MONASH University

INVESTIGATING THE EFFECT OF PROTEIN QUALITY AND ITS MOLECULAR SENSORS ON LIFE-HISTORY TRAITS IN DROSOPHILA MELANOGASTER

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A thesis submitted for the degree of Master of Philosophy at
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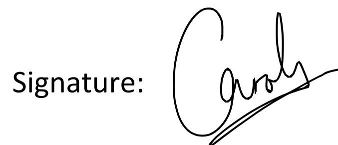
Abstract

Life history traits are greatly influenced by both environment and genes. Diet can determine the physiology e.g. lifespan and reproduction in model organisms and humans (Simpson and Raubenheimer, 2012, Fontana and Partridge, 2015). Also, lifespan and reproductive success can be determined by the genetic makeup of the organism (Merila and Sheldon, 2000, Vijg and Suh, 2005). The length of lifespan and reproductive performance are known to be greatly modified by the balance of dietary proteins (P) and carbohydrates (C), with a growing emphasis on protein as a key player. Amino acids are the building blocks of proteins, and several studies have demonstrated the importance of protein quality, the amino acid ratio, as well as their total concentration in influencing life history trait expression. Here, I investigated the effect of protein quality, as well as different P:C ratios and total P and C concentrations on reproduction of the vinegar fly *Drosophila melanogaster*. Furthermore, I looked into the role of the understudied amino acid sensing kinase, Gcn2, and its effects on lifespan and reproduction of vinegar flies in response to dietary changes.

I found that protein quality can dramatically change the nutrient requirement for peak egg laying. Flies on a low-quality protein diet required greater protein concentration to reach peak egg laying compared with those on a high-quality protein diet. My data indicate that rather than changing the total P concentration to improve reproductive yield of an animal, increasing protein quality at a lower P concentration would be equally as effective. These data could be useful for those in the field of agriculture or wildlife conservation. I also found that the nutrient sensing kinase Gcn2 was important for modifying reproduction and lifespan. In the absence of Gcn2, flies laid less eggs than controls and required a higher P concentration to reach peak egg laying. And though Gcn2 did not affect lifespan in response to nutrition, the presence of Gcn2 was still key for normal lifespan. My results provide further understanding in how Gcn2 is important for modifying reproduction in response to protein levels. These new fundamental insights point further studies to focus on the tissue-specific functions of Gcn2, which would allow us to isolate more mechanistic information about how Gcn2 activity is important to support life history traits in response to diet balance.

Declaration

This thesis is an original work of my research and contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature: 

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1. Chapter I: Introduction

1.1. Diet modifies life history traits

Detecting and ingesting nutrients of the right quantity and quality is important for optimising fitness-related traits. In early studies, a moderate reduction of food ingested was associated with increased lifespan (McCay et al., 1935, Weindruch, 1985), but decreased reproduction (Carey et al., 2002). These effects of reducing diet intake were considered to be due to calorie reduction, and the effect was termed calorie restriction (CR), which was defined as a 25%-60% reduction of calorie intake compared to *ad libitum* controls without nutrient imbalance (Weindruch et al., 1986, Weindruch, 1996, Bartke et al., 2001, Speakman et al., 2016). CR has shown effectiveness in modulating lifespan and reproduction of many organisms, for example reduced calorie intake has shown to extend the lifespan in flies (Min et al., 2007) as well as reducing reproductive output in mice (by arresting follicular cycles in females) (Nelson et al., 1985). However the effect of the same CR protocol on life-history traits does not appear to benefit all species, like the housefly (*Musca domestica*), in the same way (Cooper et al., 2004). Other studies have demonstrated that individual dietary components, such as protein or amino acids, are instead most effective at modulating lifespan and reproduction (Min and Tatar, 2006, Hall et al., 2008, Lee et al., 2008, Grandison et al., 2009, Lee, 2015, Arganda et al., 2017, Piper et al., 2017, Cerrate et al., 2019). For example, when supplementing the diet of vinegar flies (*Drosophila melanogaster*) with live-yeast, their only source of protein, egg production dramatically increased and this was coupled with a shortening of lifespan (Bradley and Simmons, 1997). Also shown in larval neriid fly (*Telostylinus angusticollis*), lifespan was extended by 73% on an intermediate concentration of protein (Runagall-McNaull et al., 2015), but female egg production was decreased in the absence of dietary protein (Adler et al., 2013).

How the levels of nutrients consumed are translated to changes in reproduction and lifespan may be explained by the Disposable Soma theory. The Disposable Soma theory, coined by Kirkwood in 1977, states that energy sources are selectively allocated to either somatic maintenance or reproduction in response to dietary energy availability (Kirkwood, 1977). When dietary energy availability is low, somatic maintenance is prioritised over reproductive

processes and when energy sources are high, reproductive processes take precedence over somatic maintenance (Kirkwood, 1977). Thus, when reproduction is strategically prioritised over somatic maintenance on rich foods, lifespan would be compromised and when somatic maintenance is prioritised over reproduction on a low energy diet, lifespan would be extended. Attempts have been made to evaluate this model experimentally by using stable isotope labelling in vinegar flies to track the utilization of dietary nitrogen and carbon that they consumed (O'Brien et al., 2008). Vinegar flies on a 'full' (high calorie) diet were shown to prioritise a greater proportion of ingested nitrogen and carbon towards reproduction than those on a more restricted diet (O'Brien et al., 2008). This higher relative level of investment in reproduction (Novoseltsev et al., 2002) than somatic maintenance in high-food fed flies was interpreted to align with the predictions of the Disposable Soma hypothesis.

A weakness of the Disposable Soma hypothesis is found in the observation that when food is increasingly restricted, but not to the point of starvation, lifespan becomes further extended. This is problematic because the Disposable Soma hypothesis predicts that because the amount of energy that is available for somatic maintenance should gradually reduce with more intense food restriction, there should be a graded shortening of lifespan. (Merry and Holehan, 1996, McCracken et al., 2020). A recent alternative hypothesis to work around this problem was proposed, called the 'clean cupboard' theory (Speakman, 2020). This theory posits that when energy resources are low, rather than the organism strategically redirecting just enough resources towards somatic maintenance over reproductive processes, the change in investment is more passive with no long-term strategy. This means that as environmental resources deplete, the organisms' cells immediately retrieve energy from stores (e.g. fat) to ensure survival as well as upregulating recycling processes (e.g. autophagy, to retrieve amino acids for de novo protein synthesis). As a side effect of this recycling effort, misfolded proteins and damaged organelles are broken down and cleaned out with the positive effect of enhanced somatic functioning and longevity. As more stores are used during food restriction, resources for other processes (e.g. reproduction) are also sequestered, thus reducing reproductive output. Thus, food restriction results in longevity benefits, but with the undesirable side effect of reduced reproduction.

Recently, evidence has accumulated for changes in life history traits from changing nutritional balance rather than the wholesale reduction in food, indicating that food quality rather than quantity is key for organismal maintenance (Mair et al., 2005, Lee et al., 2008, Skorupa et al., 2008, Grandison et al., 2009). These data indicate that consuming an imbalanced ratio of protein (P) and carbohydrates (C) might vary nutrient availability for maintaining the soma but not due to total dietary energy investment, but as a result of macronutrient imbalance. This requires that diet is considered in more complex terms than a single budget item.

1.2. Nutritional Geometry

Determining the best ratios and concentrations of macronutrients for lifespan or reproduction can be difficult due to the number of interacting factors involved. Observing the effects of nutritional components by varying one at a time is possible, however this does not permit a systematic investigation of both the main and interactive effects of macronutrients on a given trait. To overcome this, Nutritional Geometry (NG) has been developed as a tool to allow visualisation and systematic testing of the effects of any dietary components on quantifiable phenotypes (Raubenheimer and Simpson, 1997).

In a typical NG experiment, an animal is exposed to one of many diets that vary systematically across a range of calorie compositions and macronutrient (usually protein and carbohydrate) ratios with no choice between diets consumed. The nutrient space is defined by axes (generally two) that represent the levels of macronutrient ingested (e.g. protein and carbohydrates). The phenotypic response under study (e.g. lifespan or reproductive output) is plotted on the z-axis within the nutrient space and, for visualisation, the data are fitted by a surface with intensities represented as a heat map. Within the nutrient space, the nutritional composition of diets can be represented as nutritional rails that radiate out from the origin, with the slope of the nutritional rail representing the ratio of macronutrients represented by the x and y axes (e.g. the protein to carbohydrate ratio (P:C ratio)). A powerful example of the NG is a study that varied the protein (*x*) and carbohydrate (*y)* ratios to study the effects on average lifespan and reproduction (*z*) in the fruit fly (Lee et al., 2008) (Figure 1.1A and 1.1B). This study clearly showed that different P:C diets were optimal for peak lifespan or reproduction and that a substantial portion, if not the majority, of variation in

these traits occurred across isocaloric diets that varied in their P:C ratio. Thus, the NG design reveals a fuller description of nutrient effects on phenotypes in a manner that can reveal shortcomings in conventional wisdom.

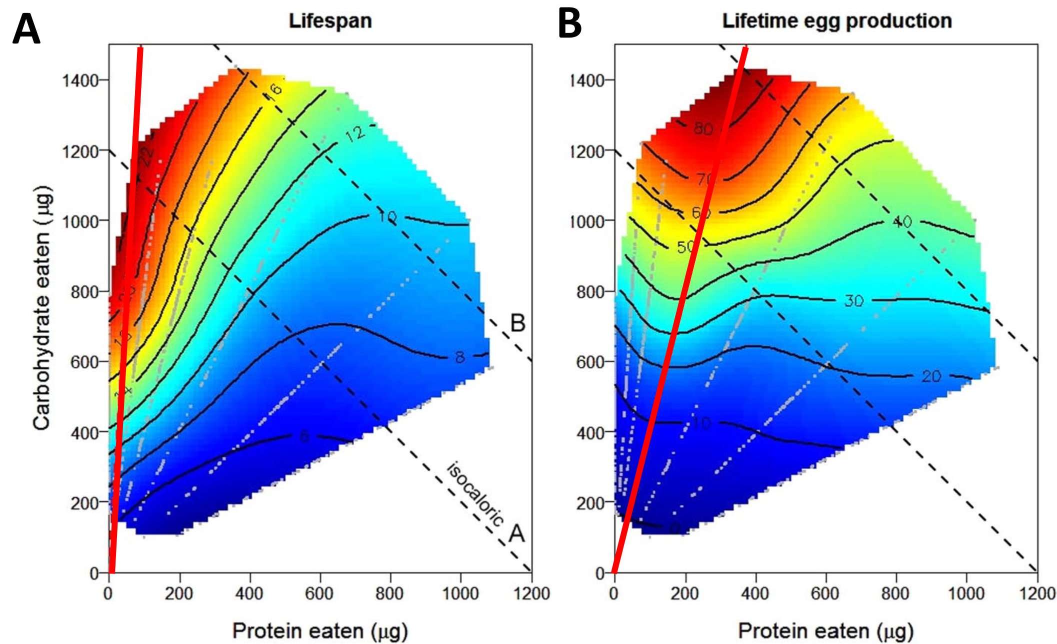


Figure 1.1. Response surface plot displaying the effect of protein and carbohydrates on **(A)** lifespan and **(B)** lifetime egg production of female vinegar flies. The red bold line represents the nutritional rail in which the fitness trait was maximised, the red colour on the surface plot represented high value and blue colour represents low value of the fitness trait measured. The grey points represent individuals that were constrained to diets whose nutritional composition is represented by the slope of the line along which they are plotted (i.e. the nutritional rail's P:C ratio). The black dashed lines indicate isocaloric points. Lifespan peaks on a diet with lower P:C ratio (1:16) than that for the peak for lifetime egg production (1:2). Modified from Lee et al. (2008).

1.3. Diet quantity and quality

Diets varying in P:C ratio can modulate lifespan and reproduction of both vertebrates and invertebrates (Table 1.1). Consistently, these studies indicate that a low P:C ratio diet supports longer life while those on a higher P:C ratio have shorter lifespan, but higher reproduction; these effects have been shown consistently to be largely independent of the total energy values of the diets on which they are maintained (Mair et al., 2005) (studies highlighted in table 1.1). Thus, these observed effects of diet balance on lifespan and

reproduction across taxa suggests that the mechanism behind these responses to diet modification are evolutionarily conserved (Moatt et al., 2019).

Protein in animals has an important function for producing muscle, enzymes and functional molecules that maintain overall body homeostasis. So, unsurprisingly changing the levels of proteins in the diet plays a role in modulating life-history traits. Although other nutrients like vitamins can modulate the length of lifespan and reproductive output in vinegar flies (Suckow and Suckow, 2006, Zou et al., 2017), reducing or elevating protein levels has been implicated as being sufficient to prolong or shorten lifespan while inversely modify egg laying in vinegar flies (Mair et al., 2005, Min and Tatar, 2006, Grandison et al., 2009, Dussutour and Simpson, 2012, Lee, 2015). A similar effect is also observed in mice (Solon-Biet et al, 2014). However, there is some debate as to whether dietary protein restriction effects the same longevity assurance as calorie restriction by intermittent feeding in rodents (Speakman et al., 2016). Thus, there could be at least two different ways by which diet modifies lifespan and maybe only one of these (i.e. protein restriction) might be conserved between insects and rodents.

Table 1.1. The P:C ratio diet for peak lifespan and reproduction of female animal model organisms. Across taxa, P:C ratio is lower for peak lifespan than the P:C ratio for peak reproduction.

Model organism	P:C ratio		References
	Peak lifespan	**Peak reproductive output**	
Flies:			
Bactrocera tryoni	1:21	1:3	Fanson and Taylor (2012)
Ceratitis cosyra	0:1	1:16	Malod et al. (2017)
Drosophila melanogaster	1:16	1:4	Lee et al. (2008)
	1:4	4:1	Lee (2015)
	1:16	1:2	Jensen et al. (2015)
	1:8	1:1.5	Semaniuk et al. (2018)
Argentine ants (*Linepitherma humile*)	1:6	Not examined	Arganda et al. (2017)
Crickets (*Gryllus veletis*)	1:8	1:3	Harrison et al. (2014)
C57BL6/J mice	1:14	Not examined	Solon-Biet et al. (2014)
	1:11	3:1	Solon-Biet et al. (2015)

In insects, the levels of specific amino acids that make up protein have been shown to be the key element for modulating life-history traits (Zimmerman et al., 2003, Mair et al., 2005, Grandison et al., 2009, Arganda et al., 2017). In vinegar flies, essential amino acids were added to restricted diets and the levels of reproductive output and lifespan were compared to those on a full, complete diet (Grandison et al., 2009). Flies that were provided a restricted diet with the addition of all 10 essential amino acids had increased egg laying and shortened lifespan, thus reproducing the effects seen when flies were fed the full diet (Grandison et al., 2009). Interestingly, adding only methionine to the restricted diet was also sufficient to increase egg laying, but in this case did so without cost to lifespan, (Grandison et al., 2009). This

demonstrated that the responses of lifespan and reproduction to nutrition are independent and are mediated by the balance of different amino acids.

