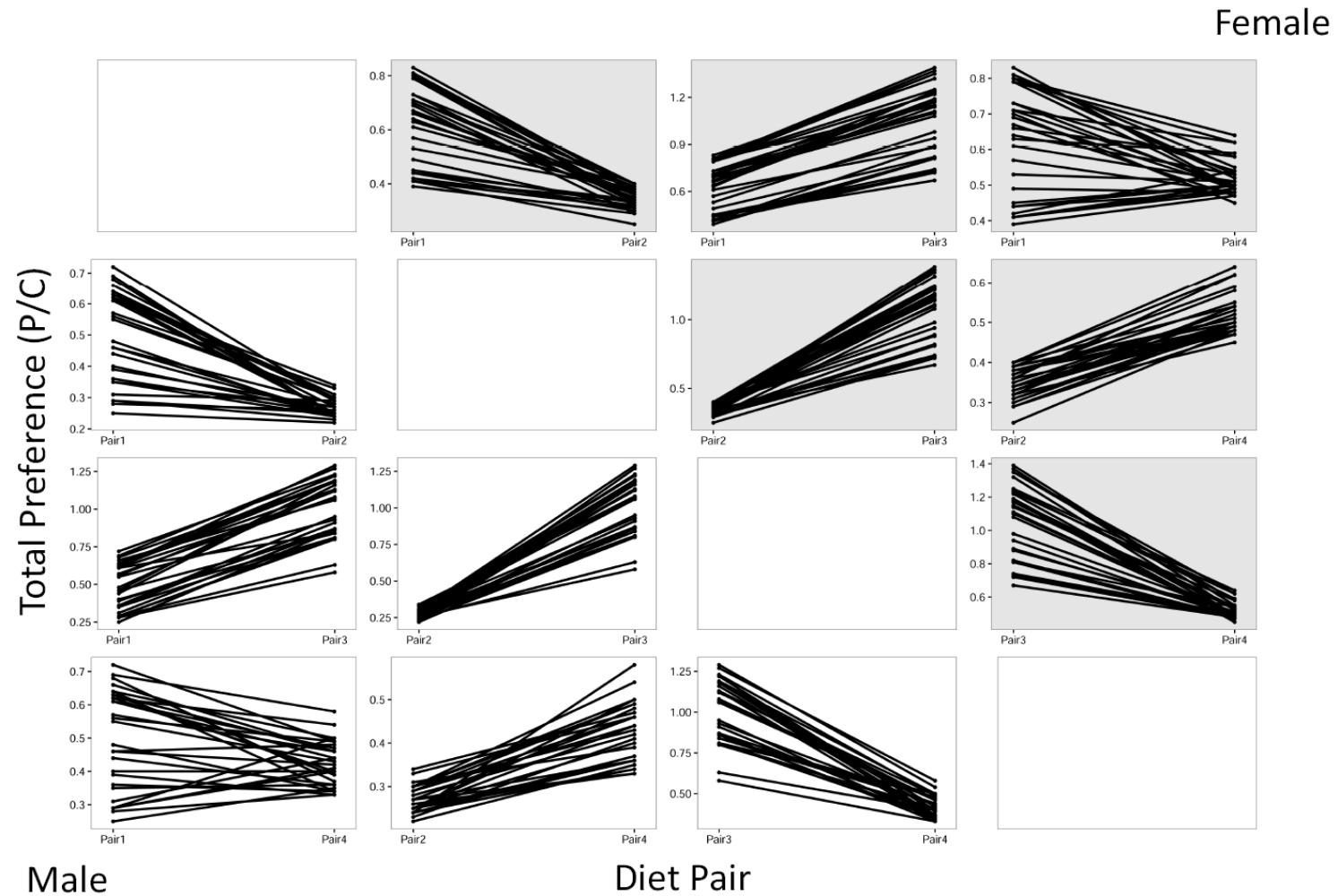


Figure 6.5. Reactions norms illustrating the genotype-by-diet pair interaction (G:DP) for the total nutrient preference (TP, defined as the intake of P divided by the intake of C) in male and female *T. commodus*. Females are presented above the diagonal (grey background) and males beneath the diagonal (white background). In each panel, lines represent the response of a given genotype across two diet pairs.

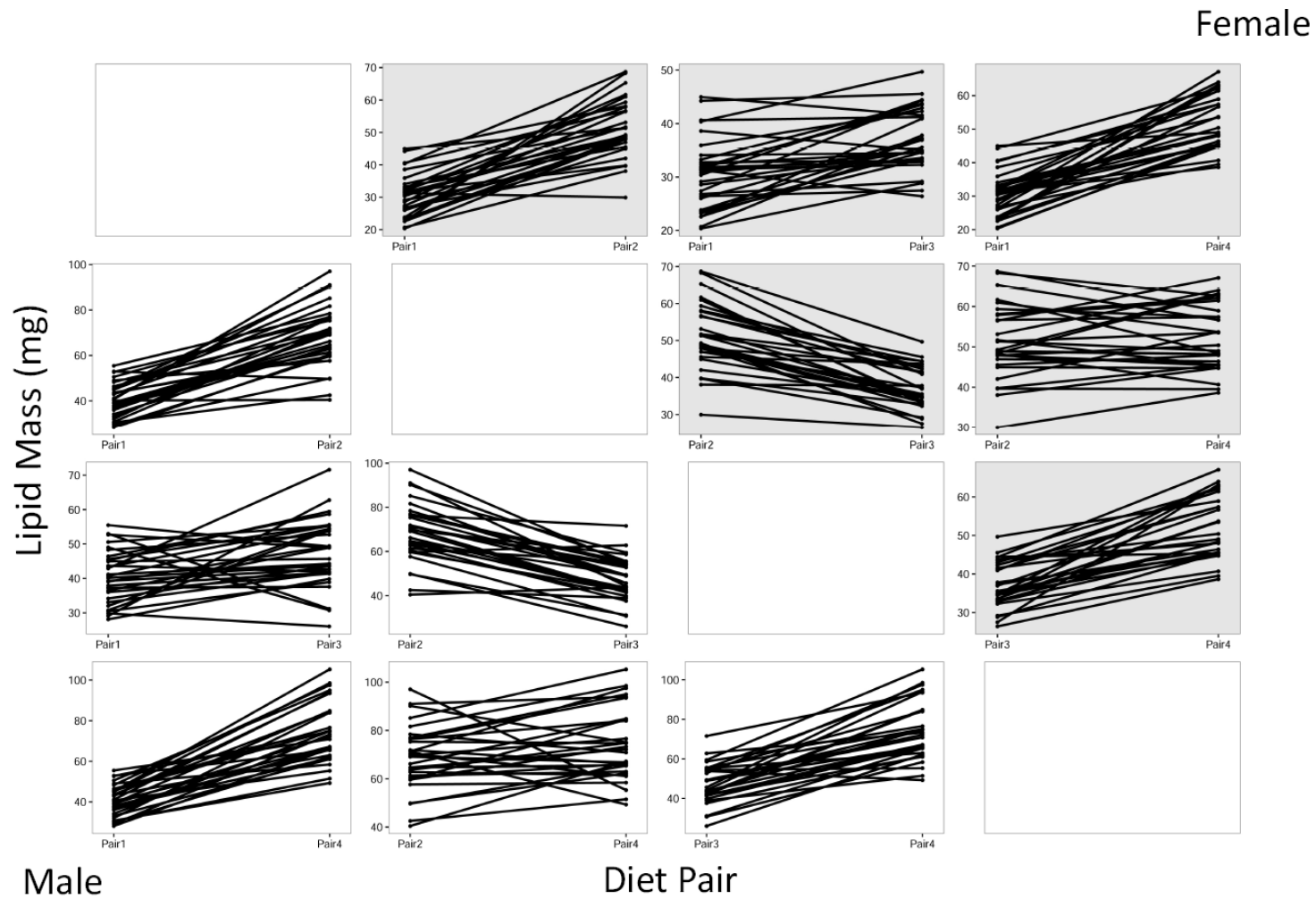


Figure 6.6. Reactions norms illustrating the genotype-by-diet pair interaction (G:DP) for lipid mass (LM) by male and female *T. commodus*. Females are presented above the diagonal (grey background) and males beneath the diagonal (white background). In each panel, lines represent the response of a given genotype across two diet pairs.

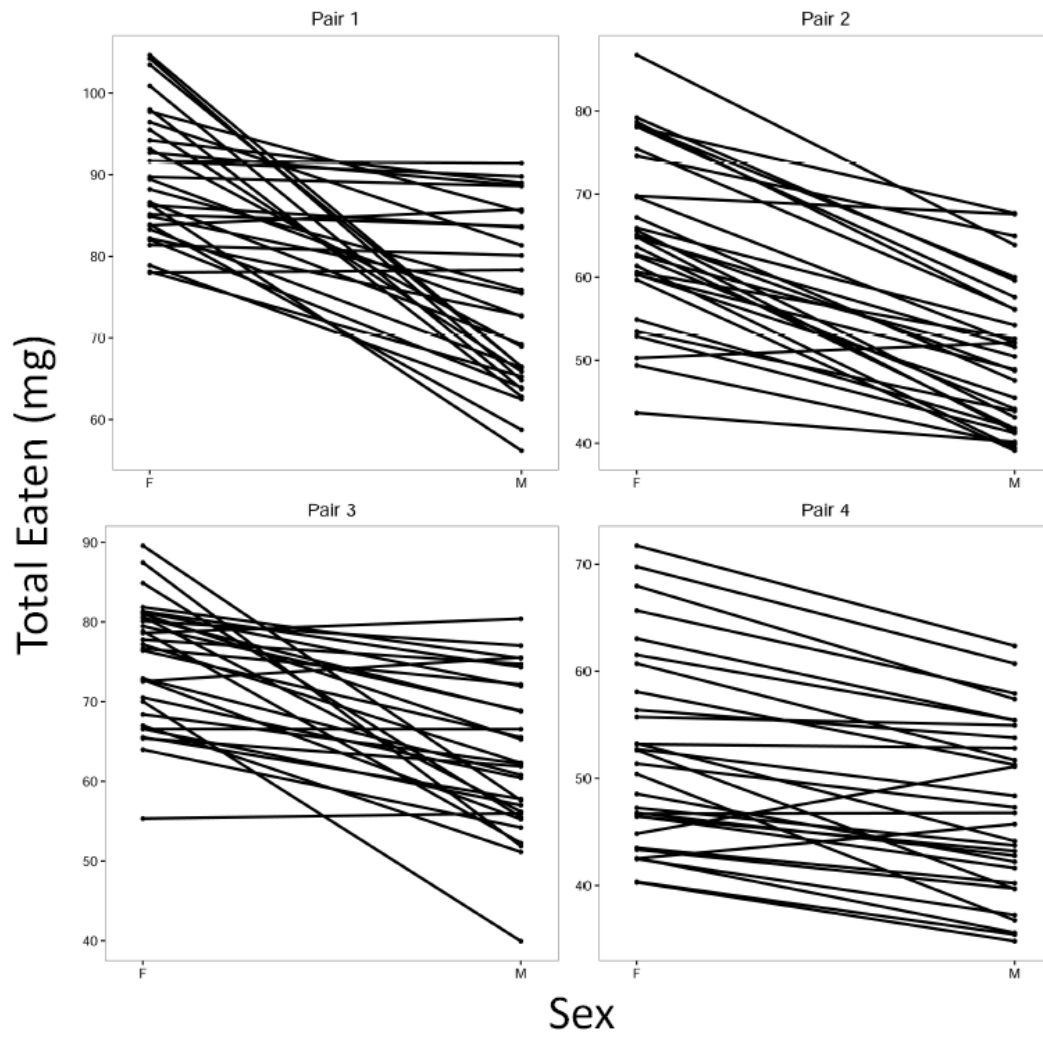


Figure 6.7. Reaction norms illustrating the genotype-by-sex interaction (G:S) for TE in the different diet pairs in *T. commodus*. In each panel, lines represent the response of a given genotype across two diet pairs.

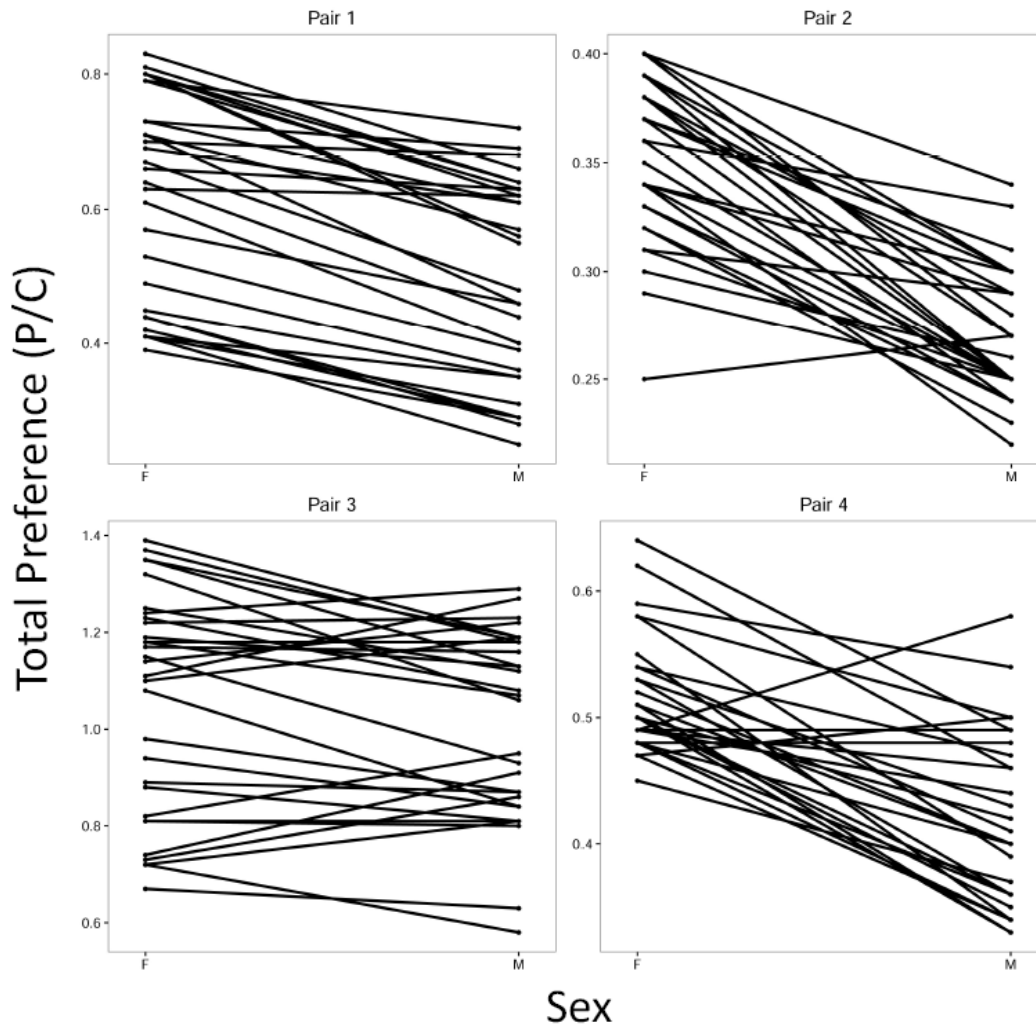


Figure 6.8. Reaction norms illustrating the genotype-by-sex interaction (G:S) for TP in the different diet pairs in *T. commodus*. In each panel, lines represent the response of a given genotype across two diet pairs.

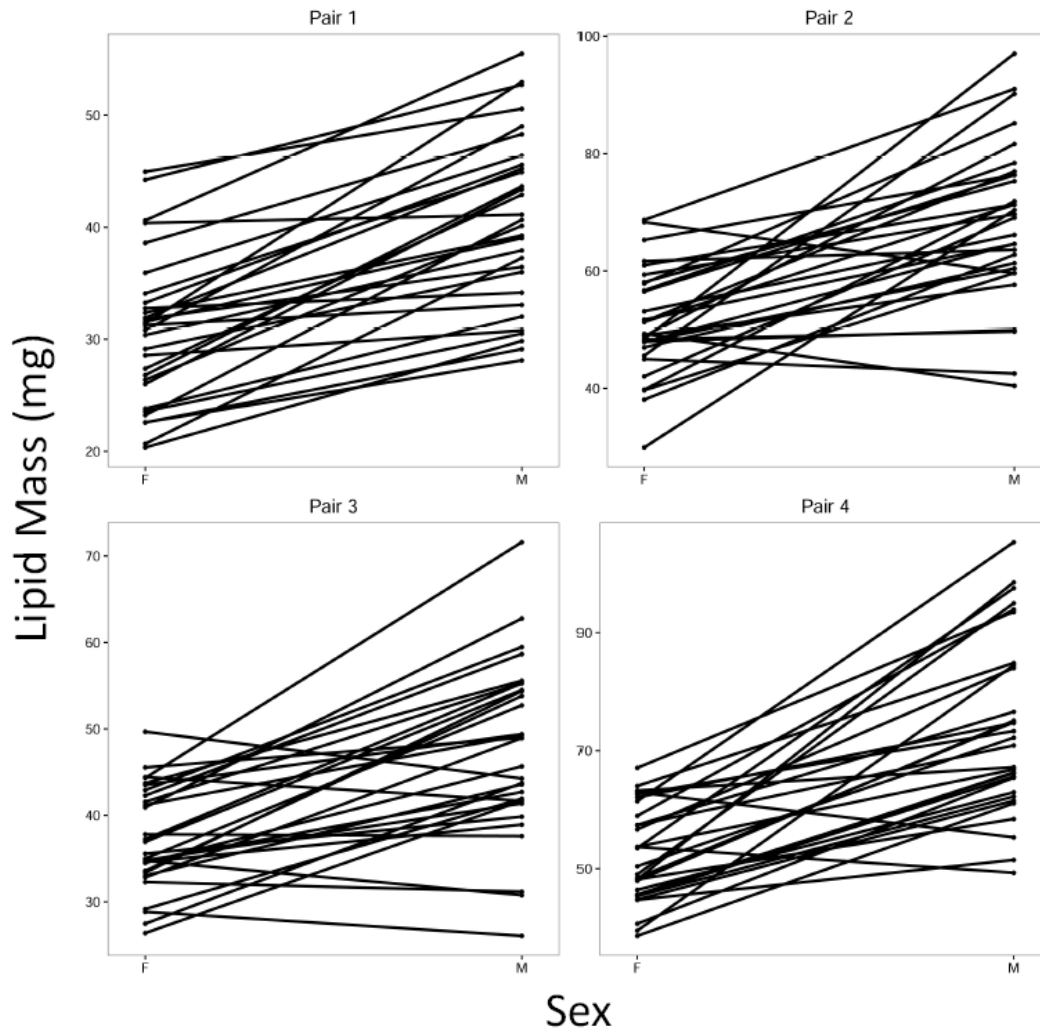


Figure 6.9. Reaction norms illustrating the genotype-by-sex interaction (G:S) for LM in the different diet pairs in *T. commodus*. In each panel, lines represent the response of a given genotype across two diet pairs.