1.4. Animal model

Flies have been used as a model organism for more than a century in genetical and developmental studies. Food for *Drosophila* husbandry is cheap to maintain in laboratories , since their standard diet is made up of yeast and sugar (see section 3.2.2.). In addition, compared to rodents the lifespan of flies is relatively short (312 to 802 days for mice vs. 50 - 90 days for flies) (Festing and Blackmore, 1971, Linford et al., 2013). For examining the effects on lifespan and reproduction, testing flies is desirable since their generation time is only 2-weeks and the population can grow exponentially with females producing >65 eggs per day (Sgrò and Partridge, 2000). Flies can be easily genetically manipulated, and with their short generation time, obtaining a population of genetically modified flies can be achieved. Compared to rodents, experimenting with flies eliminates obstacles related to ethical approvals and requires less resources and time to produce transgenic animals. Though flies are not as closely related to humans than rodents, over half of the human genes (61%) implicated for human diseases have homologs in the fly genome (Rubin et al., 2000) and molecular signalling pathways e.g. the nutrient signalling pathway the general control nonderepressible 2 (Gcn2) kinase are conserved in both flies and mammals (Zhang et al., 2000). Together, these make flies an extremely valuable animal model to extrapolate findings for humans, but with caution.

Here, I used vinegar flies as the animal model, maintained on holidic media, which is a diet made entirely from chemically purified ingredients (Piper et al, 2014). In previous studies using flies, these diets have proven to be effective for studying the effects of individual amino acids on reproduction and lifespan (Piper et al., 2014, Piper et al., 2017). To investigate further how different ratios of amino acids, as well as protein and carbohydrate ratio and concentrations, affect reproduction and lifespan, I utilised the diets from Piper et al. (2014).

1.5. 'Holidic' versus whole diet

Whole proteins from natural sources (e.g. yeast and casein) are commonly used to represent dietary proteins in nutritional studies. Nutrients like yeast are accessible and cost-effective to buy in bulk in laboratories, and the effects of yeast concentration and quality on the development of vinegar flies has been extensively documented (Bass et al., 2007, Grangeteau et al., 2018, Murgier et al., 2019) and shown to be a suitable rearing substrate. However, whole foods and their fractions contain other macronutrients and micronutrients that may have complicated interactive effects on physiology. Even different sources of the same wholefood can have different effects on lifespan, as was shown for *Drososphila* maintained on different yeasts (Bass et al., 2007). One way to avoid these unintended nutritional complications is to use synthetic diets in which all components are chemically defined ('holidic' diets) and can be manipulated independently of each other. Early studies have used holidic diets to examine the effects of diet composition on lifespan and reproduction in the walnut husk fly (*Rhagoletis complete*), in which the levels of amino acid tryptophan were found to be important for successful egg laying (Tsiropoulos, 1978).

1.6. Exome matching

What is the ideal balance of amino acids in protein for flies? Recent work has used the consumer's genome as the basis for dietary amino acid design - a technique called exome matching. For this method, the protein coding region of the organism's genome (its exome) is sequenced, *in silico* translated and the count of each amino acid is summed across all proteins. Following this, the value for each amino acid is divided by the sum of all amino acids to determine the relative proportion of each of the 20 amino acids in the expressed exome. This information is then used to construct diets in which the exact proportion of each amino acid is mixed with other nutrients e.g. carbohydrates, vitamins, minerals and lipids, thus producing the holidic media containing the optimal amino acid composition. This technique has been used in invertebrates, including ants and vinegar flies (Arganda et al., 2017, Piper et al., 2017) in which the effects of optimal amino acid composition on life-history traits was studied. The downside to mapping the organism's dietary requirement via exome matching is that sequencing exome is limited to our current knowledge of the genome, therefore

potential genome regions that may be of importance will be missed by exome sequencing. Furthermore, the current implementation does not incorporate gene expression into the degree to which each gene contributes to the exome-averaged amino acid profile. Nevertheless, exome matching has proven to be a valuable tool to find the optimum protein quality for improving life-history traits.

In vinegar flies, Piper et al. (2017) developed an exome matched diet that can enhance reproduction output with such small amounts of protein that there was no cost to lifespan. This exome matched amino acid profile was referred to as FLYaa (Piper et al., 2017). The effect of FLYaa on lifespan and egg laying was compared to flies on a diet with an amino acid composition that was mismatched to the flies' exome, termed MMaa (Hunt, 1970, Piper et al., 2017). This MMaa diet was still adequate for sustaining adult lifespan (Figure 1.2A), but much more of the MMaa mixture was required to optimise reproduction, and at these higher levels of dietary amino acids, lifespan was compromised (Figure 1.2B). Thus, the apparent trade-off between reproduction and lifespan is caused by animals consuming different amounts of protein that contains amino acid profiles not matched to consumer requirements.

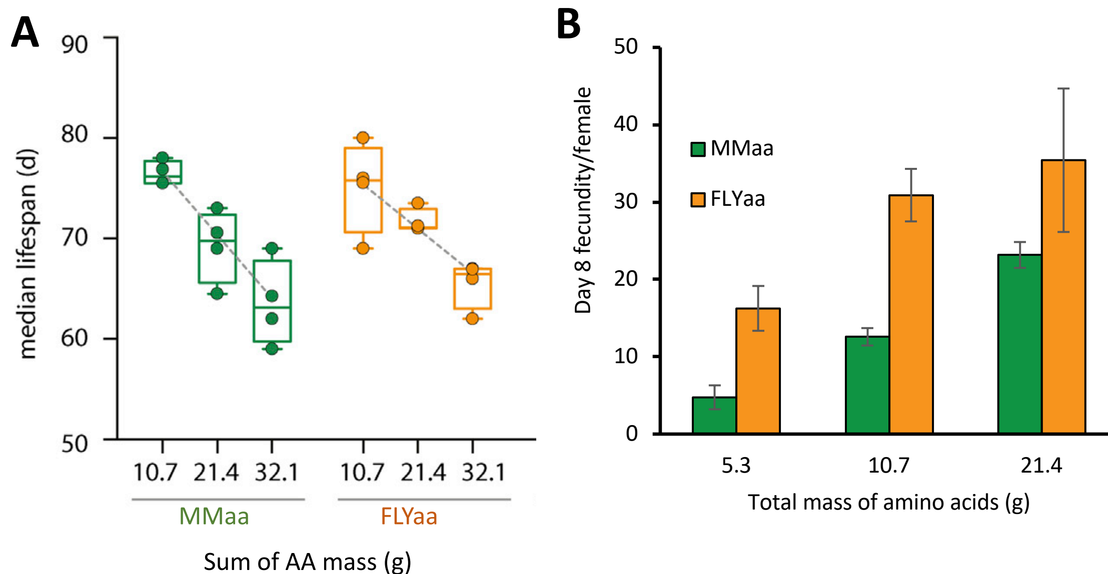


Figure 1.2. (A) The median lifespan of adult vinegar flies maintained on varying doses of a mismatched amino acid ratio (MMaa) diet and a matched amino acid ratio (FLYaa). The effect of amino acid concentration on lifespan was not different between amino acid ratios, with both lifespans optimized at 10.7 g. Bold dots presents the median lifespan from four trials for each diet treatment. **(B)** The mean number of eggs laid by flies on the MMaa and FLYaa diets at three nutrient densities. At each nutrient density, flies on FLYaa diet laid more eggs than those on the MMaa diet. Egg laying reached its maximum level at 10.7 g FLYaa, which is the same concentration at which maximum lifespan was achieved. In contrast, flies on MMaa reached their highest levels of egg laying at 21.5 g/L, at which point lifespan was significantly shorter than the flies on lower protein diets. Reproduced from Piper *et al*. (2017).

1.7. Nutrient sensing pathways and their role in shaping life-history traits

Animals possess post-ingestive mechanisms to detect the levels of nutrition they have consumed. In the case of protein, the two signalling pathways, Mechanistic Target of Rapamycin (mTOR) and the General Control Non Derepressible 2 (Gcn2) kinase respond to intracellular amino acid availability (Beugnet et al., 2003, Deval et al., 2009). They both play a key role in regulating fundamental cellular processes like metabolism, growth and autophagy

via downstream effectors so as to match cellular metabolism to the available supply of amino acids (Kapahi and Zid, 2004, Hu et al., 2018). With the right balance and quality of nutrients, protein synthesis and turnover are balanced such that a high level of function is maintained. The sum of these processes that maintain the proteome has been termed 'proteostasis' (Balch et al., 2008). When nutrient stress occurs (e.g. restricted nutrient availability), proteostasis is maintained by decreasing protein synthesis and enhancing protein turnover via autophagy, which recycles cytoplasmic components, and so makes some amino acids available for essential protein synthesis (Mortimore and Poso, 1987, Kuma et al., 2004). mTOR and Gcn2 kinase play important complementary roles in growth and maintaining proteostasis, which may ultimately affect the organisms' lifespan and reproduction levels.

1.7.1. mTOR

Mechanistic Target of Rapamycin (mTOR), formerly known as mammalian TOR, is a protein kinase that is part of the phosphatidylinositol-4,5-bisphoshate 3-kinase (PI3K)-related family. mTOR coordinates and promotes cell growth and proliferation in response to amino acids and growth factors (Saxton and Sabatini, 2017). mTOR is a core subunit of a complex that also includes raptor and GβL to form the mTOR complex 1 (mTORC1) (Laplante and Sabatini, 2012). mTORC1 is activated by amino acid availability and is translocated to be adjacent to the lysosome by a process involving a family of GTP-ases called RAG GTP-ases (Hara et al., 1998, Kim et al., 2008, Sancak et al., 2008, Laplante and Sabatini, 2009, Sancak et al., 2010)

When mTORC1 is active, it phosphorylates a number of proteins that promote protein synthesis such as the translational regulators 4E-BP (Hay and Sonenberg, 2004) and S6K (Hara et al., 1998). During amino acid restriction in *Drosophila*, mTORC1 becomes inactive as it dissociates from the lysosomal membrane (Figure 1.3). The downstream effects are the inhibition of mRNA translation by the dephosphorylation and inhibition of 4E-BP and S6K, and increased protein turnover via enhanced autophagy (Figure 1.3). mTOR has been shown to control autophagy by negatively regulating ATG1 kinase (Matsuura et al., 1997), which plays a role in the formation of autophagosomes, a double membrane vesicle that fuses with lysosomal membrane to degrade the content (Seglen and Bohley, 1992). The reduced protein

synthesis (Vabulas and Hartl, 2005) and enhanced levels of autophagy (Kuma et al., 2004), restoring homeostasis by degrading and recycling the components of damaged organelles.

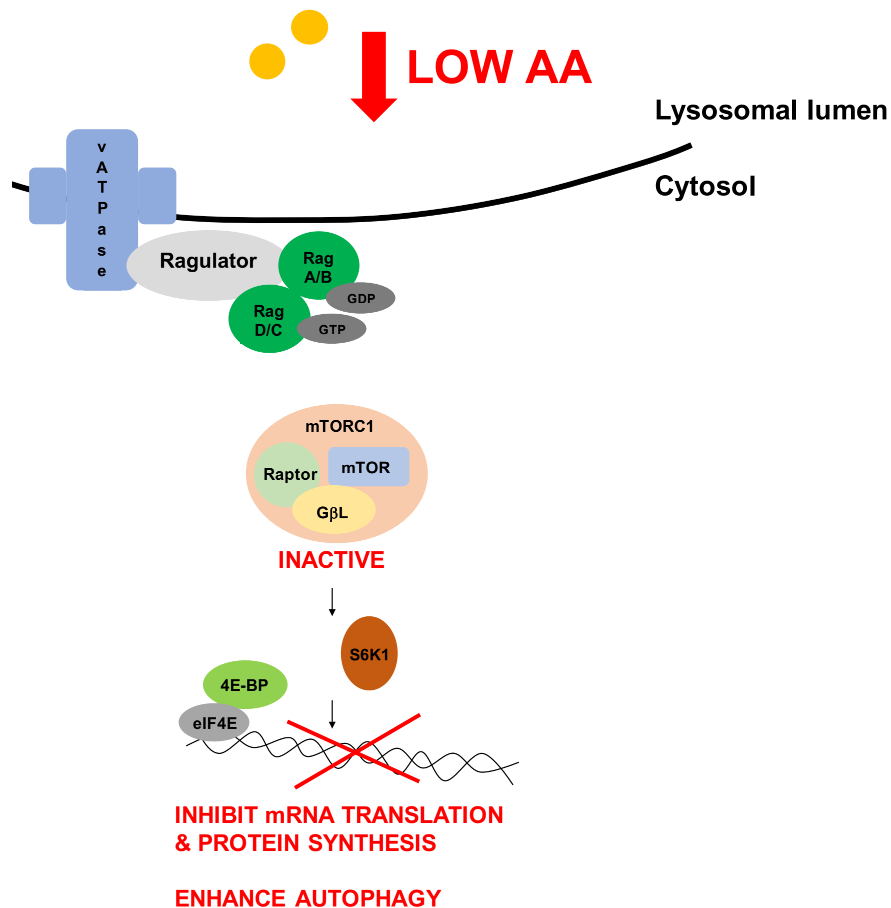


Figure 1.3. The mTOR pathway responds to intracellular amino acid levels. When nutrient levels are low, the RagA and RagC proteins are GDP and GTP bound, respectively. This causes inactive mTORC1 to dissociate from the lysosome and causes reduced phosphorylation of 4E-BP and S6K1. This results in a reduction of protein synthesis (reduce mRNA translation and protein synthesis) and increase in the level of autophagy activity.

In reproduction, mTOR has been shown to regulate follicle stem cell growth and assist in the formation of mature oocyte in the ovaries of vinegar flies (LaFever et al., 2010). Inhibition of mTOR using rapamycin (an inhibitor of mTOR) has been shown to reduce female reproduction and extend lifespan in flies (Bjedov et al., 2010), which has been attributed to enhanced proteostasis via reduced protein synthesis. Protein synthesis is highly energy consuming (Buttgereit and Brand, 1995) and inefficient, with over 30% of newly formed proteins immediately degraded after synthesis (Schubert et al., 2000). Reducing protein synthesis and the associated reduction in reproduction saves on metabolic building blocks and energy which can then be diverted towards cell maintenance and repair with the knock-on effect of promoting longevity (Syntichaki et al., 2007, Hu et al., 2018). In addition, protein turnover can increase via autophagy, which might reduce levels of harmful misfolded or aggregated proteins (Balch et al., 2008) thus "cleaning out" the cell and so enhancing its function (Speakman, 2020).