CHAPTER 7:
GENERAL DISCUSSION

The relationship between diet and sexual selection has long been recognised through studies of sexual selection acting on condition dependent sexual traits (Kirkpatrick 1982; Andersson 1994; Rowe and Houle 1996; Andersson and Simmons 2006; Cotton *et al.*, 2006; Kuijper *et al.*, 2012) and dietary trade-offs between traits as predicted by life history theory (Van Noordwijk and de Jong 1986; Partridge and Harvey 1988; Stearns 1989; Stearns 1992; Stearns 2000; Zera 2001). Typically these types of studies have relied upon simple “good vs bad” or “restricted vs *ad libitum*” dietary manipulations. While studies of this type have highlighted the importance of diet on sexual and life history traits, these dietary treatments only offer a crude understanding as to the specific nutrients that are mediating any dietary effects (Hunt *et al.*, 2004; Piper and Partridge 2007; Zajitschek *et al.*, 2009; Piper *et al.*, 2011).

There is a growing understanding of the importance of taking a multifaceted view of diet and nutrition (Maklakov *et al.*, 2008; Archer *et al.*, 2009; Simpson and Raubenheimer 2012). Furthermore, researchers are starting to appreciate that it is the intake of specific nutrients that is important for resource investment in various life history traits and that it is the specific balance of these nutrients that underlies conditional-dependent sexual traits (Lee *et al.*, 2008; Fanson *et al.*, 2009; Cotter *et al.*, 2011; South *et al.*, 2011; Solon-Biet *et al.*, 2014; Bunning *et al.*, 2015; House *et al.*, 2015; Ponton *et al.*, 2015). My work shows that the two macronutrients, protein (P) and carbohydrate (C), are central to the expression of a number of pre- and post-copulatory sexually selected traits that influence male fitness. In the decorated cricket, *Gryllodes sigillatus*, P and C intake is important in determining a male’s attractiveness to a female through their effect on his cuticular hydrocarbon (CHC) expression (Chapter 2). This finding is supported by a large number of published works showing dietary effects on male CHCs and sex pheromones (e.g. Liang and Silverman 2000; Ingleby *et al.*, 2014) but represents the only empirical study, to date, showing the specific nutrients responsible for the dietary effect (but see South *et al.*, 2011). Specifically, I show that CHC expression and male mating success are both maximised at a moderate to high intake of nutrients in a P:C ratio of 1:1.5 (Chapter 2). However, a structural equation modelling approach showed that condition-dependent variation in CHC expression was not the only trait responsible for mediating the relationship between nutrient intake and mating success (Chapter 2). Therefore, the intake of P and C has multiple pathways to influence male mating success in *G. sigillatus* which can potentially lead to nutritional trade-offs if

these different traits require different intakes of these nutrients for maximal expression. Interestingly, male *G. sigillatus* almost perfectly regulated their intake of P and C when given dietary choice to maximise mating success; that is, the regulated intake point for males resided on the peak of the nutritional landscape for mating success. This suggests that when given dietary choice, males are optimising their intake of nutrients to maximise their pre-copulatory attractiveness through multiple pathways of influence, including CHC expression.

In Chapter 3, I showed that the intake of P and C can also have important implications for the outcome of post-copulatory sexual conflict in *G. sigillatus*. The size and amino acid composition of the nuptial gift (the spermatophylax) in *G. sigillatus* is known to increase its gustatory appeal to the female which prevents her from discarding this gift during mating (Gershman *et al.*, 2012). This has important implications for male fitness because as soon as the spermatophylax is discarded, the female removes the external sperm-containing ampulla, terminating mating and thus the transfer of sperm into the female's reproductive organs (Sakaluk 1984, 1985, 2000). The number of sperm transferred to a female is therefore directly proportional to ampulla attachment time (Sakaluk 1984). As sperm competition conforms to a lottery in this species (Eggert *et al.*, 2003), optimizing the appeal of the spermatophylax and the subsequent effects on ampulla attachment time and sperm transfer, has important consequences for male fitness. However, discarding the spermatophylax and prematurely removing the ampulla provides a powerful mechanism for females to exert post-copulatory mate choice (Ivy & Sakaluk 2007). Consequently, producing a large spermatophylax with an appealing amino acid composition is good for male fitness but is likely to come at a cost to female fitness by reducing the capacity to exert an important form of mate choice. This sets the stage for sexual conflict, with the male spermatophylax taking a central role in regulating this process. My empirical work showed that the intake of P and C significantly influences the size and amino acid composition of the spermatophylax, as well as the attachment time of the ampulla (Chapter 3). Importantly, each of these traits (and therefore male fitness) were maximised at a high intake of nutrients in an approximate P:C ratio of 1:1.3. Thus, males consuming this optimal balance of P and C have the potential to bias the outcome of sexual conflict in their favour by regulating the intake of the nutrients. However, when given the choice between alternate diets males regulated their intake of nutrients to be slightly more C biased (at a P:C ratio of approximately 1:1.74) than optimal. This shows that while the intake of nutrients is key to

the outcome of post-copulatory sexual conflict in *G. sigillatus*, males are not optimally regulating their intake of nutrients to maximise the traits that help them win this conflict. It also suggests that males are prioritizing their intake of nutrients to either maximise other important life-history trait(s) or to attempt to balance the maximisation of several of these traits. Chapter 2 demonstrates that CHC expression and traits that determine pre-copulatory mating success are likely candidates.

Trade-offs (a negative covariance between phenotypic traits) are central to life history theory and the existence and magnitude of trade-offs between important life-history traits is often viewed as the result of how individuals acquire resources and allocate them to different, competing traits (Van Noordwijk and de Jong 1986; Stearns 1989; Roff 2002; Flatt and Heyland 2011). When resource acquisition is low, there are less resources to allocate to competing traits leading to trade-offs between life-history traits (Van Noordwijk and de Jong 1986; Stearns 1989; Roff 2002; Flatt and Heyland 2011). The “resource” most commonly studied in life-history studies is nutrients provided through the diet (Stearns 1989; Roff 2002; Flatt and Heyland 2011). However, most life-history studies using dietary manipulation either contrast a good and bad diet or provide restricted access to a single diet. In both cases, the nutrient composition and caloric content of the diets used are often poorly defined and the intake of diets is not accurately measured. This makes it difficult (if not impossible) to relate the acquisition and allocation of key nutrients to life-history. The Geometric Framework (GF) offers a novel method for quantifying how the exact intake of nutrients influences the expression of life-history traits and how they may be subject to nutritionally-based trade-offs and has already been used to demonstrate that nutrients play an important role in regulating life-history trade-offs (e.g. Lee *et al.*, 2008; Maklakov *et al.*, 2008; Jensen *et al.*, 2015). However, there is currently no robust theoretical framework for formally documenting the existence or strength of nutritionally-based trade-offs when using the GF.

In Chapter 4, I started by developing a framework for quantifying the strength of nutritionally-based trade-offs between life-history traits and provide the statistical tools (in R language) needed to apply this framework. I show that a nutritionally-based trade-off occurs when the 95% confidence region (CR) of the global optima on the nutritional landscape for each trait are not overlapping. Furthermore, I demonstrate that the magnitude of this trade-off can be quantified using the angle (θ) between the linear

nutritional vectors and the distance (d) between the global maxima for each life-history trait. Next, I use this framework to empirically examine the nutritionally-based trade-off between immune function and reproductive effort in male and female *G. sigillatus*. I show that the intake of P and C has significant yet divergent effects on these important life-history traits in the sexes. In females, encapsulation ability and egg production both peaked at a high intake of nutrients with a P:C ratio of 1.04:1 and 1:1.17, respectively. In contrast, encapsulation ability and nightly calling effort in males peaked at a high intake of nutrients in a P:C ratio of 5.14:1 and 1:7.08, respectively. Consequently, as females are able to maximise both immune function and reproductive effort at the same intake of nutrients, the trade-off between these traits is weak, whereas because males are unable to maximise immune function and reproductive effort at the same intake of P and C, the trade-off between these traits is much larger. This sexual dimorphism in the magnitude of the trade-off between immune function and reproductive effort is also verified from my theoretical framework with males (but not females) showing non-overlapping 95% CRs on the global maxima for these two traits and larger values of θ and d . When given dietary choice, neither sex optimally regulated their intake of nutrients, although the regulated intake point of females (P:C = 1:1.84) was more closely aligned with the optima for immune function and reproductive effort than in males (P:C = 1:2). This poor alignment suggests that males may be attempting to regulate their intake of nutrients to balance the expression of both immune function and reproductive effort (i.e. giving neither trait exclusive priority). Collectively, my work provides a novel and robust framework that has the potential to revolutionize the way that nutritionally-based life-history trade-offs are studied. As my framework provides parameters (θ and d) that quantify the magnitude of any trade-off between traits in standardized units, they are directly comparable across different traits or species (or sexes within species) being studied.

In most species, the sexes have divergent reproductive strategies that leads to sex differences in the intensity of sexual selection (Trivers 1972) which, in turn, is known to drive the evolution of sex differences in life-history strategies (Andersson 1994; Andersson and Simmons 2006; Hosken and House 2011) that place different nutritional demands on males and females (Simpson & Raubenheimer 2012). Indeed, sex-specific nutritional optima have now been shown for a number of important life-history traits, including reproductive effort (Maklakov *et al.*, 2008; Jensen *et al.*, 2015; Chapter 4), lifespan (Maklakov *et al.*, 2008)

and immune function (Chapter 4). The existence of sex-specific nutritional optima should, according to optimal foraging theory (Stephens & Krebs 1986), select for males and females to independently regulate their intake of nutrients to reach these divergent optima. This will not be the case, however, if the genes that control nutrient regulation in males and females are linked. Whenever there is divergent selection on shared traits in the sexes (such as nutrient intake), yet the genes governing these traits are linked across the sexes, intralocus sexual conflict (ISC) will occur and this may prevent the sexes from evolving independently to their sex-specific optima (Bonduriansky and Chenoweth 2009).

While many empirical examples of ISC exist (e.g. Lewis *et al.*, 2011; Mills *et al.*, 2012; Tarka 2013; Berger *et al.*, 2014; Buzatto *et al.*, 2015), very little is known about the potential for ISC over nutrient intake to influence the evolution of key life-history traits. Indeed, to my knowledge, only three studies have currently examined this topic, with each being limited in their scope (Maklakov *et al.*, 2008; Reddiex *et al.*, 2013; Jensen *et al.*, 2015). Both Maklakov *et al.* (2008) and Jensen *et al.* (2015) showed sex-specific nutritional optima for lifespan and reproductive effort in the black field cricket (*Teleogryllus commodus*) and *D. melanogaster* respectively, as well as demonstrating that the sexes have a common regulated intake point (i.e. no sexual dimorphism) in these species. While this suggests the potential for ISC over the optimal intake of nutrients, neither study examined the underlying genetics of nutrient intake in the sexes and therefore cannot unambiguously demonstrate the operation of this process. Reddiex *et al.* (2013) examined the potential for ISC over the optimal intake of nutrients for reproduction in *D. melanogaster*. Despite showing sex-specific nutritional optima for reproduction and significant genetic (co)variance between the sexes for the intake of P and C, Reddiex *et al.* (2013) concluded that there was little scope for ISC in this species. This study, however, only examined the genetics of dietary choice for a single diet pair and only for a short, 4 day period which limits the scope of these findings. More research is clearly needed to determine the potential for ISC over nutrient intake to influence the evolution of differences in important phenotypic traits shared by the sexes.

In Chapter 5, I examined the potential for ISC over the optimal intake of P and C for lifespan and reproduction in male and female *T. commodus*. Compelling evidence for ISC over the optimal intake of nutrients requires that the sexes show sex-specific nutritional optima for lifespan and reproduction and that the intake of P and C are positively genetically correlated across the sexes. In agreement with this, I found that male and female *T.*

commodus have different nutritional optima for both lifespan and reproduction. Males were shown to maximise lifespan and nightly calling effort at a high intake of nutrients in a P:C ratio of 1:3 and 1:8, respectively. In contrast, females were shown to maximise lifespan and daily egg production at a P:C ratio of 1:5 and 1:1, respectively. Furthermore, using a split-brood, half-sib quantitative genetic breeding design I showed that the regulated intake point for P and C (calculated as the mean intake of these nutrients across diet pairs) under dietary choice contained significant genetic (co)variation within the sexes and there was also significant positive genetic covariance between the sexes. Despite, demonstrating these key parameters of ISC, I show that the across-sex genetic covariance (**B**) had very little effect on the predicted response of nutrient regulation in the sexes and that rather than constraining the predicted response, **B** accelerated the predicted response of nutrient regulation. The structure of the additive genetic variance-covariance matrix for the regulated intake of nutrients within males and females (**G_m** and **G_f**, respectively) appeared to play more of a role in constraining the predicted response of nutrient regulation in the sexes. Furthermore, I found evidence of independent nutrient regulation between the sexes. This suggests that while the potential for ISC over the optimal intake of nutrients in *T.commodus* exists, it is likely to be weak, with the finding of divergent nutrient regulation in the sexes suggesting that ISC may be in the initial steps of resolution. Collectively, my work represents the most complete study to date showing the importance of nutrients in determining the evolution of sex differences in lifespan and reproduction.

In general, our understanding of the genetics of nutrient regulation is still incredibly limited. Optimal foraging theory predicts that animals will regulate their intake in a nutritionally imbalanced environment through compensatory feeding on a given diet or foraging from multiple sources (Stephens and Krebs 1986) but few studies have looked at how genes, and especially the interaction between genes and the nutritional environment, may regulate this behaviour and the consequences this has for the expression of important traits that are contingent on this behaviour. For example, the protein leverage hypothesis (PLH) theorises that animals increase their intake of C to maintain a constant intake of P when P is a limited nutrient in the diet (Simpson and Raubenheimer 2005) and studies in mice (Sorensen *et al.*, 2008) and humans (Martens *et al.*, 2013; Gosby *et al.*, 2014) have shown that this increased consumption of C can have numerous long-term health risks linked to obesity (Raubenheimer *et al.*, 2015). Whether or not individuals are genetically

pre-disposed to over-consume C when presented with a nutritionally imbalanced diet and whether these genes are linked to those for obesity has not yet been examined.

In Chapter 6, I use a half-sib breeding design to examine the quantitative genetics of total dietary consumption (TE), nutrient preference (TP) and lipid mass (LM) when male and female *T. commodus* from the same families were split and presented with alternate pairs of imbalanced diets. Using an animal model, this enabled me to examine genotype-by-diet pair and genotype-by-sex interactions for these three traits, as well as estimates of heritability (h^2) and genetic correlations (r_A) between traits, within each diet pair and for each sex. I demonstrated significant genotype-by-diet pair and genotype-by-sex-by-diet pair interactions for all three traits and significant genotype-by-sex interactions for TE and LM but not TP. There was also abundant genetic variance in these traits that differed across diet pairs and the sexes. There was also significant genetic covariance between traits within diet pairs and the sexes suggesting that these traits are genetically linked. Importantly, there were negative genetic correlations between the total amount of diet consumed and the total preference for nutrients in a diet pair (with negative values meaning more C chosen) and the extent of these genetic correlations was larger in males than females. Collectively, my work shows that lipid mass (as a proxy for obesity) is genetically determined but the extent depends on the nutritional environment and the sex of the individual. Furthermore, the significant genetic correlations between lipid accumulation and feeding behaviours (especially the preference and total consumption of C) indicate a mechanistic link between the genes that govern these traits. This ultimately means that although individuals are genetically susceptible to obesity, the expression of this trait is also determined by the underlying genes that control feeding behaviours.

Collectively, my thesis is novel in bringing together several core evolutionary fields (nutritional ecology, sexual selection and conflict, life history theory and quantitative genetics) to demonstrate the multidimensional complexity of nutrition and the far reaching evolutionary consequences that nutrition may have. However, there is still much scope for further investigation to build upon the findings of this work. One avenue that needs further work is the expansion of the GF used in this thesis. My work has focused on two macronutrients: P and C, but dietary intake in both the wild and many laboratory settings are not limited to just these two nutrients. A simple expansion would be the use of the third major macronutrient, lipid (L), especially in conjunction with P. Such an expansion could be

particularly useful when investigating nutritionally imbalanced environments and the underlying genetic architecture of obesity and its associated health problems. P and L combinations have been used successfully in previous geometric studies that have looked at the optimal intake of nutrients in predatory ground beetles (Jensen *et al.*, 2012), mink (Mayntz *et al.*, 2009), and wolf spiders (Jensen *et al.*, 2013). The addition of L into the GF would provide further insight into the nature of an animal's dietary preference and would expand our understanding into the effect of an additional macronutrient on life history-traits. However, at present these dietary manipulations are still limited to comparing the intake of just two macronutrients. A specific focus for future development of the GF should, therefore, be the design and production of diets that vary in all three macronutrients, with corresponding analyses that examine the potentially complex interactions between these macronutrients.