1.7.2. Gcn2

The General Control Nonderepressible 2 (Gcn2) is a eukaryotic translation initiation factor 2α (eIF2α) kinase. Gcn2 is universally conserved in all eukaryotic organisms (Berlanga et al., 1999). The general outline of the Gcn2 kinase signalling pathway is displayed in Figure 1.4. Gcn2 responds to the presence of uncharged transfer RNAs (tRNA), whose concentration increases with reduced intracellular amino acid levels (Gallinetti et al., 2013). These uncharged tRNAs are thought to bind to a protein domain similar to a histidyl-tRNA synthase on Gcn2 (Wek et al., 1995). When activated, Gcn2 phosphorylates eIF2α (Zhang et al., 2002), which leads to a global reduction in efficiency of translational initiation, alleviating nutrient stress by reducing protein synthesis (Hu et al., 2018). Although general translation is reduced, some transcripts (e.g. transcription factors like Gcn4 in yeast and ATF4 in mammals) contain a series of short ORFs in their 5'-UTRs that actually result in an enhancement of their translation when translational efficiency is lowered (Lu et al., 2004). These transcription factors activate the expression of genes involved in cellular protection against nutrient stress e.g. upregulating genes involved in resistance to oxidative stress (Harding et al., 2003) and stimulating enzymes responsible for synthesizing amino acids (Hinnebusch, 1997). Gcn2 kinase has also been shown to modify behaviour in response to dietary imbalance of amino

acids. For example, *Gcn2* knockout mice were more delayed in averting chow with imbalanced amino acids compared to controls (Maurin et al., 2005).

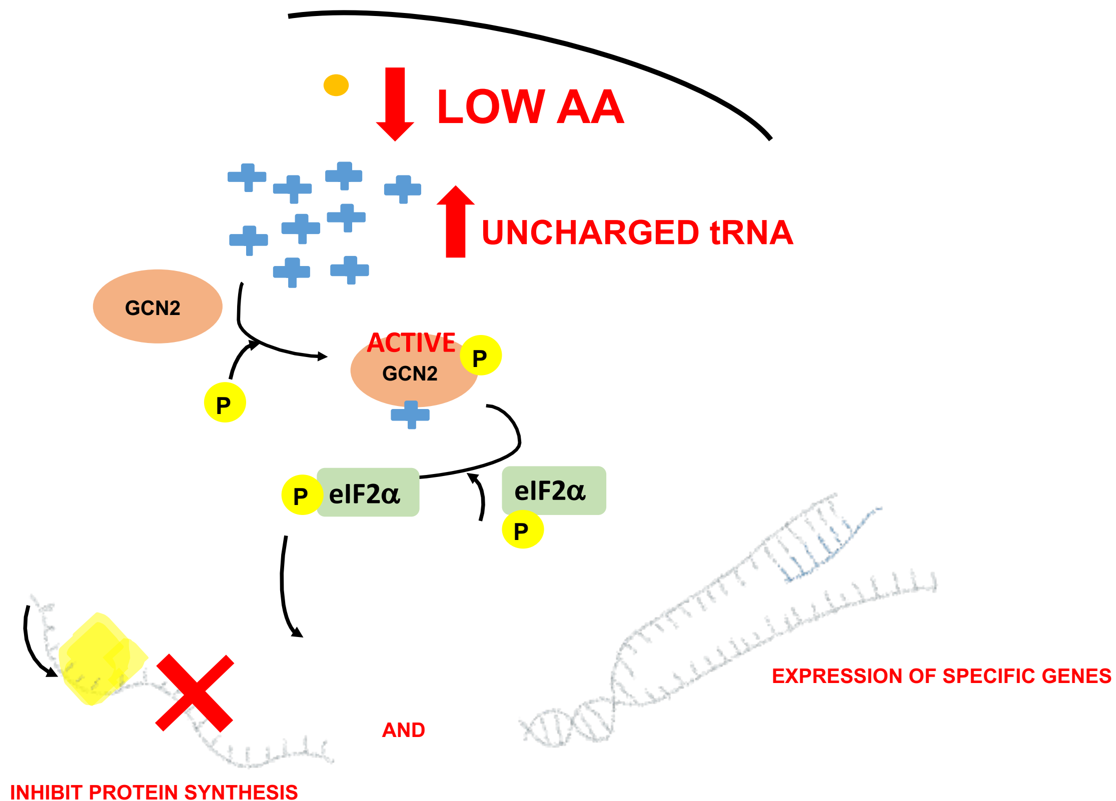


Figure 1.4. The Gcn2 nutrient signalling pathway is activated by low amino acid levels. Under low amino acid conditions, the levels of uncharged tRNAs (in blue) increase. Gcn2 kinase becomes phosphorylated, leading to the phosphorylation of the eIF2α subunit. This results in the inhibition of protein synthesis, as well as the upregulation of expression of specific genes.

Both Gcn2 and mTOR regulate translation via the regulator of eukaryotic translation elongation 4E-BP (Hay and Sonenberg, 2004). During nutrient abundance, mRNA translation and protein synthesis is promoted by phosphorylation and inhibition of 4E-BP, which is a negative regulator of translation (Hay and Sonenberg, 2004). Interestingly, this protein has a role in lifespan extension in response to dietary protein manipulation (Zid et al., 2009). A recent study in vinegar flies showed that under low nutrient conditions transcription of *4E-BP* increased in the intestine and fat bodies, however when *Gcn2* was knocked down the

transcription of *4E-BP* was suppressed (Kang et al., 2017). This result indicated that Gcn2 promoted the expression of *4E-BP* under dietary restricted conditions (Kang et al., 2017). Modulating the activity of downstream effector molecules like 4E-BP by more than one nutrient signalling pathway is a reminder of the broader signalling network that regulates life-history traits.

The effect of Gcn2 on lifespan has been documented in animal models. When Gcn2 is absent, lifespan is shortened, by ~10-15% in worms (Rousakis et al., 2013), but when over expressed in yeast, they had an extended replicative lifespan (Hu et al., 2018). These results may be due to reduced levels of protein translation (Hu et al., 2018). The activity of Gcn2 also has a diet-dependent effect on reproduction in mice: *Gcn2* knock-out females on a nutrient-deficient diet had more pre- and post-natal defects and a significant increase in still-born pups compared to those on a synthetic amino acid complete diet (Zhang et al., 2002).

Evidence shows that inhibiting mTOR using pharmaceutical interventions can extend lifespan and reduce reproductive capacity in vinegar flies (Bjedov et al., 2010, Suganya et al., 2010) and that mTOR signalling is essential for the effect of diet restriction (Emran et al., 2014). However, the role of Gcn2 in response to changes in nutrient balance, is currently not characterised.

1.8. General aim

In my project, I aim to investigate the effect of protein quantity and quality in regulating reproduction and lifespan, and how this effect interacts with the Gcn2 nutrient sensing pathway. The fruit fly, *Drosophila melanogaster*, was used as the animal model because it has a short lifespan and generation time, and so is ideal for studying the effects of diet on lifespan. My thesis reports the outcomes from two studies: (1) chapter II focuses on the interactive effects of carbohydrate level with protein quantity and quality in modulating reproduction in wild type flies, while (2) chapter III focuses on the way Gcn2 kinase modulates reproduction and lifespan responses to changing diet composition. In both experiments, I adopted a Nutritional Geometry approach in order to systematically visualise and analyse the effects of both protein and carbohydrate concentrations, as well as nutrient density, on these traits.

My hypothesis for chapter II was that the nutritional optimum for peak female egg laying would vary when two different protein qualities were used to make the diets. Specifically, I predicted that lower amounts of a higher quality protein would be required to maximise egg laying than when using lower-quality protein. This has important implications for the way we understand how fitness traits respond to diet when studies and environments differ in the quality of dietary components that are available. In chapter III my hypothesis was that the overall lifespan between wildtype and flies with non-functional Gcn2 would differ, where transgenic flies would die quicker due to their inability to respond to amino acid dilution. But that the nutrient optima for the traits would be similar between wildtype and flies with non-functional Gcn2, since Gcn2 is assumed to be non-functional, and therefore not necessary, when nutrition is sufficient. For the same reasons, I also hypothesised that there would no difference in egg laying between wildtype and flies with non-functional Gcn2. These data are important to our understanding of how key evolutionarily conserved nutrient signalling pathways are involved in life history trait responses to diet.

2. Chapter II: Protein quality modifies the response of reproduction

Abstract

Diet composition, especially the relative abundance of key macronutrients, is well known to affect animal wellbeing by changing reproductive output, metabolism and length of life. However, less attention has been paid to the ways the quality of these nutrients modify these macronutrient interactions. Nutritional Geometry can be used to model the effects of multiple dietary components on life-history traits and to compare these responses when diet quality is varied. Previous studies have shown that dietary protein quality can be increased for egg production in *Drosophila melanogaster* by matching the dietary amino acid proportions to the balance of amino acids used by the sum of proteins in the fly's *in silico* translated exome. Here, we show that dietary protein quality dramatically alters the effect of protein quantity on female reproduction across a broad range of diets varying in both protein and carbohydrate concentrations. These data show that when sources of ingredients vary, their relative value to the consumer can vastly differ and yield very different physiological outcomes. Such variations could be particularly important for meta analyses that look to draw generalisable conclusions from diverse studies.

2.1. Introduction

To optimise fitness, organisms must consume a sufficient quantity and quality of nutrients to suit their needs (Hall et al., 2008, Lee et al., 2008, Bong et al., 2014). This is demonstrated by the dramatic changes in reproductive function seen when flies or rodents are subjected to food restriction or changes in diet balance, particularly when the relative proportion of protein to carbohydrates is changed (Widdowson and Cowen, 1972, Good and Tatar, 2001, Carey et al., 2002, Liang and Zhang, 2006, Lee et al., 2008, Skorupa et al., 2008, Simpson and Raubenheimer, 2012, Solon-Biet et al., 2015, Camus et al., 2019).

Food is comprised of dozens of nutrients that interact to modify animal physiology. Understanding how nutritional interactions affect the consumer is complex but can be facilitated using a structured approach such as Nutritional Geometry (Raubenheimer and Simpson, 1997). Nutritional Geometry maps the responses of life history traits to quantitative variations of two or more macronutrients. This is typically performed by exposing animals to one of many diets that vary across a range of calorie compositions and protein to carbohydrate ratios. A nutrient space is defined by axes (generally two) that represent the quantity of nutrients that an organism has eaten – thus any point in space can represent the status of an organism according to its nutritional history. By mapping an array of organisms with different dietary histories into this space, their collective phenotypic responses can be fitted by an overlaid surface represented by a heat map (*z*- axis). This allows for the modelling of the interactive effects of nutrients on phenotypes of interest. Although conceptually simple, assessing phenotypic responses to nutrition through the perspective of Nutritional Geometry has revealed new understanding of biology and, in some cases, has unified apparently conflicting interpretations about the way organisms respond to diet change (Solon-Biet et al., 2016).

Nutritional Geometry experiments have shown that variation in two of the energy-yielding macronutrients, protein and carbohydrate, affect the expression of many traits. In particular the lifespan and reproduction of adult vinegar flies (*Drosophila melanogaster*) are shaped by the interactive effects of dietary protein and carbohydrate (Lee et al., 2008, Skorupa et al., 2008, Jensen et al., 2015). In the case of protein, more recent work has shown that the

proportion of its constituent amino acids has an important role to play in protein's effects on these traits (Grandison et al., 2009, Piper et al., 2017).

The dietary amino acid requirements of female flies for optimal egg production can be determined from its genome by a process termed exome matching (Piper et al., 2017). To exome match a diet, we *in silico* translate the exome of the consumer, sum the abundance of each amino acid across all proteins, and find the relative proportion of each amino acid. We then use this proportion as the basis for the abundance of each amino acid in the food. By matching the dietary protein quality to the fly exome in this way, we found that for a fixed mass of amino acids, exome matched diets supported higher levels of reproduction than diets that were mismatched (Piper et al., 2017). Together, these data show that dietary amino acid balance is important for determining fitness outcomes.

Although dietary amino acid balance is important, Nutritional Geometry experiments that measure fitness responses to diets invariably treat protein as a single nutrient dimension with a fixed proportion of all 20 amino acids. This is reasonable for experiments in which the protein source is held constant across all diets. However, proteins vary in quality when attained from different sources and, like many natural ingredients, the same type of protein may vary in quality between locations and seasons. Given this, it may be difficult to directly compare the effects of consumed "protein" on a trait when the data are from different studies. To examine these effects, we designed an experiment using a Nutritional Geometry design to assess how changing dietary protein quality modifies the interactive effects of the amino acid (protein; P) to carbohydrate (C) ratio on egg laying. We designed an array of diets varying in P:C ratio for each of two different amino acid (protein) mixtures. These mixtures varied in the relative proportion of each amino acid, as well as the identity of the most limiting essential amino acid and the degree to which it is predicted to be limiting.

2.2. Method

2.2.1. Animal husbandry

We used the Dahomey outbred population of *Drosophila melanogaster* (Mair et al., 2005). Routine rearing and maintenance of flies employed the techniques and sugar/yeast (SY) diet described in Bass et al. (2007). All flies were maintained at 25°C with a 12 hr: 12 hr light dark photoperiod. For the experiment, a population of flies was age synchronised as in Piper and Partridge (2016).

2.2.2. Experimental diets

Completely defined synthetic (holidic) diets were made according to Piper et al. (2014), in which free amino acids are used to make up protein equivalents. To convert amino acids to protein equivalents, we used the molar quantities of nitrogen and the assumption that N makes up 16% of whole proteins (Sosulski and Imafidon, 1990). Two protein qualities, defined by their amino acid ratios, were compared: FLYaa (matched to the amino acid ratio of the exome of adult flies), and MMaa (a ratio considered mis-matched to the flies' requirements). The relative proportion of each amino acid within each amino acid ratios are displayed in Table 2.1. From this point on, the concentration of total amino acids is referred to as protein (P), calculated according to the above method.

For both amino acid ratios, we generated diets that were one of five P:C ratios (1:3.6, 1:1.8, 1:1.1, 1:0.8, 1:0.6) and one of four caloric densities (66.8 kcal/L, 111.3 kcal/L, 155.8 kcal/L, 200.3 kcal/L), where dietary energy densities were estimated by calculation, using a value of 4 kcal/g for both protein and carbohydrates (Table 2.2.). Thus, 20 diets were employed to test the effect on egg laying of each amino acid ratio.

Table 2.1. The relative proportions of each amino acid in the two amino acid ratios tested, MMaa and FLYaa.

	Amino acids		**Ratio**	
			MMaa	**FLYaa**
Essential amino acids	Phenylalanine	F	0.027	0.037
	Histidine	H	0.022	0.026
	Isoleucine	I	0.077	0.052
	Lysine	K	0.044	0.057
	Leucine	L	0.052	0.094
	Methionine	M	0.018	0.025
	Arginine	R	0.016	0.057
	Threonine	T	0.057	0.056
	Valine	V	0.081	0.062
	Tryptophan	W	0.008	0.010
Non-essential amino acids	Alanine	A	0.133	0.075
	Cysteine	C	0.001	0.017
	Aspartate	D	0.043	0.053
	Glutamate	E	0.058	0.063
	Glycine	G	0.144	0.062
	Asparagine	N	0.044	0.047
	Proline	P	0.044	0.052
	Glutamine	Q	0.058	0.046
	Serine	S	0.061	0.079
	Tyrosine	Y	0.013	0.031

Table 2.2. The equivalent protein: carbohydrate (P:C) ratio, displayed with the nutrient densities.

P:C equivalent	Sum mass of amino acids (g/L)	Equivalent protein (g/L)	Equivalent carbohydrate (g/L)[a]	Estimated caloric content (kcal/L)
1:3.6	3.92	3.54	12.77	66.8
1:3.6	6.53	5.91	21.29	111.3
1:3.6	9.14	8.27	29.81	155.8
1:3.6	11.75	10.63	38.32	200.3
1:1.8	6.42	5.81	10.27	66.8
1:1.8	10.7	9.68	17.12	111.3
1:1.8	14.98	13.55	23.97	155.8
1:1.8	19.26	17.42	30.82	200.3
1:1.1	8.32	7.52	8.38	66.8
1:1.1	13.86	12.54	13.96	111.3
1:1.1	19.41	17.55	19.54	155.8
1:1.1	24.95	22.57	25.13	200.3
1:0.8	9.57	8.66	7.12	66.8
1:0.8	15.95	14.43	11.87	111.3
1:0.8	22.33	20.20	16.62	155.8
1:0.8	28.71	25.97	21.37	200.3
1:0.6	10.99	9.94	5.70	66.8
1:0.6	18.32	16.57	9.50	111.3
1:0.6	25.64	23.20	13.30	155.8
1:0.6	32.97	29.65	17.10	200.3

[a] Carbohydrate is added to the diet as sucrose.

2.2.3. Experimental set-up

Flies were housed in devices called dFlats, which are made up of a block of Perspex with 12-wells drilled into them, such that the drilled wells have a volume the same as standard fly vials (FS32, Pathtech) and their arrangement is such that the 12 openings match the position of wells in a 12-well plate (based on the design: https://www.flidea.tech/projects). 3ml food was dispensed into each of the wells in a 12-well plate. Each dFlat well contained 10 mated female flies and we maintained one dFlat for each of the 20 holidic experimental diets (12 replicate wells x 10 flies = 120 flies per diet).

2.2.4. Reproduction experiment

Once a week for 3 weeks the number of eggs laid on the media over an 18-hour period was counted and recorded. Measuring reproductive output during the first weeks of egg laying has shown to be representative of life-long reproduction of flies (Chapman and Partridge, 1996, Muller et al., 2001). For each well in each dFlat device, the number of eggs laid per female on each experimental day (day 8, 15 and 22) was calculated. The value for eggs laid by an average female in a well was summed across days and used to calculate the cumulative egg laying of an average female in a food type. We call this value the index of reproduction. The number of eggs in each well and food type was obtained by taking images using a web camera attached to a stereomicroscope. The images were then processed in Image J to acquire the correct image size, which were then automatically counted in the software called QuantiFly (Waithe et al., 2015).

2.2.5. Statistical analyses

All analyses were conducted using R (version 3.3.0, available from http://www.R-project.org/). To analyse the relationship between our index of reproduction and the protein (P) and carbohydrate (C) concentration, we generated separate response surfaces for each of the two amino acid ratios. Each surface was estimated using multivariate second-order polynomial regression, whereby the linear, quadratic and cross-product terms from this model capture the linear and non-linear effects of P and C concentration on fly reproduction.

For each amino acid ratio, the minimum adequate model for each linear model was found by determining if eliminating the most complex parameter significantly reduced the model fit. We visualised the response surface of each amino acid ratio using predictions derived from thin-plate splines from the fields package (see www.github.com/NCAR/Fields) and subsequently visualised via the ggplot package.

2.3. Results

To capture the effects of differences in protein quality on egg production of *D. melanogaster*, we made media containing two different amino acid ratios, one known to be mismatched to fly requirements for egg laying (MMaa) and the other known to be matched precisely to the fly's exome (FLYaa) (Piper et al., 2017). For each amino acid ratio, an array of 5 different P:C ratios, each at 4 different nutrient densities, was tested. Thin-plate splines were used to visualise egg laying response surfaces of flies maintained on the different diets (Figure 2.1A and 2.1B).

Across all food types, flies maintained on the MMaa diets laid fewer eggs per female per day than those on the FLYaa diets (MMaa diet, 24.07 ± 1.42; FLYaa 31.69 ± 2.14; one-way ANOVA: $F_{1,33} = 11.205$, $p < 0.001$). For flies on both the MMaa and the FLYaa diet, there was a significant effect of the linear component of protein, such that egg laying increased with increasing dietary protein concentration (Table 2.3.). For FLYaa, we also found the quadratic term for protein concentration to be significant, which is shown in the plots as the peak of egg laying in FLYaa occurred at intermediate concentrations of protein (P:C of 1:0.8) (Figure 2.1.; Table 2.3.). From this point, egg laying dropped away as the protein concentration either increased or decreased (Figure 2.1A; Table 2.3.). In contrast, peak egg laying on MMaa occurred on the food with the highest nutrient density with P:C of 1:0.6. There was no detectable effect of carbohydrate concentration on egg production across foods of both amino acid ratio and there was also no significant interaction between carbohydrate and protein concentration detected (Table 2.3.). These results show that for the range of diets we tested, protein was the principle determinant of egg production, and that per gram of amino acids supplied, FLYaa supported higher levels of egg laying than MMaa.

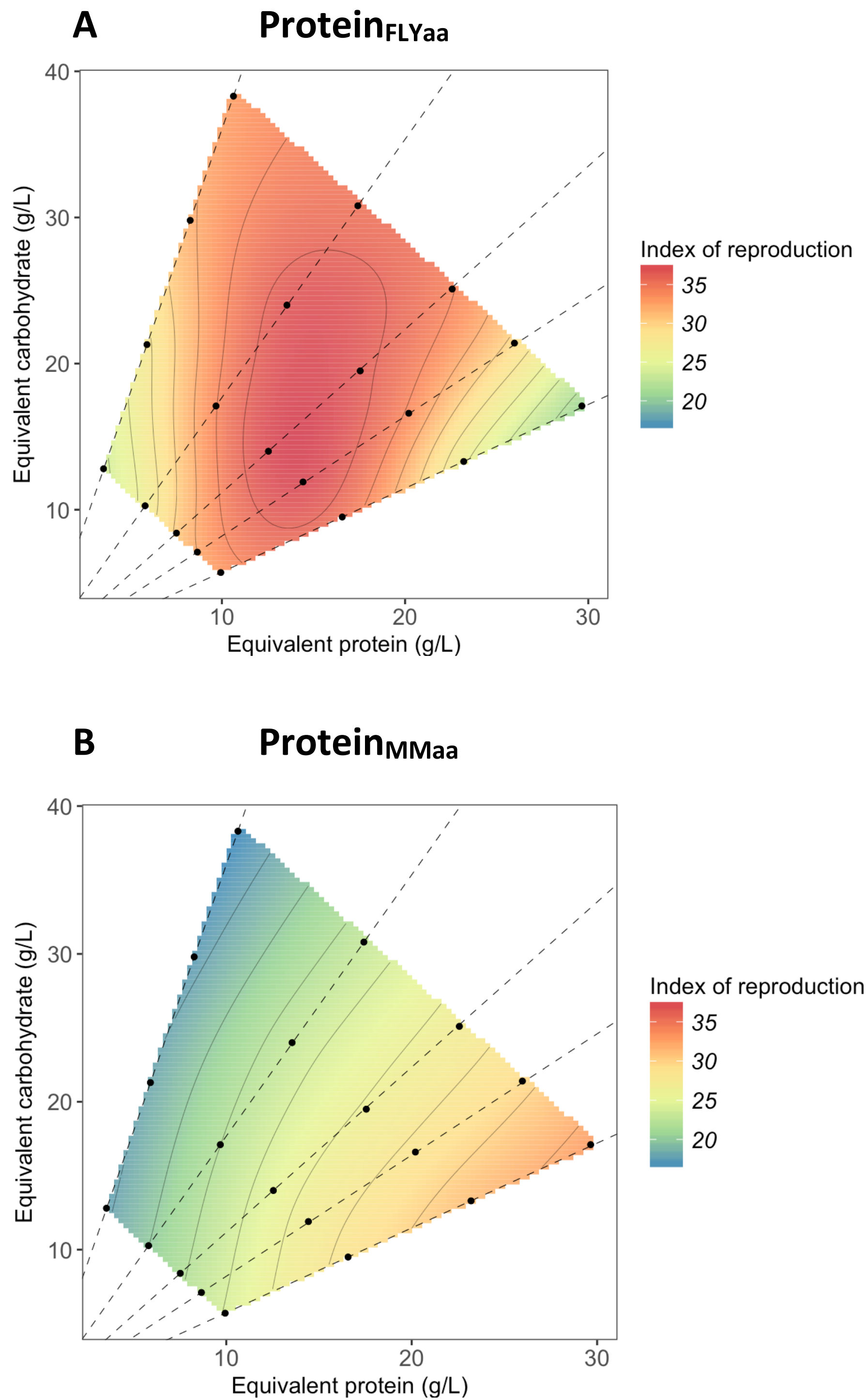


Figure 2.1. Egg laying varies with the quantity of carbohydrates and the quantity and quality of protein. Flies were maintained on diets containing amino acids in the ratio of either **(A)** FLYaa (fly exome matched amino acid ratio) or **(B)** MMaa (mis-matched amino acid ratio). The black dots represent the individual 20 diets from which the index of reproduction was assessed and the dashed lines represent nutritional rails of fixed P:C.

Table 2.3. Egg production on each amino acid ratio (FLYaa and MMaa) was modelled using linear predictors for protein (P) and carbohydrate (C) and their interaction (P x C). Because the data appear non-linear, we also assessed the quadratic terms of protein (P^2) and carbohydrates (C^2). Minimum adequate models are reported. β, indicates the the slope estimate for each variable, and; SE, the standard error. Highlighted bold signifies significance, * p<0.05; ** p<0.001.

Amino acid ratio		P	C	P^2	C^2	P x C
FLYaa	β	**3.250**	-0.206	**-0.127**	-0.007	0.042
	SE	**1.393**	1.203	**0.037**	0.024	0.050
	t-value	**2.332***	-0.171	**-3.464****	-0.301	0.846
MMaa	β	**1.186**	0.129	N/A[b]	N/A[b]	-0.032
	SE	**0.502**	0.341			0.027
	t-value	**2.365***	0.377			-1.184

[b] N/A, not applicable. For the MMaa model, P^2 and C^2 were not significant in the model and so were removed during model reduction.

2.4. Discussion

The ratio of dietary protein to carbohydrate in the diet has an important role in determining reproduction in *Drosophila melanogaster*: higher density diets with greater P:C ratios support higher female egg laying (Mair et al., 2005, Lee et al., 2008, Skorupa et al., 2008, Jensen et al., 2015). Here we show that varying the protein quality of a diet can also dramatically alter its bioavailability for reproduction of female *D. melanogaster*. These data demonstrate how diverse outcomes in important fitness traits can occur when the quality of food ingredients vary.

Protein is often a limiting component of the diet for terrestrial animals (White, 1993), which means that changes in the quality of dietary protein consumed should have observable effects on fitness for many animals in diverse settings. Indeed, supplementing the diet of wild cotton rats and cottontail rabbits with an essential amino acid (methionine) can improve their reproductive success and increase population size (Lochmiller et al., 1995, Webb et al., 2005), while maintaining blue tits on a diet supplemented with amino acids matched for egg protein formation laid more eggs per clutch than those that received a mismatched balance of amino acids (Ramsay and Houston, 1998). Similarly, female copepods fed on a diet containing essential amino acid profiles that were closely matched to female body composition had higher reproductive success (Guisande et al., 1999) and, supplementing amino acids in the diet of livestock, like boars and chickens, can increase reproductive output and yield more lean muscle (Dong et al., 2016, Cerrate et al., 2019). Finally, recent work in the lab on flies and mice has shown that differences in protein quality, specified at the level of essential amino acid balance, can have dramatic effects on animal reproduction and feeding behaviour (Leitao-Goncalves et al., 2017, Piper et al., 2017, Solon-Biet et al., 2019). Thus, changing the quality of dietary protein by altering the proportion of amino acids can have important effects on fitness traits of both laboratory-reared and wild animals.

In our experimental diets, we found large changes in egg output because the amino acid ratio was altered so that the bioavailability of the nitrogen source varied 2.5-fold (Piper et al., 2017). In other words, the level of FLYaa that was required to support maximal egg output was ~15 g/L, which is 2.5-times less than the amount of MMaa (37 g/L) that would be required

to achieve the same output. Thus, smaller amounts of high-quality food are required for optimal egg laying. With defined diets, this large difference in protein quality is easily generated because each amino acid can be independently manipulated over a wide range of concentrations. Interestingly, similarly large differences in the relative abundance of amino acids (i.e. changes in protein quality) can also be found between natural proteins in whole foods. For example, when comparing the average amino acid profiles across food groups published in an FAO report (Food Policy and Food Science Service, 1970), we found that for a fixed quantity of protein, the average amino acid proportions in meat represent ~2-fold higher quality than the average legume-based protein. This is because the limiting essential amino acid in legume protein, methionine, is more deficient compared to the limiting essential amino acid, leucine, in meat protein. Thus, we predict that using different dietary components to feed animals or humans will result in dramatic changes to physiology and behaviour. Furthermore, without explicit knowledge of the source of ingredients and their quality, it will be difficult to extract generalisable conclusions from meta-analyses that incorporate diverse studies.

It is important to note that protein is not the only compound axis in most published Nutritional Geometry experiments. For example, the other energy yielding macronutrients, carbohydrate and fat, also exist as a diverse array of molecules in food. This can be even more complicated since in typical studies with flies, sucrose and yeast are often used the sources of carbon and protein, respectively (Bass et al., 2007, Min et al., 2007, Lee et al., 2008), and yeast contains various types of carbohydrates as well as other nutritional components, such as sterols, nucleic acids, vitamins, and minerals (Lange and Heijnen, 2001). In addition to our findings about protein quality, other work has shown that varying the identity of the carbohydrate component of the diet from sucrose to fructose can alter fly physiology (Lushchak et al., 2014). Thus, it will be important in future studies to understand the relative contribution of different carbohydrates, fats and other nutrients in modifying fitness traits.

2.5. Conclusion

By showing that the precise amino acid composition of dietary protein is key for dictating female fecundity, we demonstrate the need for more information to be provided when labelling composite nutritional axes in Nutritional Geometry experiments. In particular, protein should be labelled with its source to indicate its quality – a metric that would be further improved if the most limiting essential amino acid were identified by referencing the dietary protein amino acid profile to the *in silico* translated exome of the consumer (Piper et al., 2017). We anticipate this will facilitate the comparability of data between studies

3. Chapter III: Gcn2 plays a role in modifying reproduction and lifespan

Abstract

After eating, digesting and absorbing food, organisms detect protein quality and quantity via the mTOR (Mechanistic Target Of Rapamycin) and Gcn2 (General control non-derepressible 2) kinase pathways to modify life-history traits. While there is a lot of work studying the role of mTOR in mediating the effect of diets on lifespan, there is much less known about Gcn2, and although Gcn2 is well-characterised in yeast, there is limited knowledge of its role in shaping physiology in higher organisms, including *Drosophila melanogaster*. To assess the role of Gcn2 in reproduction and lifespan in response to nutrient changes systematically, we compared the responses of *Gcn2Δ* flies to wild type flies, when maintained on an array of diets designed using the principles of Nutritional Geometry. These incorporated five different protein (P): carbohydrate (C) ratios, each at four nutrient densities. We found that when the *Gcn2* gene was knocked out, the nutritional optimum for egg laying moved towards diets with a greater nutrient density. For lifespan, both wild type and *Gcn2Δ* flies were longest lived on low P:C diets, but lifespan of *Gcn2Δ* fell away more steeply than wild type as P:C ratio increased. For both genotypes, the dietary optimum for lifespan diverged from that for reproduction – principally due to changes in carbohydrate concentration. Thus, this study highlights the importance of study designs incorporating a greater range of diet balance variations. It also outlines how *Gcn2Δ* modifies the response of flies to altered nutrient balance.