The expansion of current analyses to incorporate three macronutrients can be easily accomplished using standard selection analyses (Lande and Arnold 1983), but it is the visualisation of the effect of all three nutrients on a given trait that remains challenging. A possible solution comes in the form of 'right-angled mixture triangles' suggested by Raubenheimer (2011) but there are a number limitations to this approach. The biggest limitation is that a mixture triangle relies on nutrient proportions, rather than using actual recorded values of nutrient intake, to visualise data. As a result, mixture triangles must take cross sections to view the different effects of nutrient concentration on traits (e.g. a low versus high nutrient concentration are often compared). This makes it difficult to visualize the important (often nonlinear) effect that nutrient concentration has on trait expression. Consequently, while mixture triangles provide an overall idea of the 'big picture' effect of nutrients on traits, such figure would lack the real data 'depth' currently seen in two nutrient analyses and conventional nutritional landscapes. One approach that is likely to prove useful is using canonical analysis of the quadratic and correlational nutritional effects (referred to as the γ matrix in Phillips & Arnold, 1989). This approach has been used with great success in multivariate selection studies (e.g. Chapter 2) and works by extracting the major vectors of selection acting on combinations of the original traits. Typically, there will be fewer vectors of significance extracted than the original numbers of traits being examined and these can be easily visualized using thin-plate splines. Consequently, this

approach could also be used to reduce the effects of more than two nutrients into fewer dimensions for visualization.

Mixture triangles, however, do have the potential to form the basis of a link between lab and field studies of nutritional ecology (Raubenheimer 2011). A common criticism of the GF, and indeed dietary manipulations in general, is that it is difficult to compare the findings of a controlled laboratory experiment to the natural feeding environments of animals in the wild. It is often impractical or impossible to collect accurate measures of nutrient intake in the wild, with field studies typically relying on measures of the proportion of nutrients utilised as the differences between the nutrient concentration of food eaten and the faeces (Crossman *et al.*, 2005; Grant and Scholes 2006; Felton *et al.*, 2009; Raubenheimer 2011). The use of mixture triangles as recommended by Raubenheimer (2011) would, therefore, provide a useful tool for these wild studies and help simplify comparisons to laboratory studies. A potential future avenue of work to improve our understanding of nutrient utilisation in the wild would be the use of stable isotope analysis to calculate the isotopic values of, for example $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in an animal's tissue and common food sources. The use of mixture models would allow one to partition the contribution of food sources to the isotopic composition of specific animal tissue (Phillips 2012) and, therefore, provide an estimate for the amount the carbon and nitrogen (proxies for carbohydrate and protein) (DeNiro 1978; DeNiro and Epstein 1981) eaten by an animal in the wild. The popularity of mixture models in field studies is steadily increasing (Lehmann *et al.*, 2013; Pacella *et al.*, 2013; Hobson and Quirk 2014; Voigt *et al.*, 2015) and provides much scope for further developments to help understand nutrient utilization on both laboratory and field studies.

Expansion of the GF does not have to be limited to just additional macronutrients, it can also be extended to include the manipulation of micronutrients. Previous studies, and the work I present in my thesis (Chapter 4 and 5), has shown that reproductive effort and lifespan can be maximised on diets with different P:C ratios and total nutrient concentrations (Lee *et al.*, 2008; Maklakov *et al.*, 2008; Jensen *et al.*, 2015). The addition of the essential amino acid methionine to diets has been shown to alter lifespan in *D. melanogaster* and some rodent species (Miller *et al.*, 2005; Zid *et al.*, 2009) and interestingly the addition of methionine has been shown to restore egg production in long lived fruit flies fed a calorie restricted diet (Grandison *et al.*, 2009). Despite evidence of interactions between macro- and micronutrients, only one study to date has specifically tested these

interactions using the powerful response surface methodologies provided by the GF (Archer *et al.*, 2015). This study, however, found that supplementation of DL-alpha-tocopherol (vitamin E) did not improve lifespan or reproductive effort in the black field cricket, *T. commodus*. Clearly further work is warranted to investigate the interactions between macro- and micronutrients, their role in sexual selection and their impact in mitigating life history trade-offs, such as between lifespan and reproduction.

In addition to examining the role of macro- and micronutrients on sexual selection and life history trade-offs, a further potential avenue for research would be to look at the specific effects of nutrients on the underlying mechanisms that regulate life history trade-offs. A number of mechanisms have been investigated for their role in trade-offs between longevity and reproductive effort (Flatt & Hayland 2011). For example, the insulin and target of rapamycin (TOR) signalling pathways have been shown to influence trade-offs between reproductive effort and lifespan (Flatt and Heyland 2011). In *Drosophila*, reduced insulin signalling has been shown to extend longevity but at the cost of reduced fertility (Clancy *et al.*, 2001) while mutations of the TOR pathway have also been shown to extend lifespan (Kapahi *et al.*, 2004), although any effect on fertility is currently unknown. Both pathways have been shown to be influenced by diet but typically such results are found through the use of dietary restriction studies (Marden *et al.*, 2003; Nässel *et al.*, 2013) and no study has yet used the GF to look for specific nutrient effects on these underlying mechanisms. Similarly, studies on oxidative damage have also highlighted the potential for increased reproductive effort to cause increased oxidative damage (Alonso-Alvarez *et al.*, 2004; Alonso-Alvarez 2006; Blount *et al.*, 2015) and thus have a negative effect on lifespan (Monaghan *et al.*, 2009; Metcalfe and Alonso-Alvarez 2010; Heidinger *et al.*, 2012). Studies looking at dietary effects are, however, limited and the only study to specifically use the GF found only limited evidence of a dietary effect on oxidative damage (Archer *et al.*, 2015).

In conclusion, my work shows the importance of taking a multidimensional view of nutrition when examining how diet can influence sexual selection and sexual conflict and the resulting consequences these processes have for sex-specific trait expression and in mediating trade-offs between key life-history traits. Empirical research has only just started to scratch the surface into this complexity and there is still incredibly scope for future development, especially using the GF as a robust guide to include the study of more macro- and micronutrients, as well as mapping key regulatory mechanisms known to influence

sexual selection and life-history trade-offs into nutrient space. The research I present in my thesis provides an important first step in understanding this complexity that I hope will inspire and catalyse further research into this area.

APPENDIX 1: PROTOCOL FOR THE MANUFACTURE OF GEOMETRIC FRAMEWORK DIETS

Each diet consisted of the three proteins; casein, albumen and peptone in a 3:1:1 ratio and the digestible carbohydrates; sucrose and dextrin in a 1:1 ratio. All diets contained Wesson's salts (2.5%), ascorbic acid (0.28%), cholesterol (0.55%) and vitamin mix (0.18%) in equal amounts. To this mixture of proteins, carbohydrates and micronutrients, each diet was diluted as required through the addition of crystalline cellulose which is indigestible to the majority of insects (Martin *et al.*, 1991).

The vitamin mix was made first by weighing out individual vitamins into a small weighing boat using a clean micro-spatula and a microbalance before mixing together using a mortar and pestle. This mixture was stored at -20°C in a sealed container until it was needed. The main body of each diet was made by adding the required amounts of cellulose and casein to large glass beakers. Equal amounts of cholesterol were then weighed out into smaller glass beakers to which linoleic acid was added using a pipette. This cholesterol/linoleic acid mixture was dissolved thoroughly in chloroform and poured into the dry cellulose/casein mix. The diets were left in a fume hood and stirred regularly over 24 hours to allow the chloroform to evaporate. After 24 hours, the required amounts of Wesson salt's, sucrose, dextrin, peptone, albumin and ascorbic acid were added to the large glass beakers. The required amount of vitamin mix was then weighed out into smaller glass beakers and dissolved in a 20% ethanol solution and poured into the dry diet mix. Each ingredient was weighed out using clean spatulas and weighing boats for each new ingredient and the diets were stirred thoroughly on the addition of each ingredient.

Diets were blended in a domestic kitchen food processor for approximately 2 minutes before being put into a Pyrex baking tray and dried in a drying oven at 30°C. Diets were removed from the drying oven and blended every 24 hours until dry. Dry diets were ground using a centrifugal mill into a homogenous fine powder and stored at -20°C in sealed containers until there were needed.