3.1. Introduction

The amount of protein an organism consumes is important for determining its capacity for reproduction and length of life (Dussutour and Simpson, 2012, Rho and Lee, 2016). Proteins are principally sensed by two evolutionarily conserved molecular pathways: mTOR (Mechanistic Target Of Rapamycin) and Gcn2 (General control non-derepressible 2). These nutrient-sensing pathways are key for matching the rate of protein synthesis, an expensive anabolic process, to the supply of its building blocks (amino acids). The responses of mTOR and Gcn2 to nutrient changes are different. On a high protein diet mTOR is activated, whilst Gcn2 is inactive, which leads to an increase in protein synthesis and cell growth (Dibble and Manning, 2013, Hu et al., 2018). However, on a low protein or an amino acid imbalanced diet, Gcn2 becomes active whilst mTOR is inactive, which protects cells by saving energy through reducing general translation and selectively upregulating the expression of key survival genes (Wek et al., 1995). mTOR has been heavily studied because of its role in growth and its associated mis-regulation in human disease, including Huntington disease (Ravikumar et al., 2004) and breast cancer (Connolly et al., 2006). Gcn2, on the other hand, has been extremely well characterised at the molecular level in yeast, but it is much less well characterised in higher organisms. Understanding the Gcn2 mechanism and how its responses to nutrition impact on lifespan are important for deeper understanding of the nutritional signalling pathways that modify health and fitness.

The role of Gcn2 in mediating the beneficial effects of dietary restriction (DR) has been touched on in studies of yeast. Under low amino acid conditions, like reduced methionine levels, yeast cells are longer-lived (Wu et al., 2013, Hu et al., 2018). However, when yeast null for Gcn2 were exposed to methionine restriction, the beneficial effect on lifespan was impaired (Wu et al., 2013), which suggests that Gcn2 is important for guarding the soma against amino acid imbalance and preserving lifespan. In other work on mice, it has also been shown that the absence of Gcn2 can affect reproductive success in food restricted mice. On a low nutrient diet, *Gcn2* knockout mice display pre- and post-natal defects including still-births, thus showing Gcn2 is important protection against a restricted diet (Zhang et al., 2002).

Experiments employing nutritional geometry (NG) are a useful tool that reveals a fuller description of nutrient effects on phenotypes that would be overlooked in the conventional manner. Experiments employing nutritional geometry (NG) designs have consistently shown that the dietary proportions of protein (P) : carbohydrate (C) modify reproduction and lifespan in many organisms, including flies. Specifically, low P:C diets tend to extend lifespan but lower reproduction, while high P:C diets tend to increase reproduction levels but shorten lifespan (Lee et al., 2008, Lee, 2015, McCracken et al., 2020). The deficiency of certain individual amino acids is detrimental for reproduction of Gcn2 mutant mice (Zhang et al., 2002). However, whether this effect is due to protein or an interaction with carbohydrates in the diet was not known. Despite the evolutionary conservation of these effects and of Gcn2, there has been no formal test of the role of Gcn2 in mediating organismal responses to altered P:C balance. Because vinegar flies can be easily genetically manipulated and reared in large numbers for diet manipulation studies, they are the ideal system in which to undertake these studies. Despite Gcn2 being conserved in all eukaryotic organisms (Berlanga et al., 1999), the role of Gcn2 in how organisms beyond yeast respond to diet is not clear.

In this chapter, I conducted a NG study to examine the lifespan and reproduction of wild-type and *Gcn2Δ* flies in response to holidic diets that differ in the concentration and ratio of protein and carbohydrate. Because Gcn2 is thought to be inactive under conditions of high dietary amino acid levels, we expected *Gcn2Δ* flies to be no different from controls on high nutrient diets. In contrast, we anticipate that *Gcn2Δ* flies on low protein diets will be severely compromised, losing the lifespan benefits of dietary restriction. If so, Gcn2 would be implicated as a key mechanism governing the response of lifespan to dietary amino acid concentrations. We found these expectations were not met, pointing to a more complex role of Gcn2 in dictating fly physiology.

3.2. Experimental procedures

3.2.1. Fly stock and husbandry

An outbred population of the *Drosophila melanogaster* strain white^{Dahomey} (wDah), was used as the wild type control group. This strain has a mutation in the *white* (*w*) gene, which produces white eyed flies instead of the normal red eyes (Bingham, 1980, Hazelrigg et al., 1984). wDah flies were created in the Partridge Lab (University College of London, UK) through multiple rounds of backcrossing of the mutated *w* gene into an outbred population of wild-type Dahomey (personal communication, M. Piper).

To investigate the role of Gcn2, I used *Gcn2Δ* transgenic flies. The *Gcn2Δ* flies were made by ends-out homologous recombination, completely replacing the *Gcn2* coding region with the mini-white gene, by Sebastian Grönke at the Max Planck Institute for the Biology of Ageing, Cologne. *Gcn2Δ* flies were backcrossed to wDah for 8 generations and stocks of homozygous mutants were maintained in standard fly vials (25mm x 95mm, FS32, Pathtech) at 18°C containing ~7 mL of sugar-yeast (SY) food (see section 3.2.2.).

To collect age-synchronised and population-density controlled flies for experiments, between 100-150 pairs of adult flies were maintained in each of a number of population bottles (250 mL volume) that contained ~70 mL of SY diet and closed with a permeable bung. These population bottles were maintained at 25°C for 2 days, a sufficient time for females to mate and lay eggs on the substrate. The resulting adults were then transferred to an egg lay cage, which was a cylindrical perspex tube capped at one end with fine gauze (8.75 cm diameter x 14.8 cm high) and capped at the other end with a removable petri dish containing apple juice agar as a substrate for egg laying (8.75 cm diameter) (apple juice agar recipe in appendix 1). Adult flies were kept in cages over two days to lay eggs, swapping the petri dish after each day. To encourage egg laying, a pea-sized amount of live yeast paste was added on top of the apple juice agar. After the second day of egg laying, the adult flies were discarded and the eggs on the agar were washed off with ~10ml of 1x PBS (100 mL of 10x PBS stock in 900 mL of deionised water) into a 15 mL Falcon tube. Once the eggs had settled, excess PBS was

poured off. 25 μL of the settled egg-PBS suspension was pipetted or 'seeded' into population bottles containing ~70 mL of SY food. To seed more than 30 population bottles, more than one egg laying plate was required to harvest sufficient egg numbers for seeding. For this, instead of using the cylindrical cages, I transferred approximately 4,000 flies into a population tank (25 cm x 25 cm x 40 cm; closable acrylic container), pooled from 27 population bottles, and placed nine apple juice agar plates inside. The nine plates were sufficient to seed more than 35 population bottles, with each bottle expected to yield ~300 flies.

After seeding, the population bottles were maintained at 25°C, relative humidity 60%, 12hr: 12hr light-dark photoperiod. The eggs developed into adult flies after approximately 9.5 days. Once the flies emerged, they were transferred to fresh SY food for 2 days at which point, the flies were anaesthetised with CO_2 and females collected for the experiment. When on their experimental diets, vials of female flies were turned onto fresh food every 2-3 days until the experiment start date. In total sufficient wDah and *Gcn2Δ* were produced to obtain 2,400 females of each genotype.

3.2.2. Rearing and stock maintenance diet

The stock populations of wDah and *Gcn2Δ* flies were maintained on a sugar-yeast SY diet containing sucrose 50 g/L (97364, Bundaberg Sugar), autolysed Brewer's yeast powder 100 g/L (290331225, MP Biomedicals), agar 10 g/L (Grade J3, Gelita), nipagin 30 mL/L (1067575000, Merck Millipore) and propanoic acid 3 mL/L (800605, Merck Pty Limited). The addition of nipagin and propanoic acid is essential to prevent fungal and bacterial growth.

3.2.3. Experimental diets

The complete defined synthetic (holidic) diets were made according to Piper et al. (2014), whereby free amino acids were used to make up protein equivalents. The ratio of the amino acids in the holidic media used in my experiments was based on Piper et al. (2017), who revealed that a dietary amino acid ratio based on the sum of amino acids required to express all proteins encoded by the fly exome (FLYaa) was optimal for reproduction in adult female

vinegar flies without cost to lifespan. The total concentration of carbohydrate and amino acids used in Piper et al. (2014) and Ma et al. (2020) were used to select the range and total concentration of protein and carbohydrates to employ in the diets. The relative ratio of each amino acid in FLYaa is displayed in table 3.1.

Table 3.1. The relative proportions of each amino acid in FLYaa.

	Amino acids		FLYaa ratio
Essential amino acids	Phenylalanine	F	0.037
	Histidine	H	0.026
	Isoleucine	I	0.052
	Lysine	K	0.057
	Leucine	L	0.094
	Methionine	M	0.025
	Arginine	R	0.057
	Threonine	T	0.056
	Valine	V	0.062
	Tryptophan	W	0.010
Non-essential amino acids	Alanine	A	0.075
	Cysteine	C	0.017
	Aspartate	D	0.053
	Glutamate	E	0.063
	Glycine	G	0.062
	Asparagine	N	0.047
	Proline	P	0.052
	Glutamine	Q	0.046
	Serine	S	0.079
	Tyrosine	Y	0.031

The range of holidic diets designed for the experiment are displayed as plotted points in figure 3.1., with diets differing systematically in P:C ratios and caloric densities. I tested the effects of twenty different diets: five different P:C ratios (1:3.6, 1:1.8, 1:1.1, 1:0.8, 1:0.6; represented by coloured lines radiating out from the origin in figure 3.1.) with each dietary P:C ratio made up at four different caloric densities (66.8 kcal/L, 111.3 kcal/L, 155.8 kcal/L, 200.3 kcal/L; represented by grey dashed lines in figure 3.1.). The total content of amino acids and sucrose (as the carbohydrates) and their nutrient densities are displayed in table 3.2. Dietary energy

densities were estimated by calculation, using a value of 4 kcal/g for both protein and carbohydrates. The crude amount of protein was calculated by using a conversion factor; that 16% of protein is nitrogen (Sosulski and Imafidon, 1990). The total content of amino acids (the equivalent proteins) will be referred to as protein in this chapter.

The recipe for each litre of each holidic diet is shown in appendix 2. The recipe for stock solutions required to make the diets (the buffer base, cholesterol, minerals, nucleic acids, lipids, vitamins, folic acid and amino acid solutions) are shown in appendix 3. For tyrosine, the maximum solubility is 0.45 g/L, however for some holidic diets with a high ratio of protein (highlighted in appendix 2) this limit was exceeded and thus it is likely that some tyrosine did not dissolve.

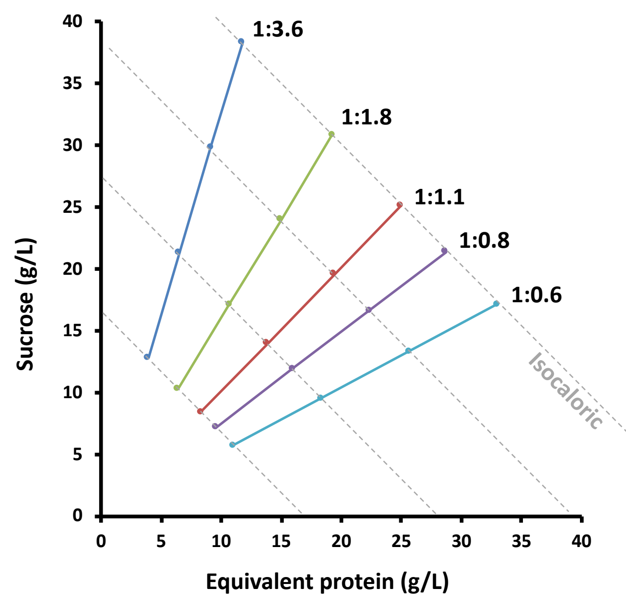


Figure 3.1. A graphical display of the experimental diets used in the study. Each dot represents one of the 20 experimental diets. The five P:C ratio are displayed in bold next to each nutritional rail (coloured lines). The grey dotted lines represent the four nutrient densities: 66.80 kcal/L, 111.30 kcal/L, 155.80 kcal/L and 200.30 kcal/L (grey dashed lines connect isocaloric diets).

Table 3.2. The ratios of protein equivalents: carbohydrate (P:C) are displayed with the nutrient densities. The equivalent proteins were calculated using a conversion factor, of which 16% of proteins is in a mass of nitrogen (Sosulski and Imafidon, 1990).

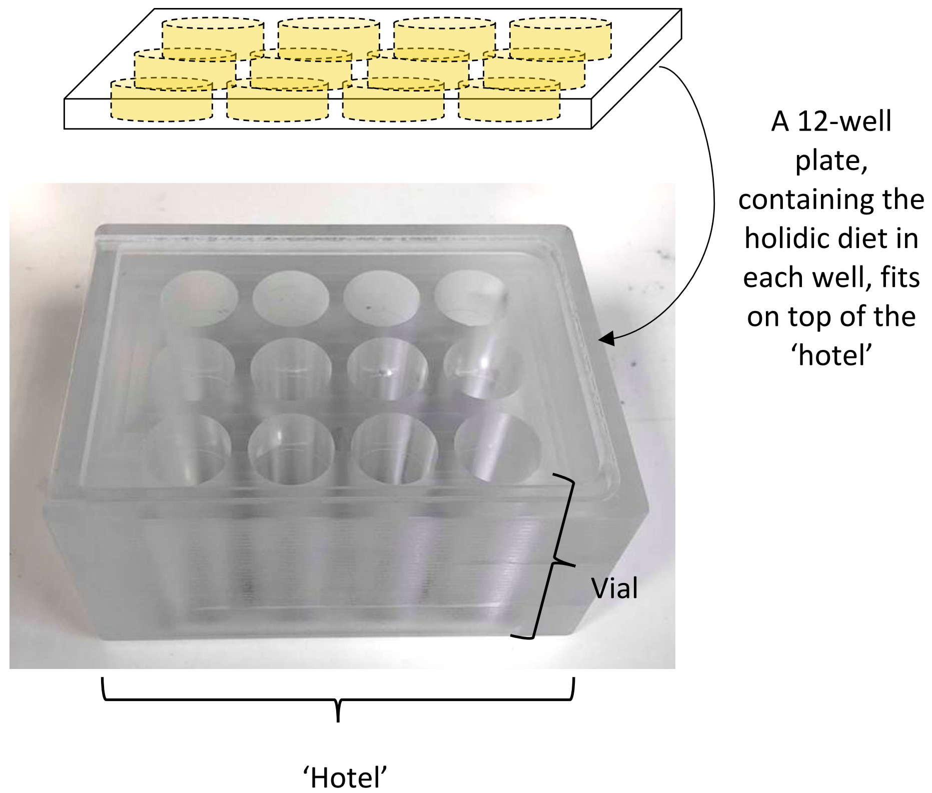
P:C equivalent	Sum mass of amino acids (g/L)	Equivalent protein (g/L)	Equivalent carbohydrate[c] (g/L)	Estimated caloric content (kcal/L)
1:3.6	3.92	3.54	12.77	66.80
1:3.6	6.53	5.91	21.29	111.30
1:3.6	9.14	8.27	29.81	155.80
1:3.6	11.75	10.63	38.32	200.30
1:1.8	6.42	5.81	10.27	66.80
1:1.8	10.7	9.68	17.12	111.30
1:1.8	14.98	13.55	23.97	155.80
1:1.8	19.26	17.42	30.82	200.30
1:1.1	8.32	7.52	8.38	66.80
1:1.1	13.86	12.54	13.96	111.30
1:1.1	19.41	17.55	19.54	155.80
1:1.1	24.95	22.57	25.13	200.30
1:0.8	9.57	8.66	7.12	66.80
1:0.8	15.95	14.43	11.87	111.30
1:0.8	22.33	20.20	16.62	155.80
1:0.8	28.71	25.97	21.37	200.30
1:0.6	10.99	9.94	5.70	66.80
1:0.6	18.32	16.57	9.50	111.30
1:0.6	25.64	23.20	13.30	155.80
1:0.6	32.97	29.65	17.10	200.30

[c] Carbohydrate is added to the diet as sucrose.

3.2.4. Fly hotels

Flies were housed in devices referred to as fly 'hotels', or dFlats which are made up of a block of Perspex with 12-wells drilled into them, such that the drilled wells have a volume the same as standard fly vials (FS32, Pathtech) and their arrangement is such that the 12 openings match the position of wells in a 12-well plate (based on the design: https://www.flidea.tech/projects) (Figure 3.2). 3 mL of food was dispensed into each of the wells in a 12-well plate, which was fitted on top of the chamber (Figure 3.2.). Although this design means that each 'hotel' contained a single diet, and this could potentially confound our interpretation of how diet modified phenotype, the scale of the experiment made it infeasible to distribute the different diets across 'hotels'. In no other assays have we found evidence for hotel-specific effects. Each well of the 'hotel' housed 10 mated female flies and I maintained one 'hotel' for each of the 20 holidic experimental diets (12 replicate wells x 10 flies = 120 flies per diet). The 12-well plates with fresh holidic media were replaced every two to three days.

Figure 3.2. A 'hotel' containing 12 vials was used in the lifespan and reproduction experiments. Each vial within a 'hotel' contained 10 female flies. A 12-well plate filled with one of the holidic diets was fitted on top of the 'hotel', which was refreshed every 2-3 days.



3.2.5. Reproduction experiment

Once a week during the first four weeks of the experiment, the number of eggs laid on the surface of the food over an 18-hour period was recorded. For each well, the number of eggs laid per female on experimental days 8, 15, 22 and 29 were summed and then averaged across the 12 wells for each diet. This gave an index of reproduction, which was expressed as the number of eggs laid per female per day.

To count eggs, images of individual wells were taken using a camera (Logitech model c920) mounted on a microscope. The eggs could be easily counted from the images on screen, however, due to the large number of images (1920 images per time point) I used an automated counting software package called QuantiFly (Waithe et al., 2015). To train QuantiFly I inputted manual counts for wells from the lowest and the highest nutrient dense holidic diet along the 1:3.6 nutritional rail on experimental day 15. From the 12 well images from each diet treatment, I randomly selected three wells to use in the training model, thus six images in total were used in the training model (Appendix 4).

QuantiFly can process 600 x 600 pixel images, therefore all original images needed to be cropped and downsized. To do this, I used a macro to automatically crop images in batches and down-size the resolution in FIJI (Schindelin et al., 2012) (Appendix 5). The macro is available at https://github.com/dwaithe/quantifly (Waithe et al., 2015). The edited images of eggs were processed and counted in QuantiFly in batch mode to yield egg numbers for each image.

3.2.6. Lifespan experiment

Before each change of holidic media, the number of deaths and censors (missing or death unrelated to diet) were observed and recorded using the software DLife, developed by Scott Pletcher (University of Michigan) (Linford et al., 2013). The software calculates the median lifespan per vial.

3.2.7. Statistical analyses

All analyses were conducted using R (version 3.3.0, available from http://www.R-project.org/). To analyse the relationship between protein (P) and carbohydrate (C) concentration and the index of reproduction and median lifespan, we estimated separate response surfaces for each of the two genotypes, wDah and *Gcn2Δ* [following Lee et al. (2008)]. Each surface was estimated using multivariate second-order polynomial regression, whereby the linear, quadratic and cross-product terms from this model capture the linear and non-linear effects of P and C concentration on fly reproduction or median lifespan. We then visualised the response surface of each genotype, using predictions derived from thin-plate splines from the fields package (see www.github.com/NCAR/Fields) and plotted using the ggplot package (Wickham, 2016).

Additionally, I compared the index of reproduction and lifespan surfaces between genotypes. The number of eggs laid per female ad the median lifespan was standardised, such that the mean equated to 0 and the standard deviation equated 1. The full model included the linear, quadratic and interaction terms of protein and carbohydrate, and the interaction of genotype. This full model was compared to another model but replacing the genotype interaction with the genotype as an additive effect. These two models were compared using type II ANOVA.

3.3. Results

3.3.1. Reproduction

To understand the role of Gcn2 in reproduction, I compared egg laying of *Gcn2Δ* females with that of wDah flies – an outbred wild-type lab strain into whose genetic background the *Gcn2Δ* flies were backcrossed. For both genotypes, an array of five different P:C ratios, each at four different nutrient densities, was tested. Thin-plate splines were used to visualise egg laying response surfaces of flies maintained on the different diets (Figure 3.3A and 3.3B). The overall egg laying across all food types was not significantly different between genotypes (Mean index of reproduction: wDah flies, 56.17 ± 5.77 eggs per female per day; *Gcn2Δ* flies, 49.43 ± 2.44 eggs per female per day; ANOVA: $F_{1,28} = 1.27$, $p = 0.27$). I did, however, find that genotype significantly altered the shape of the reproduction response surface (ANOVA: $F_{5,468} = 4.17$, $p < 0.01$).

In *Gcn2Δ* flies, the diet for peak egg laying occurred at intermediate nutrient density of the highest P:C ratio (P:C 1:1.1) (Figure 3.3). There was a significant effect of P on egg laying, where egg laying generally increased with increasing dietary P concentration ($\beta = 3.570$, *t*-value = 2.625, $p < 0.05$; table 3.3). There was also a significant effect of P^2, which indicates egg laying dropped away as concentration of P either increased or decreased from the peak at ~20 g/L dietary protein ($\beta = -0.108$, *t*-value = -3.033, $p < 0.01$; table 3.3). There was no detectable effect of the linear and quadratic component of C or the P x C interaction term (Table 3.3). In wDah flies, the diet for peak egg laying was different from that for Gcn2*Δ* flies and was at a low nutrient density with high P:C ratio (P:C 1:0.8) (Figure 3.3). However, we did not observe a significant effect of either P or C to change egg laying (Table 3.3), indicating that little variation in egg laying was explain by dietary macronutrients in the controls.

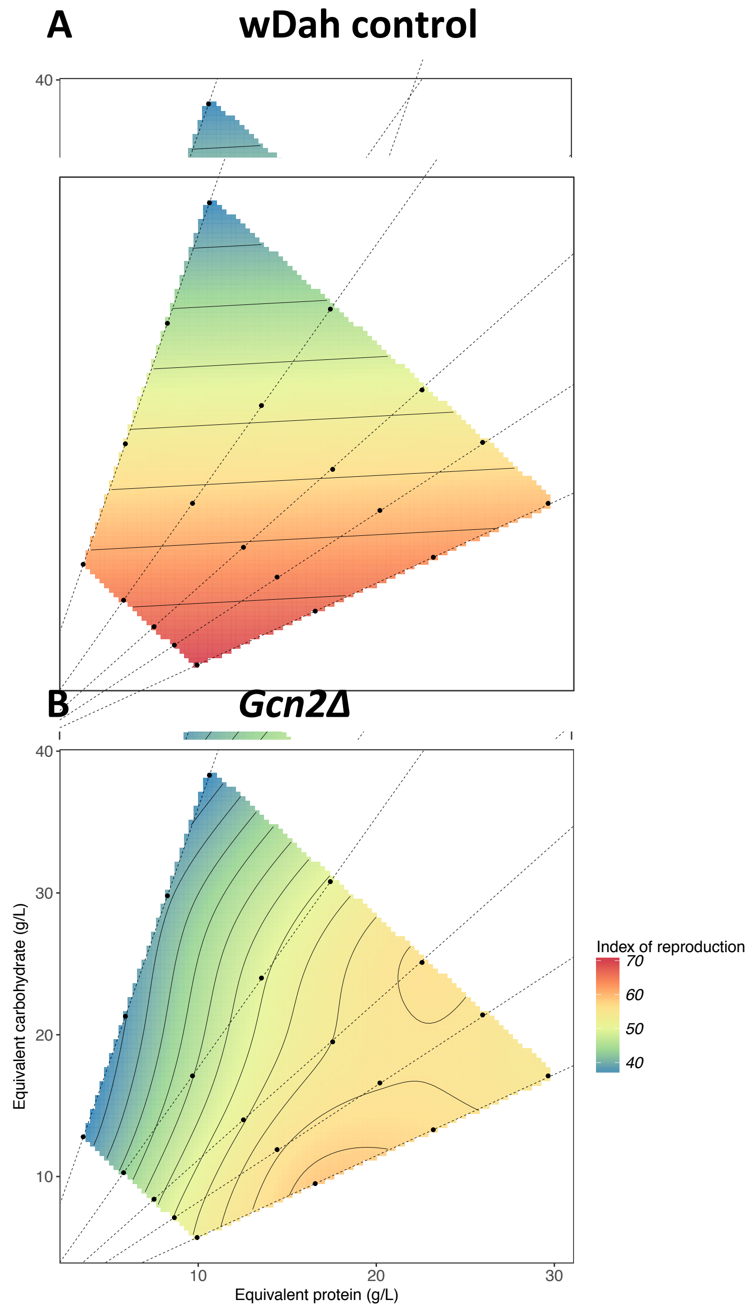


Figure 3.3. Egg laying responses of wDah and *Gcn2Δ* flies to changes in dietary carbohydrates and protein. **(A)** wDah flies and **(B)** *Gcn2Δ* flies were maintained on diets systematically varying in P:C ratio and overall nutrient density. The black dots represent the individual 20 diets from which the index of reproduction was assessed, and the dashed lines represent nutritional rails of fixed P:C. In control wDah flies **(A)**, the nutrient optimum for egg laying in wDah appeared to be at low nutritional density with P:C ratio 1:0.8, but in *Gcn2Δ* flies **(B)** the nutrient optimum appeared shifted towards a more nutrient dense, but a lowed P:C ratio (1:1.1 diet).

Table 3.3. The effects of the linear components of protein (P), carbohydrates (C), the quadratic term of protein (P^2) and carbohydrates (C^2) and the protein and carbohydrate cross-product interaction (P x C) on egg production of wDah and *Gcn2Δ* flies. For each genotype, the response surface was estimated using multivariate second-order polynomial regression SE, standard error. Highlighted bold signifies significance. p <0.05*; <0.01**.

Genotype		P	C	P^2	C^2	P x C
wDah	Parameter	-0.682	-5.597	-0.075	0.049	0.194
	SE	4.396	3.796	0.115	0.077	0.157
	t-value	-0.155	-1.474	-0.654	0.639	1.237
	p-value	0.879	0.163	0.524	0.533	0.236
Gcn2Δ	Parameter	**3.570**	-0.844	**-0.108**	-0.003	0.042
	SE	**1.360**	1.174	**0.036**	0.024	0.049
	t-value	**2.625**	-0.719	**-3.033**	-0.136	0.859
	p-value	*	0.484	**	0.894	0.405

3.3.2. Lifespan

I also investigated the role of Gcn2 in lifespan by comparing *Gcn2Δ* flies with wDah controls, using the same flies and across the same set of diets as for reproduction (section 3.2.3.) Thin-plate splines were used to visualise the response of median lifespan to each of the different diets (Figure 3.4A and 3.4B). To note, one vial in the diet group P:C 1:1.8 of the most nutrient dense group was discarded from the analyses due to loss of data.

Across all diets, wDah flies were significantly longer lived than *Gcn2Δ* flies (Mean lifespan: wDah flies, 51.27 ± 0.87 days; *Gcn2Δ* flies, 48.08 ± 1.05 days; ANOVA: $F_{1,28} = 5.79$, $p < 0.05$). Genotype also significantly changed the shape of the surface describing the response of lifespan to changes in dietary P and C levels (ANOVA: $F_{5,467} = 3.24$, $p < 0.01$).

When looking at each of the response surfaces for wDah and *Gcn2Δ* flies, lifespan of both genotypes peaked at the highest nutrient density of the lowest P:C ratio of 1:3.6 (Figure 3.4). However, the main effects of P and C were found not to affect lifespan significantly in wDah flies (Table 3.4). Similarly, in *Gcn2Δ* flies there was no significant effect of either P or C on lifespan (Table 3.4).

Table 3.4. The effects of the linear components of protein (P), carbohydrates (C), the quadratic term of protein (P^2) and carbohydrates (C^2) and the protein and carbohydrate interaction (P x C) on lifespan of wDah and *Gcn2Δ* flies. For each genotype, the response surface was estimated using multivariate second-order polynomial regression. SE, standard error. Highlighted bold signifies significance. p <0.05*; <0.01**.