APPENDIX 2: SEQUENTIAL MODEL BUILDING APPROACH TO COMPARE THE NUTRITIONAL LANDSCAPES OF MEASURED TRAITS

I used a sequential model building approach to assess whether the linear and nonlinear effects of protein and carbohydrate ingestion differed for my response variables (Draper and John 1988; Chenoweth and Blows 2005). As the different response variables (spermatophylax weight, spermatophylax attractiveness and ampulla attachment time) were measured in different scales, it was necessary to standardize them for statistical comparison. Prior to comparison, I therefore standardized each response variable and nutrient intake to a mean of zero and standard deviation of one using a Z- transformation. I then included a dummy variable, response type (RT), in a reduced model containing only the standardized linear terms:

$$R = \beta_0 + \alpha_0 RT + \sum_{i=1}^n \beta_i N_i + \varepsilon \quad (\text{Eq.1})$$

where R is the standardized response variables, N_i refers to the intake of the i th nutrient, n represents the number of nutrients contained in the model and ε is the unexplained error. From (1), the unexplained (i.e. residual) sums of squares for this reduced model (SS_r) was compared to the same quantity (SS_c) from a second (complete) model that included all of the terms in (1) with the addition of the terms $\alpha_i N_i RT$ which represents the linear interaction of RT and the i th nutrient.

$$R = \beta_0 + \alpha_0 RT + \sum_{i=1}^n \beta_i N_i + \sum_{i=1}^n \alpha_i N_i RT + \varepsilon \quad (\text{Eq.2})$$

A partial F -test (Bowerman and O'Connell 1990) was used to compare SS_r and SS_c from (Eq.1) and (Eq.2) respectively:

$$F_{a,b} = \frac{(SS_r - SS_c)/a}{SS_c/b} \quad (\text{Eq.3})$$

where a is the number of terms that differ between the reduced and complete model and b is the error degrees of freedom for SS_c .

To test whether the quadratic effect of nutrient intake differed between response variables, the SS_r from the reduced model:

$$R = \beta_0 + \alpha_0 RT + \sum_{i=1}^n \beta_i N_i + \sum_{i=1}^n \alpha_i N_i RT + \sum_{i=1}^n \beta_i N_i^2 + \varepsilon \quad (\text{Eq.4})$$

was compared to the SS_c of the complete model:

$$R = \beta_0 + \alpha_0 RT + \sum_{i=1}^n \beta_i N_i + \sum_{i=1}^n \alpha_i N_i RT + \sum_{i=1}^n \beta_i N_i^2 + \sum_{i=1}^n \alpha_i N_i^2 RT + \varepsilon \quad (\text{Eq.5})$$

using (Eq.3).

To test whether correlational effects of nutrient intake on response variables differed, the SS_r from the reduced model:

$$R = \beta_0 + \alpha_0 RT + \sum_{i=1}^n \beta_i N_i + \sum_{i=1}^n \alpha_i N_i RT + \sum_{i=1}^n \beta_i N_i^2 + \sum_{i=1}^n \alpha_i N_i^2 RT + \sum_{i=1}^n \sum_{j \geq 1} \beta_{ij} N_i N_j + \varepsilon \quad (\text{Eq.6})$$

was compared to the SS_c of the complete model:

$$R = \beta_0 + \alpha_0 RT + \sum_{i=1}^n \beta_i N_i + \sum_{i=1}^n \alpha_i N_i RT + \sum_{i=1}^n \beta_i N_i^2 + \sum_{i=1}^n \alpha_i N_i^2 RT + \sum_{i=1}^n \sum_{j \geq 1} \beta_{ij} N_i N_j + \sum_{i=1}^n \sum_{j \geq 1} \alpha_{ij} N_i N_j RT + \varepsilon \quad (\text{Eq.7})$$

using (Eq.3).

In summary, the comparison of model (Eq.1) versus (Eq.2), (Eq.4) versus (Eq.5), and (Eq.6) versus (Eq.7) provides a test for the overall significance of the interaction between response type and the linear, quadratic and correlational effects of nutrient intake, respectively. Therefore, significant differences in these model comparisons (as detected with a partial F -test) demonstrate that the linear, quadratic and/or correlational effects of nutrient intake on the response variables differ, respectively. I also inspected the interaction of individual nutrients with the response variable terms from the full model (Eq.7) to determine which of the nutrients were responsible for the significance of the overall partial F -test.

APPENDIX 3: CALCULATING THE ANGLE (θ) BETWEEN NUTRITIONAL VECTORS AND 95% CONFIDENCE INTERVALS

I calculated the angle (θ) between the linear vectors for the two response variables being compared as:

$$\theta = \cos^{-1} \left(\frac{a \cdot b}{\|a\| \|b\|} \right) \quad (1)$$

where a is the linear effects of P and C intake in the first response variable being compared, b is the linear effects of these nutrients for the second response variable, $\|a\| = \sqrt{a \cdot a}$ and $\|b\| = \sqrt{b \cdot b}$. When $\theta = 0^\circ$ the vectors are perfectly aligned and the optima for the two response variables reside in the same location in nutrient space, whereas $\theta = 180^\circ$ represents the maximum possible divergence between these vectors. To determine the significance of θ , I estimated the 95% confidence intervals (CI) of this angle using a Bayesian approach implemented in the 'MCMCglmm' package of R (version 2.15.1, www.r-project.org). Using the linear models from the response surface approach above (Appendix 1), Bayesian inference was used to generate posterior distributions for each response variable and these were used to estimate the 95% CI for θ .

Annotated exemplar R code used to estimate the angle (θ), and 95% CIs, between linear vectors for the effects of nutrients on the weight and gustatory appeal of the spermatophylax and ampulla attachment time from Chapter 4:

```
#Load the package (MCMCglmm)

library(MCMCglmm)

# read in nutritional data for the first male trait (e.g. spermatophylax
#weight)

angle.data<-read.table("weight.txt",h=T)
attach(angle.data)
str(angle.data)

#define a non-informative prior

prior<-list(R=list(V=1,nu=0.02))
```

```

# str(angle.data) should give a column for spermatophylax weight, P intake
# and C intake)
# Bayesian linear regression to estimate beta for each variable
# posterior distribution based on 15200 estimates of each parameter:

angle.model.1<-MCMCglmm(weight~P+C-1,data=angle.data,
prior=prior,nitt=400000,burnin=20000,thin=25)

# and again for second male trait (e.g. spermatophylax gustatory appeal):

angle.data2<-read.table("appeal.txt",h=T)
attach(angle.data2)
str(angle.data2)
angle.model.2<-MCMCglmm(appeal~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.weight<- angle.model.1$Sol[i,1:2]
  b.appeal<- angle.model.2$Sol[i,1:2]

# creates a vector of beta estimates for each variable for each row of the
# posterior distribution (and the loop runs through all rows)

  angles[i]<- acos((t(b.weight) %*% b.appeal) / ((sqrt(t(b.weight)
%*% b.weight)) * (sqrt(t(b.appeal) %*% b.appeal)))) * (180/pi) }

# calculates the angles between spermatophylax weight and spermatophylax
#gustatory appeal betas for each row of the posterior distribution

summary(angles)

# to examine angle estimates which are now stored in the vector called
#'angles'

HPDinterval(as.mcmc(angles),0.95)

# provides the 1st and 3rd quantiles which are functionally equivalent to
# to the 95% CIs. Used the median and 95% CIs in this thesis for theta

```

APPENDIX 4: EXEMPLAR R CODE FOR CALCULATING LINEAR NUTRITIONAL EFFECTS OF P AND C, ADDITIVE GENETIC VARIANCE-COVARIANCE (G) MATRIX, THE PREDICTED RESPONSE TO SELECTION ($\Delta\bar{z}$), THE ANGLE (θ) BETWEEN THE PREDICTED RESPONSE TO SELECTION AND THE VECTOR OF LINEAR NUTRITIONAL EFFECTS, THE *R* MEASURE OF CONSTRAINT AND THE CORRESPONDING 95% CONFIDENCE INTERVALS FOR EACH MEASURE