Genotype		P	C	P^2	C^2	P x C
wDah	Parameter	-0.523	-0.041	0.012	0.005	0.006
	SE	0.653	0.564	0.017	0.011	0.023
	t-value	-0.801	-0.073	0.729	0.445	0.256
	p-value	0.436	0.943	0.478	0.663	0.802
Gcn2Δ	Parameter	-0.750	0.605	0.014	-0.009	0.000
	SE	0.778	0.672	0.020	0.014	0.028
	t-value	-0.964	0.900	0.707	-0.653	0.013
	p-value	0.351	0.384	0.491	0.524	0.990

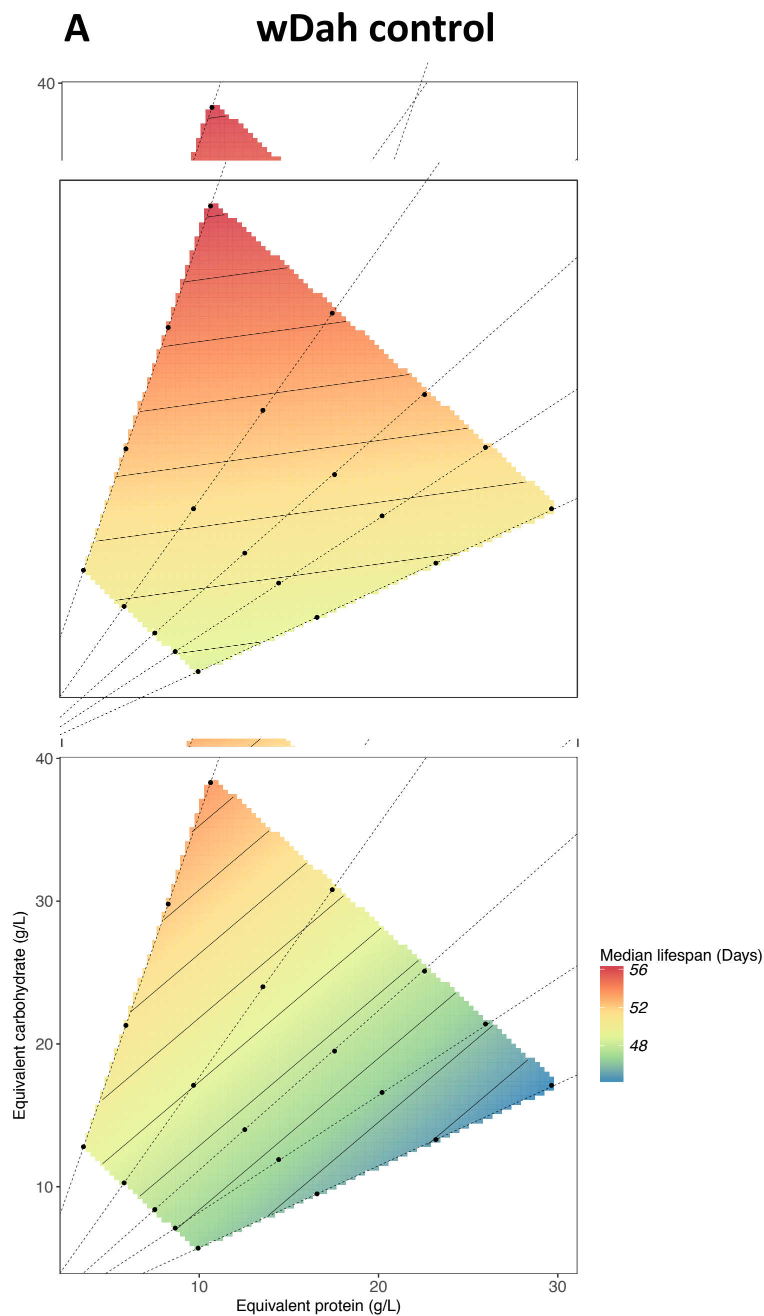


Figure 3.4. Lifespan response to changes in dietary carbohydrates and protein concentrations. **(A)** wDah flies and **(B)** *Gcn2Δ* flies were maintained on 20 diets varying in P:C ratio and nutrient density. The black dots represent the individual 20 diets and the dashed lines represent nutritional rails of fixed P:C. The nutritional optima for median lifespan of wDah control and *Gcn2Δ* flies were at the highest nutrient density of the lowest P:C (1:3.6) diet. For *Gcn2Δ* flies, the median lifespan appeared to fall away towards the higher P:C (1:0.6) ratio diet.

3.4. Discussion

Nutrients play an important role in modulating fitness-related traits. There is a great deal of evidence that points towards increasing protein and amino acid levels in the diet as major contributors to the nutritional drive to promote egg laying performance as well as reduce lifespan outcomes (Lee et al., 2008, Hoedjes et al., 2017, Piper et al., 2017). Investigating how modifications to nutrient signalling pathways can change the responses of fly lifespan and reproduction to nutrition is important for understanding the mechanisms by which these phenotypes are controlled.

In yeast, increasing levels of uncharged tRNAs have been shown to activate Gcn2, a situation that also led to a significant increase in replicative lifespan, as well as reduced overall protein synthesis (Hu et al., 2018). Gcn2 is responsible for enhanced cellular proteostasis and lifespan under these conditions by ensuring that protein production matches the available supply of amino acids (Hu et al., 2018). However, the amino acid sensor Gcn2 kinase is not well characterised beyond yeast and so I sought to characterise the role of the Gcn2 kinase nutrient sensing pathway in modulating *Drosophila* egg laying and lifespan in response to nutrient restriction.

3.4.1. Gcn2 affect reproduction in response to low protein

There was no significant difference in egg lay between wDah and *Gcn2Δ* flies when comparing index of reproduction from all diet treatment. However, in *Gcn2Δ* flies, the number of eggs laid was affected by the concentration of protein, where lower protein concentrations compromised reproduction to a greater extent than for controls. Although our diets do not provide information on the response of *Gcn2Δ* flies to specific amino acid deficiencies, they do indicate an evolutionarily conserved role for Gcn2 to support normal reproduction when protein levels are low.

Under amino acid deficient conditions, Gcn2 is important for adapting the levels of protein synthesis to the amount of amino acids in the cells (Dever et al., 1992) and is important for regulating specific genes that are involved in adapting to amino acid deprivation (Hinnebusch,

1996). One of the set of functionally related genes that is regulated under amino acid deprivation is the autophagy (*Atg*) genes, whose expression is triggered via the Gcn2-ATF4 pathway (Ye et al., 2010). Autophagy is important for restoring amino acid homeostasis, since it allows cells to reutilize resources by degrading intracellular proteins and organelles (Singh and Cuervo, 2011). In *Drosophila*, autophagy has also been found to be important for oogenesis during starved conditions (Barth et al., 2011). During starvation, autophagy genes (*Atg 5* and *Atg8*) were upregulated in the germline cells and follicle cells in the ovaries, which has an important role in egg development (Barth et al., 2011). Lacking Gcn2 may have inhibited the process of autophagy activation on low protein diets, and so had a generally detrimental impact on reproduction when compared to wDah flies. It would be interesting to see the regulation of *Atg* genes in response to low proteins in our *Gcn2Δ* flies.

3.4.2. Gcn2 is required for normal lifespan, but not in response to nutrition

The lifespan of *Gcn2Δ* flies were shorter than the controls across diets, and genotype modified the shape of the response surface (Figure 3.4B). The plots do indicate that *Gcn2Δ* were shorter lived particularly on higher protein diets. This might suggest that Gcn2 plays a protective role against potential damage from high protein levels. If true, a possible explanation involves a deficit in purine levels, the building blocks of DNA and RNA, which would be increasingly required with higher egg laying at high protein levels. In yeast, it has been shown that purine deprivation, as well as amino acid deprivation can activate Gcn2. In our experiment, high amino acid levels drive higher production and this could create a purine deficit that activates Gcn2, which then activates the transcription factor ATF4 (Activating transcription factor 4), to stimulate purine biosynthesis to supply building blocks for cell growth (Vattem and Wek, 2004). So, in absence of Gcn2, mated female flies maintained on high dietary P:C ratios may critically deplete their purine stores, due to high rates of egg production, which is not matched by an increase in purine biosynthesis, and so shortens lifespan. Although this explanation is plausible and interesting, it is worth highlighting again that we did not have sufficient power in this experiment to detect significant effects of protein or carbohydrate on lifespan. An alternative explanation is that flies without functional Gcn2 may well be generally

sick (as reflected by their shortened lifespan on average), and so be vulnerable to any diet change in a non-specific way.

3.4.3. Lifespan and egg laying have divergent nutritional optima for both genotypes when more than one nutrient dimension is considered

The dietary P:C balance that was optimal for lifespan differed from that which was optimal for egg laying in both wDah and *Gcn2Δ* flies, which is consistent with these two traits having distinct nutritional optima. This was one of the key findings of the original studies using nutritional geometry designs to study lifespan in *Drosophila* (Lee et al., 2008, Skorupa et al., 2008). These past studies used complex media with yeast as the source of protein. More recently, Piper et al. (2017) using holidic diets found that the divergence of nutritional optima for these traits may not be necessary when the dietary amino acid proportions were varied to maximise protein quality for reproduction. Critically, the increase in protein quality that was reported meant that flies could be fed smaller amounts of high-quality protein to maximise reproduction and these low levels of high-quality protein imposed no cost to lifespan. This indicated that a single nutritional optimum was possible for these two traits (Piper et al., 2017). My experiment differed from that of Piper et al by incorporating more diet treatments, varying levels of both sugar and exome matched protein simultaneously. The outcome of testing this broader range of nutrient space is that the lifespan and reproduction optima for both genotypes diverged, mainly along the carbohydrate dimension. This observation was not possible in the study of Piper et al (2017) and shows how a broader view of nutrient space using the NG can reveal additional physiological effects that are hidden by studies that manipulate one variable (e.g. protein) at a time. In combination with the important point above about the flies potentially suffering a deficit of purines on high protein diets, my work underscores the importance of incorporating nutrient interactions into studies that examine the effects of macronutrients on fitness-related traits.

3.5. Conclusion

In this study, I found that the absence of Gcn2 in vinegar flies affected the shape of the reproduction and lifespan response surfaces to changes in dietary protein and carbohydrate concentrations. The absence of Gcn2 shifted the peak of reproduction towards a higher protein and more nutrient dense diet, and reduced lifespan across the diets, particularly at high protein and more nutrient dense diets. Though Gcn2 did not affect lifespan in response to nutrition, the presence of Gcn2 was still key for normal lifespan. This study is the first to record the effect of Gcn2 using a NG approach, giving an in-depth insight into the interaction of GCN2 and macronutrients on life-history traits.

4. Chapter IV: Discussion

Organisms live in an imperfect environment, where their sources of food are nutritionally imbalanced relative to their requirements. This means that organismal fitness in the wild is constrained by the nutrition that they consume (Simpson and Raubenheimer, 2012). For this reason, animals have evolved behavioural and physiological adaptations that enable them to mix diets and differentially metabolise nutrients so that they better match up to requirements. (Mayntz et al., 2005, Jensen et al., 2011).

Protein is an important nutritional determinant of fitness (Goodrick, 1978, Min and Tatar, 2006, Fanson et al., 2012, Pan et al., 2014, Arganda et al., 2017). Numerous studies have reported the effect of modifying protein intake quantity on growth and the number of offspring animals can produce (McCay et al., 1935, Mair et al., 2005, Min and Tatar, 2006, Grandison et al., 2009, Dussutour and Simpson, 2012, Lee, 2015). Protein quality, which is dictated by its amino acid composition, can modify the biological availability of protein and this has been shown to modify life history traits (Piper et al., 2017). However, how the ratio of dietary amino acids affects these traits in the broader context of macronutrient interactions, and by what mechanisms, is unknown. In this thesis, I have investigated how the relative abundance of dietary carbohydrate and protein modify the impact of protein quality on egg laying in vinegar flies, *Drosophila melanogaster,* and also how these effects are altered by one of the main, but understudied, amino acid signalling pathways.

In fly studies, it is common to study the effects of protein and carbohydrate variation on lifespan and/or reproduction through the use of two or a few diets (Min and Tatar, 2006, Min et al., 2007, Piper et al., 2017). However, this under-represents the complexity of how nutrition interacts to modify physiology. By using Nutritional Geometry (NG), we can study these interactive effects on traits in a more powerful way. Using NG, I found that the nutrient optimum for reproduction of vinegar flies changed depending on protein quality (Chapter II). This demonstrated how nutrient balance beyond just protein and carbohydrate interactions can affect a major component of fitness. Following this, I demonstrated how the effects of protein and carbohydrate variation on egg laying and lifespan was modified by mutation in

one of the two main amino acid sensors, Gcn2 (Chapter III). Thus, giving a further insight into the understudied nutrient sensing pathway.

4.1. Protein quality and protein quantity interact to modify *Drosophila* egg laying

In nutritional studies on *D. melanogaster*, the main macronutrients examined for their effects on life-history traits are predominantly carbohydrates and proteins. Yeast is a common source of protein, but dry yeast contains about 40% protein, with carbohydrates, fats and essential micronutrients making up the remaining 60% (Majara et al., 1998). Different sources of yeast can have measurable differences in their impact on lifespan and reproduction (Bass et al., 2007). This presents an issue for investigating the effects of proteins and carbohydrates on phenotypes when using yeast, since there are nutrient variations that are not within the control of the experimenter. Using synthetic diets in which every component is added as a purified ingredient (i.e. a holidic diet) allows the experimenter to manipulate each dietary component independently of all others, thus avoiding nutrient variation that is found in natural ingredients.

The diets that I tested used the Piper lab's holidic medium recipe (Piper et al., 2014), which incorporates the dietary amino acid ratio (FLYaa) that maximises reproduction at low concentrations without shortening lifespan. In my experiments, I observed that egg laying drops away at both lower and higher doses of protein and that enhancing protein quality by improving its amino acid proportions lowered the concentration of dietary protein at which optimal egg production was achieved. Protein sources are not equal in their amino acid ratio, and since I found that a change in ratio can drastically alter the reproduction of an organism, protein quality should be specified and emphasised when data are reported in the literature – perhaps being explicitly highlighted on the protein axes of NG graphs in nutritional studies.

4.2. Gcn2 pathway affects reproduction in vinegar flies

Different genotypes can respond differently to diets, for example the diet for optimal reproduction can change depending on the sex of vinegar flies (Camus et al., 2017). The Gcn2

kinase pathway can detect depletion of individual amino acids (Murguia and Serrano, 2012), due to its sensitivity to the presence of uncharged tRNAs (Zaborske et al., 2010). This signalling pathway has an important control over physiology (Zhang et al., 2002) and behaviour in mice (Maurin et al., 2005). However, in vinegar flies, changes in reproduction and lifespan in response to nutrition via Gcn2 has been understudied.

I showed that when Gcn2 was deleted, flies required a higher concentration of protein to increase egg laying than for flies with functional Gcn2. In addition, I found that genotype modified the shape of the surface response to diet. Interestingly, the diet I used to study the egg laying response of *Gcn2Δ* flies contained the higher quality protein (FLYaa) (see chapter III; figure 3.3B), and yet the egg laying response was visually comparable to the effect of nutrition on egg laying when wildtype vinegar flies were maintained on the poorer quality protein diet (MMaa) (see chapter II; figure 2.1B). The visual comparison between these surfaces indicates that having in-tact Gcn2 signalling and high protein quality is important for egg production. To look into the mechanism behind the role of Gcn2 on reproduction in flies, it would be interesting to locate the tissue-specific requirement for this effect. This could be done by artificially increasing the levels of uncharged tRNAs by knocking down one or more tRNA synthetases in specific tissues (Armstrong et al., 2014). This would activate Gcn2 in specific tissues and so would provide a step toward identifying their role(s) in reproduction in response to dietary protein changes.