Please note that this code has been reduced to focus on the calculation of all these measures for only male lifespan. Calculation of daily reproductive effort and lifetime reproductive effort as well as the female equivalent would only require a rewording of the appropriate sections of code.

```
#Read in the data

Data <- as.data.frame(read.table(file="./Intralocus_Daily.txt", header=T))

#Change the first column from ID to Animal

names(Data)[1] <- "animal"

#Specify factors and numerics from the data, include NA's

Data$animal<-as.factor(Data$animal)
Data$Sex<-as.factor(Data$Sex)
Data$DietPair<-as.factor(Data$DietPair)
Data$Total_P_Eaten<-as.numeric(Data$Total_P_Eaten)
Data$Total_P_Eaten_Daily<-as.numeric(Data$Total_P_Daily)
Data$Total_C_Eaten<-as.numeric(Data$Total_C_Eaten)
Data$Total_C_Eaten_Daily<-as.numeric(Data$Total_C_Daily)
Data$Total_P_Eaten_Male<-as.numeric(Data$Total_P_Eaten_Male, na.rm = F)
Data$Total_P_Eaten_Male_Daily<-as.numeric(Data$Total_P_Male_Daily, na.rm=F)
Data$Total_P_Eaten_Female<-as.numeric(Data$Total_P_Eaten_Female, na.rm = F)
Data$Total_P_Eaten_Female_Daily<-as.numeric(Data$Total_P_Female_Daily,
na.rm=F)
Data$Total_C_Eaten_Male<-as.numeric(Data$Total_C_Eaten_Male, na.rm = F)
Data$Total_C_Eaten_Male_Daily<-as.numeric(Data$Total_C_Male_Daily, na.rm=F)
Data$Total_C_Eaten_Female<-as.numeric(Data$Total_C_Eaten_Female, na.rm = F)
Data$Total_C_Eaten_Female_Daily<-as.numeric(Data$Total_C_Female_Daily,
na.rm=F)
head(Data)

#Read in the raw data values to get trait means

RawData <- read.csv("RawData.csv", header=T)
names(RawData)
MaleP <- mean(RawData$Male_P, na.rm=T)
MaleC <- mean(RawData$Male_C, na.rm=T)
FemaleP <- mean(RawData$Female_P, na.rm=T)
FemaleC <- mean(RawData$Female_C, na.rm=T)

#Read in the pedigree file
```

```

#Specify columns as factors

Ped <- as.data.frame(read.table(file = "./qg_pedigree.txt", header = T))
for (x in 1:3) Ped[,x] <- as.factor(Ped[,x])
head(Ped)

#Load packages

library(MCMCglmm)
library(Matrix)

#Specify the prior for the MCMC model, G is the G Matrix and R is the
Residual Matrix

prior1.1 <- list(G = list(G1 = list(V = diag(4), n = 1.002)), R = list(V =
diag(4), n = 1.002))

# Specify the model structure
# Specify the structure of the data e.g. normal, poisson, gaussian
# Include the terms to compare, random effects, and covariance effects
# Specify number of iterations, thinning number and burnin number
# Specify the prior, pedigree and data

# Model1.1 is the standard model with all variance and co-variances
calculated

modell1.1 <- MCMCglmm(cbind(Total_P_Eaten_Male_Daily,
Total_C_Eaten_Male_Daily, Total_P_Eaten_Female_Daily,
Total_C_Eaten_Female_Daily) ~ trait -1, random = ~us(trait):animal,
rcov = ~us(trait):units, family = rep("gaussian", times=4),
pedigree = Ped, data = Data, nitt=15000, thin = 50, burnin=100,
prior = prior1.1, verbose = T)

# This is a model test, it tests the validity of the variance/covariance
# matrix's posterior distributions
# Best result is to be close to 0
autocorr(modell1.1$VCV)
# Save the G matrix to a .csv file
modell1.1_G <- modell1.1$VCV
write.csv(modell1.1_G, file="modell1.1_G.csv")

# Create an object for the variance covariance matrix of model_1.1
model_VCV<-modell1.1$VCV [1:2998, 1:16]

# Set up Model 1.2 Where the off-diagonal elements of G are set to 0

data2 <- data.frame(Data$animal, Data$Sex, Data$Total_P_Eaten_Daily,
Data$Total_C_Eaten_Daily)

names(data2) <- c("animal", "sex", "Total_P", "Total_C")
data2$animal <- as.factor(data2$animal)
data2$sex <- as.factor(data2$sex)

prior1.4<-list(R = list(V = diag(2), n = 1.002), G=list(G1=list(V=diag(2),
nu=1.002), G2=list(V=diag(2), nu=1.002)))

modell1.2 <- MCMCglmm(cbind(Total_P, Total_C) ~ trait -1, random =
~us(trait:at.level(sex,"M")):animal + us(trait:at.level(sex, "F")):animal,
rcov = ~us(trait):units, family = rep("gaussian", times=2),
pedigree=Ped, data = data2, nitt=15000, thin = 50, burnin=100,

```

```

prior=prior1.4, verbose = T)

# Create an object for the variance covariance matrix of model_1.2
model_1.2_VCV<-model1.2$VCV [1:2998, 1:8]

# Read in the data for Male Beta
# Bayesian linear regression to estimate beta for each male trait, produces
# posterior distribution for each parameter:

Male.Data <- read.table("Male_Beta.txt", header=T)
str(Male.Data)

# Beta for Male Lifespan

modelBM1 <- MCMCglmm(Z_MLS ~ Z_MP + Z_MC-1, data = Male.Data, nitt=15000,
thin=50, burnin=100)

summary(modelBM1)

# Read in data for Female Beta
# Bayesian linear regression to estimate beta for each female trait,
# produces posterior distribution for each parameter:

Female.Data <- read.table("Female_Beta.txt", header=T)
str(Female.Data)

# Beta for Female Lifespan

modelBF1 <- MCMCglmm(Z_FLS ~ Z_FP + Z_FC-1, data = Female.Data, nitt=15000,
thin=50, burnin=100)

summary(modelBF1)

#Create a vector the same life as the posterior and fill it with 0's to
represent no intersexual correlation

x = 298
MP_FP_Blank <- rep(0, x)
MP_FC_Blank <- rep(0, x)
MC_FP_Blank <- rep(0, x)
MC_FC_Blank <- rep(0, x)
FP_MC_Blank <- rep(0, x)
FC_MC_Blank <- rep(0, x)

#Calculation for delta z bar
#Calculate deltaZ for Male Protein_LS

MP_LS_delta_z<-numeric(298)

# creates an empty vector the same length as the posterior distribution
#((nitt-burnin)/thin) to calculate length of vector
# distribution will be stored as follows:
# Fill the empty vector
# Filled from the variance/covariance matrix and beta
# This fills the empty vector in row order but MCMC created the rows
# randomly during its iterative run
#Confirm this with a simple linear regression on one column of the
# posterior distribution outputs

for(i in 1:298){

```

```

MP_MP <- modell.1$VVCV[i, 1]
MP_MC <- modell.1$VVCV[i, 2]
MP_FP <- modell.1$VVCV[i, 3]
MP_FC <- modell.1$VVCV[i, 4]
MP_LS <- modelBM1$Sol[i, 1]
MC_LS <- modelBM1$Sol[i, 2]
FP_LS <- modelBF1$Sol[i, 1]
FC_LS <- modelBF1$Sol[i, 2]

# Calculate deltaz with below formula

MP_LS_delta_z[i]<-
0.5*((MP_MP*MP_LS)+(MP_MC*MC_LS)+(MP_FP*FP_LS)+(MP_FC*FC_LS))

# Summary of deltaz including confidence

summary(MP_LS_delta_z)
HPDinterval(as.mcmc(MP_LS_delta_z),0.95)

# HPDinterval is an interval in which most of the distribution lies

# Calculate deltaZ for Male Carb_LS

MC_LS_delta_z<-numeric(298)

# creates an empty vector the same length as the posterior distribution
# distribution will be stored as follows:

for(i in 1:298){
  MP_MC <- modell.1$VVCV[i, 2]
  MC_MC <- modell.1$VVCV[i, 6]
  FP_MC <- modell.1$VVCV[i, 7]
  FC_MC <- modell.1$VVCV[i, 8]
  MP_LS <- modelBM1$Sol[i, 1]
  MC_LS <- modelBM1$Sol[i, 2]
  FP_LS <- modelBF1$Sol[i, 1]
  FC_LS <- modelBF1$Sol[i, 2]

  MC_LS_delta_z[i]<-
0.5*((MP_MC*MP_LS)+(MC_MC*MC_LS)+(FP_MC*FP_LS)+(FC_MC*FC_LS)) }

summary(MC_LS_delta_z)
HPDinterval(as.mcmc(MC_LS_delta_z),0.95)

#####
#Calculate deltaZ_RB=0 for Male Protein_LS

MP_LS_delta_z_RB0<-numeric(298)

# creates an empty vector the same length as the posterior distribution
# distribution will be stored as follows:

for(i in 1:298){
  MP_MP_RB0 <- modell.2$VVCV[i, 1]
  MP_MC_RB0 <- modell.2$VVCV[i, 2]
  MP_FP_RB0 <- MP_FP_Blank [i]
  MP_FC_RB0 <- MP_FC_Blank [i]
  MP_LS_RB0 <- modelBM1$Sol[i, 1]
  MC_LS_RB0 <- modelBM1$Sol[i, 2]
  FP_LS_RB0 <- modelBF1$Sol[i, 1]
  FC_LS_RB0 <- modelBF1$Sol[i, 2]

```



```

MP_LS_delta_z_RB0[i]<- 0.5*((MP_MP_RB0 * MP_LS_RB0)+(MP_MC_RB0 *
MC_LS_RB0)+(MP_FP_RB0 * FP_LS_RB0)+(MP_FC_RB0 * FC_LS_RB0))

summary(MP_LS_delta_z_RB0)
HPDinterval(as.mcmc(MP_LS_delta_z_RB0),0.95)

#Calculate delta_Z_RB=0 for Male Carb_LS

MC_LS_delta_z_RB0 <-numeric(298)

# creates an empty vector the same length as the posterior distribution
# distribution will be stored as follows:

for(i in 1:298){
  MP_MC_RB0 <- model1.2$VCV[i, 2]
  MC_MC_RB0 <- model1.2$VCV[i, 4]
  FP_MC_RB0 <- FP_MC_Blank [i]
  FC_MC_RB0 <- FC_MC_Blank [i]
  MP_LS_RB0 <- modelBM1$Sol[i, 1]
  MC_LS_RB0 <- modelBM1$Sol[i, 2]
  FP_LS_RB0 <- modelBF1$Sol[i, 1]
  FC_LS_RB0 <- modelBF1$Sol[i, 2]

  MC_LS_delta_z_RB0[i]<- 0.5*((MP_MC_RB0 * MP_LS_RB0)+(MC_MC_RB0 *
MC_LS_RB0)+(FP_MC_RB0 * FP_LS_RB0)+(FC_MC_RB0 * FC_LS_RB0))

summary(MC_LS_delta_z_RB0)
HPDinterval(as.mcmc(MC_LS_delta_z_RB0),0.95)

##### Calculate the Angle between Delta Z and Beta for Male LS
#Create a vector of n-length for the sum of Betas squared

Sum_Beta_LS<-numeric(298)
for(i in 1:298){
  MP_LS <- modelBM1$Sol[i, 1]
  MC_LS <- modelBM1$Sol[i, 2]

  Sum_Beta_LS[i]<- ((MP_LS * MP_LS) + (MC_LS * MC_LS))}

#Calculate the square root of the Beta squared

Sqrt_Sum_Beta_LS <- sqrt(Sum_Beta_LS)

#Calculate delta_Z squared

MP_LS_squared <- (MP_LS_delta_z * MP_LS_delta_z)
MC_LS_squared <- (MC_LS_delta_z * MC_LS_delta_z)

#Sum of the delta_Z squared

Sum_Delta_z_LS_squared <- MP_LS_squared + MC_LS_squared

#Square root of sum of delta_z squared

Sqrt_Sum_Delta_z_LS <- sqrt(Sum_Delta_z_LS_squared)

#Calculate delta_Z * beta

Z_B1 <- MP_LS_delta_z * MP_LS

```

```

Z_B2 <- MC_LS_delta_z * MC_LS

#Sum of delta_Z * beta

Sum_Z_B_LS <- (Z_B1 + Z_B2)

#Calculate R - Sum of delta_z * beta divided by square root of sum delta_z
# * square root of sum beta

r_LS <- Sum_Z_B_LS/(Sqrt_Sum_Delta_z_LS*Sqrt_Sum_Beta_LS)

#Calculate the angle of r

angle_LS <- acos(r_LS) * (180/pi)

#Summary of angle data
summary(angle_LS)
HPDinterval(as.mcmc(angle_LS),0.95)

#####Calculate the R value using delta_Z and delta_Z when intersex
# correlation is 0 for Lifespan
# R=0 the adaptation is completely stalled by genetic correlation
# R=0.5 then the covariance structure causes the fitness of the mean
# phenotype to increase only 50% as quickly as expected if traits
# were genetically independent
# R=2 then genetic covariances accelerate evolution such that adaptation
# occurs twice as fast as expected under genetic independence

#Male Protein and Lifespan
MPLS_R <- function(MPLS_R){

MPdZLS <- sample(MP_LS_delta_z, size=1, replace=T)
MPdZ0LS <- sample(MP_LS_delta_z_RB0, size=1, replace=T)

MPLSdWC <- ((MaleP + MPdZLS) - MaleP)
MPLSdWI <- ((MaleP + MPdZ0LS) - MaleP)

MPLSR <- (MPLSdWC/MPLSdWI)
return(MPLSR) }

MPLS__R <- replicate(1000, MPLS_R())

summary(MPLS__R)
HPDinterval(as.mcmc(MPLS__R),0.95)

#Male Carb and Lifespan
MCLS_R <- function(MCLS_R){

MCdZLS <- sample(MC_LS_delta_z, size=1, replace=T)
MCdZ0LS <- sample(MC_LS_delta_z_RB0, size=1, replace=T)

MCLSdWC <- ((MaleC + MCdZLS) - MaleC)
MCLSdWI <- ((MaleC + MCdZ0LS) - MaleC)

MCLSR <- (MCLSdWC/MCLSdWI)
return(MCLSR) }

MCLS__R <- replicate(1000, MCLS_R())

summary(MCLS__R)
HPDinterval(as.mcmc(MCLS__R),0.95)

```

APPENDIX 5: EXEMPLAR R CODE FOR CALCULATING THE UNCONDITIONAL EVOLVABILITY ($e(\beta)$)

Please note that this example code has been reduced to only look at lifespan. To calculate daily reproductive effort and lifetime reproductive effort would only require amending this code to the required traits.

```
#Before using this code you must have calculated G and B using the code in
#Appendix S4

#Get the mean from the raw data for each nutrient trait

RawData <- read.csv("RawData.csv", header=T)
names(RawData)
MaleP <- mean(RawData$Male_P, na.rm=T)
MaleC <- mean(RawData$Male_C, na.rm=T)
FemaleP <- mean(RawData$Female_P, na.rm=T)
FemaleC <- mean(RawData$Female_C, na.rm=T)

#Calculate the product of the means of traits i and j to mean standardize G

MPXMP <- (MaleP * MaleP)
MPXMC <- (MaleP * MaleC)
MPXFP <- (MaleP * FemaleP)
MPXFC <- (MaleP * FemaleC)
MCXMC <- (MaleC * MaleC)
MCXFP <- (MaleC * FemaleP)
MCXFC <- (MaleC * FemaleC)
FPXFP <- (FemaleP * FemaleP)
FPXFC <- (FemaleP * FemaleC)
FCXFC <- (FemaleC * FemaleC)

#Call the evolve function for lifespan

ls_evolve<- function (ls_evolve){

#Randomly sample with replacement from each separate part of the G matrix
# and divide by the product of the means of traits
#i and j to mean standardize G then create its own object

MPMP <- ((sample(modell1.1$VCV [1:298, 1], size=1, replace=T)) / MPXMP)
MCMP <- ((sample(modell1.1$VCV [1:298, 2], size=1, replace=T)) / MPXMC)
FPMP <- ((sample(modell1.1$VCV [1:298, 3], size=1, replace=T)) / MPXFP)
FCMP <- ((sample(modell1.1$VCV [1:298, 4], size=1, replace=T)) / MPXFC)
MPMC <- ((sample(modell1.1$VCV [1:298, 5], size=1, replace=T)) / MPXMC)
MCMC <- ((sample(modell1.1$VCV [1:298, 6], size=1, replace=T)) / MCXMC)
FPMC <- ((sample(modell1.1$VCV [1:298, 7], size=1, replace=T)) / MCXFP)
FCMC <- ((sample(modell1.1$VCV [1:298, 8], size=1, replace=T)) / MCXFC)
MPFP <- ((sample(modell1.1$VCV [1:298, 9], size=1, replace=T)) / MPXFP)
MCFP <- ((sample(modell1.1$VCV [1:298, 10], size=1, replace=T)) / MCXFP)
FPFP <- ((sample(modell1.1$VCV [1:298, 11], size=1, replace=T)) / FPXFP)
FCFP <- ((sample(modell1.1$VCV [1:298, 12], size=1, replace=T)) / FPXFC)
MPFC <- ((sample(modell1.1$VCV [1:298, 13], size=1, replace=T)) / MPXFC)
MCFC <- ((sample(modell1.1$VCV [1:298, 14], size=1, replace=T)) / MCXFC)
FPFC <- ((sample(modell1.1$VCV [1:298, 15], size=1, replace=T)) / FPXFC)
FCFC <- ((sample(modell1.1$VCV [1:298, 16], size=1, replace=T)) / FCXFC)

#Randomly sample with replacement from each separate part of the Beta
```

```

# outputs and multiply this by the mean of the trait,
#then create its own object

LSMP <- ((sample(modelBM1$Sol[1:298, 1], size=1, replace=T)) * MaleP)
LSMC <- ((sample(modelBM1$Sol[1:298, 2], size=1, replace=T)) * MaleC)
LSFP <- ((sample(modelBF1$Sol[1:298, 1], size=1, replace=T)) * FemaleP)
LSFC <- ((sample(modelBF1$Sol[1:298, 2], size=1, replace=T)) * FemaleC)

#Start to make up G into a 4 x 4 matrix
#Start by making each row of the matrix

G1 <- matrix(c(MPMP, MCMP, FPMP, FCMP), nrow=1, ncol=4)
G2 <- matrix(c(MCMP, MCMC, FPMC, FCMC), nrow=1, ncol=4)
G3 <- matrix(c(FPMP, FPMC, FPFPP, FCFPP), nrow=1, ncol=4)
G4 <- matrix(c(FCMP, FCMC, FCFPP, FCFC), nrow=1, ncol=4)

#Make a matrix from the 4 rows

G <- as.matrix(rbind(G1, G2, G3, G4))

#Make a one column vector of the Beta outputs

B <- as.matrix(c(LSMP, LSMC, LSFP, LSFC), nrow=4, ncol=1)

#Make a transpose of B

tB <- t(B)
tB

#Calculate the length of vector B

norm_vec <- function (x) { sqrt(sum(x^2)) }
b <- norm_vec(B)

#Square the length of vector B

b2 <- (b*b)

#Calculate the unconditional evolvability

ls_ev <- (((tB %*% G) %*% B)/b2)
return(ls_ev)}

#Repeat the ls_evolve function 1000 times

ls_evolv <- replicate(1000, ls_evolve())

#Give a summary of the results

summary(ls_evolv)
HPDinterval(as.mcmc(ls_evolv),0.95)

```

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