4.3. *white* gene as a potential confounding factor

A comparison of the egg laying output for wild-type flies on high quality protein in chapter II (Figure 2.1A) to that of the controls in chapter III (Figure 3.3A) reveals potential differences where none were expected because they share the same genetic background. The obvious difference between the control strains in the two chapters is that the flies in chapter II had wild type red eyes, while those in chapter three carried a mutation in the white gene, which gave the flies white eyes. While this difference is not predicted to yield different reproduction outcomes between the lines, the peak egg laying of rDah flies was at an intermediate concentration of protein and carbohydrate, while the peak egg laying of wDah flies was at the lowest carbohydrate and at a less nutrient dense level. We also observed that the average

egg count across all diet types was higher for the white eyed flies than the red eyed flies, an effect that could be accounted for by inter-trial variation in overall egg laying rates (which can vary by more than two-fold (Piper et al, 2014)). While overall average differences can vary substantially, the trends of change across diet types is normally preserved indicating that the change in egg laying peak could mean that eye colour influences the flies' responses to diet for reproduction.

The white gene encodes a transporter that loads pigment granules for deposit into cells in the compound eyes, the ocelli, as well as being found expressed in the Malpighian tubules and the testis (O'Hare et al., 1984, Hazelrigg, 1987). My data indicate that eye colour may have had a role in modifying the effects of both protein and carbohydrate on egg laying. Recently, it was shown that the white gene can control copulation success in vinegar flies (Xiao et al., 2017), indicating it may have further additional roles that are not yet described. One possibility for its role in modulating nutrient availability is via a potential function in solute transport in the malpighian tubules, which are equivalent to the kidneys. It would be interesting to conduct a direct comparison of the two genotypes in which they are assessed contemporaneously to see if the white gene is required for normal reproduction responses to diet balance. If so, it could have large implications for the many studies that use transgenic *Drosophila*, which often carry a functional copy of the white gene as a visible marker and are compared to the white-eyed controls. If the change in *white* alone modifies how diet influences reproduction, it would call for a reassessment of the best control line to use when studying the role of transgenes on life history traits.

4.4. Conclusion

My experiments are the first to examine the effect of protein quality on reproduction of female vinegar flies using the Nutritional Geometry approach, and how this is modified by the nutrient sensing kinase, Gcn2. I found that changes as small as altering amino acid ratios can dramatically alter physiology, meaning these levels need to be carefully controlled and described in experimental studies seeking to understand the role of diet modification on reproduction. I have found that Gcn2 plays a role in determining how life-history traits

respond to nutritional variation. Together, these data improve our understanding of the dietary changes that modify fitness and the way they are encoded genetically.

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Appendices

Appendix 1. Apple juice agar recipe. The final volume of the following recipe was sufficient to produce approximately nine egg lay plates. The plates were taped and kept at 4°C until used.

Ingredients	Amount
Nipagin	10.5 mL
Agar	12.5 g
Apple juice	150 mL
Water	*250 mL
	**25 mL

Preparation:

1. Stir agar and water (amount*) until boil.
2. Add apple juice and stir well.
3. Bring to a boil.
4. Turn off the heat.
5. Pour the extra cold water (amount **)
6. Add nipagin, mix well and pour.

Appendix 2. The recipe for each litre of holidic diet with the FLYaa and MMaa ratio for each nutrient density. Yellow highlight with red text indicates that the maximum solubility of tyrosine (0.45 g/L) was exceeded.

P:C equivalent		Components	Nutrient density (kcal/L)							
			FLYaa ratio				MMaa ratio			
			66.8	111.3	155.8	200.3	66.8	111.3	155.8	200.3
1:3.6		Agar (g)	7.000	7.000	7.000	7.000	7.000	7.000	7.000	7.000
		Isoleucine (g)	0.205	0.342	0.478	0.615	0.333	0.555	0.777	0.999
		Leucine (g)	0.373	0.622	0.871	1.120	0.221	0.369	0.517	0.664
		Tryosine (g)	0.168	0.281	0.393	0.505	0.077	0.128	0.179	0.231
		Sucrose (g)	12.775	21.291	29.807	38.324	12.775	21.291	29.807	38.324
		20 mg/mL absolute EtOH Cholesterol (mL)	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000
		x1000 CaCl2 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
		x1000 MgSO4 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
		x1000 CuSO4 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
		x1000 FeSO4 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
		x1000 MnCl2 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
		x1000 ZnSO4 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
		MilliQ water (mL)	822.815	805.359	787.903	770.446	824.077	807.461	790.846	774.230
			Autoclave step (sterilisation time 11 minutes at 121°C)							
		x10 Buffer (mL) *	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000
		Nucleic acid and lipid solution (mL) *	8.000	8.000	8.000	8.000	8.000	8.000	8.000	8.000
	Amino acid solutions	Essential amino acid (mL)*	11.079	18.464	25.850	33.236	11.077	18.461	25.846	33.230
		Non-essential amino acid (mL) *	11.079	18.464	25.850	33.236	11.077	18.461	25.846	33.230
		100 mg/mL Glutamate (mL)	2.779	4.631	6.484	8.336	2.770	4.616	6.463	8.309
		50 mg/mL Cysteine (mL)	1.248	2.081	2.913	3.745	0.000	0.000	0.000	0.000
		Vitamins (mL)*	21.000	21.000	21.000	21.000	21.000	21.000	21.000	21.000
		Folic acid (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
		Propionic acid (mL)	6.000	6.000	6.000	6.000	6.000	6.000	6.000	6.000
		Nipagin (mL)	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000

P:C equivalent		Components	Nutrient density (kcal/L)							
			FLYaa ratio				MMaa ratio			
			66.8	111.3	155.8	200.3	66.8	111.3	155.8	200.3
1:1.8		Agar (g)	7.000	7.000	7.000	7.000	7.000	7.000	7.000	7.000
		Isoleucine (g)	0.336	0.560	0.784	1.008	0.333	0.555	0.777	0.999
		Leucine (g)	0.612	1.020	1.428	1.836	0.221	0.369	0.517	0.664
		Tryosine (g)	0.276	0.460	0.644	0.828	0.077	0.128	0.179	0.231
		Sucrose (g)	10.272	17.120	23.968	30.816	12.775	21.291	29.807	38.324
		20 mg/mL absolute EtOH Cholesterol (mL)	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000
		x1000 CaCl2 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
		x1000 MgSO4 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
		x1000 CuSO4 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
		x1000 FeSO4 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
		x1000 MnCl2 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
		x1000 ZnSO4 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
		MilliQ water (mL)	806.088	777.480	748.872	720.264	824.077	807.461	790.846	774.230
			Autoclave step (sterilisation time 11 minutes at 121°C)							
		x10 Buffer (mL) *	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000
		Nucleic acid and lipid solution (mL) *	8.000	8.000	8.000	8.000	8.000	8.000	8.000	8.000
	Amino acid solutions	Essential amino acid (mL)*	18.156	30.260	42.364	54.468	11.077	18.461	25.846	33.230
		Non-essential amino acid (mL) *	18.156	30.260	42.364	54.468	11.077	18.461	25.846	33.230
		100 mg/mL Glutamate (mL)	4.554	7.590	10.626	13.662	2.770	4.616	6.463	8.309
		50 mg/mL Cysteine (mL)	2.046	3.410	4.774	6.138	0.000	0.000	0.000	0.000
		Vitamins (mL)*	21.000	21.000	21.000	21.000	21.000	21.000	21.000	21.000
		Folic acid (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
		Propionic acid (mL)	6.000	6.000	6.000	6.000	6.000	6.000	6.000	6.000
		Nipagin (mL)	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000

*for further recipe see appendix 3.

Appendix 2 continued.

P:C equivalent	Components	Nutrient density (kcal/L)							
		FLYaa ratio				MMaa ratio			
		66.8	111.3	155.8	200.3	66.8	111.3	155.8	200.3
1:1.1	Agar (g)	7.000	7.000	7.000	7.000	7.000	7.000	7.000	7.000
	Isoleucine (g)	0.435	0.725	1.016	1.306	0.707	1.179	1.650	2.122
	Leucine (g)	0.793	1.321	1.850	2.378	0.470	0.784	1.097	1.411
	Tryosine (g)	0.358	0.596	0.834	1.073	0.163	0.272	0.381	0.490
	Sucrose (g)	8.375	13.959	19.542	25.126	8.375	13.959	19.542	25.126
	20 mg/mL absolute EtOH Cholesterol (mL)	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000
	x1000 CaCl2 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	x1000 MgSO4 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	x1000 CuSO4 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	x1000 FeSO4 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	x1000 MnCl2 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	x1000 ZnSO4 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	MilliQ water (mL)	793.411	756.351	719.291	682.232	796.088	760.814	725.539	690.265
		Autoclave step (sterilisation time 11 minutes at 121°C)							
	x10 Buffer (mL) *	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000
	Nucleic acid and lipid solution (mL) *	8.000	8.000	8.000	8.000	8.000	8.000	8.000	8.000
	Amino acid solutions: Essential amino acid (mL)*	23.520	39.200	54.880	70.559	23.516	39.193	54.870	70.548
	Amino acid solutions: Non-essential amino acid (mL) *	23.520	39.200	54.880	70.559	23.516	39.193	54.870	70.548
	Amino acid solutions: 100 mg/mL Glutamate (mL)	5.899	9.832	13.765	17.698	5.880	9.800	13.720	17.640
	Amino acid solutions: 50 mg/mL Cysteine (mL)	2.650	4.417	6.184	7.951	0.000	0.000	0.000	0.000
	Vitamins (mL)*	21.000	21.000	21.000	21.000	21.000	21.000	21.000	21.000
	Folic acid (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	Propionic acid (mL)	6.000	6.000	6.000	6.000	6.000	6.000	6.000	6.000
	Nipagin (mL)	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000

P:C equivalent	Components	Nutrient density (kcal/L)							
		FLYaa ratio				MMaa ratio			
		66.8	111.3	155.8	200.3	66.8	111.3	155.8	200.3
1:0.8	Agar (g)	7.000	7.000	7.000	7.000	7.000	7.000	7.000	7.000
	Isoleucine (g)	0.501	0.835	1.169	1.502	0.814	1.356	1.899	2.441
	Leucine (g)	0.912	1.520	2.128	2.737	0.541	0.902	1.262	1.623
	Tryosine (g)	0.411	0.686	0.960	1.234	0.188	0.313	0.438	0.563
	Sucrose (g)	7.123	11.871	16.620	21.368	7.123	11.871	16.620	21.368
	20 mg/mL absolute EtOH Cholesterol (mL)	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000
	x1000 CaCl2 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	x1000 MgSO4 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	x1000 CuSO4 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	x1000 FeSO4 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	x1000 MnCl2 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	x1000 ZnSO4 (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	MilliQ water (mL)	785.038	742.397	699.756	657.115	788.119	747.532	706.945	666.358
		Autoclave step (sterilisation time 11 minutes at 121°C)							
	x10 Buffer (mL) *	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000
	Nucleic acid and lipid solution (mL) *	8.000	8.000	8.000	8.000	8.000	8.000	8.000	8.000
	Amino acid solutions: Essential amino acid (mL)*	27.062	45.103	63.145	81.186	27.058	45.096	63.134	81.173
	Amino acid solutions: Non-essential amino acid (mL) *	27.062	45.103	63.145	81.186	27.058	45.096	63.134	81.173
	Amino acid solutions: 100 mg/mL Glutamate (mL)	6.788	11.313	15.838	20.364	6.766	11.276	15.786	20.297
	Amino acid solutions: 50 mg/mL Cysteine (mL)	3.050	5.083	7.116	9.149	0.000	0.000	0.000	0.000
	Vitamins (mL)*	21.000	21.000	21.000	21.000	21.000	21.000	21.000	21.000
	Folic acid (mL)*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	Propionic acid (mL)	6.000	6.000	6.000	6.000	6.000	6.000	6.000	6.000
	Nipagin (mL)	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000

*for further recipe see appendix 3.

Appendix 2 continued.

P:C equivalent	Components		Nutrient density (kcal/L)							
			FLYaa ratio				MMaa ratio			
			66.8	111.3	155.8	200.3	66.8	111.3	155.8	200.3
1:0.6	Agar (g)		7.000	7.000	7.000	7.000	7.000	7.000	7.000	7.000
	Isoleucine (g)		0.575	0.959	1.342	1.726	0.935	1.558	2.181	2.804
	Leucine (g)		1.048	1.746	2.445	3.143	0.621	1.036	1.450	1.864
	Tryosine (g)		0.472	0.787	1.102	1.417	0.216	0.359	0.503	0.647
	Sucrose (g)		5.702	9.503	13.304	17.105	5.702	9.503	13.304	17.105
	20 mg/mL absolute EtOH Cholesterol (mL)		15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000
	x1000 CaCl2 (mL)*		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	x1000 MgSO4 (mL)*		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	x1000 CuSO4 (mL)*		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	x1000 FeSO4 (mL)*		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	x1000 MnCl2 (mL)*		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	x1000 ZnSO4 (mL)*		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	MilliQ water (mL)		775.539	726.566	677.592	628.618	779.078	732.463	685.848	639.233
			Autoclave step (sterilisation time 11 minutes at 121°C)							
	x10 Buffer (mL) *		100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000
	Nucleic acid and lipid solution (mL) *		8.000	8.000	8.000	8.000	8.000	8.000	8.000	8.000
	Amino acid solutions	Essential amino acid (mL)*	31.081	51.802	72.523	93.243	31.076	51.793	72.511	93.228
		Non-essential amino acid (mL) *	31.081	51.802	72.523	93.243	31.076	51.793	72.511	93.228
		100 mg/mL Glutamate (mL)	7.796	12.993	18.191	23.388	7.770	12.950	18.131	23.311
		50 mg/mL Cysteine (mL)	3.503	5.838	8.173	10.508	0.000	0.000	0.000	0.000
	Vitamins (mL)*		21.000	21.000	21.000	21.000	21.000	21.000	21.000	21.000
	Folic acid (mL)*		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	Propionic acid (mL)		6.000	6.000	6.000	6.000	6.000	6.000	6.000	6.000
	Nipagin (mL)		15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000

*for further recipe see appendix 3.

Appendix 3. Recipe for stock solutions that were added after the autoclaving step.

Other solutions

Stock solution		Final Volume (mL)	Method
10x Buffer		1000	• 30 mL of acetic acid and 30 g of monopotassium phosphate was added to ~500 mL of milliQ water. • 10 g of sodium bicarbonate was added slowly. • The pH was adjusted to 4 accordingly. • The solution was made up to 1000 mL of milliQ water. • The buffer was autoclaved at 121°C for 11 mins. • Stored at 4°C until use.
20 mg/mL absolute EtOH Cholesterol		50	• 1 g of cholesterol was dissolved in 50 mL of absolute ethanol. • 50 mL was aliquoted and stored at 4°C until use.
Trace elements	$CaCl_2$	1000	• 250 g was dissolved in 1000 mL of milliQ water. • Filter sterilised through 0.20 µm pore size, aliquoted and stored at room temperature.
	$MgSO_4$		• 250 g was dissolved in 1000 mL of milliQ water. • Filter sterilised through 0.20 µm pore size, aliquoted and stored at room temperature.
	$CuSO_4$		• 2.5 g was dissolved in 1000 mL of milliQ water. • Filter sterilised through 0.20 µm pore size, aliquoted and stored at room temperature.
	$FeSO_4$		• 25 g was dissolved in 1000 mL of milliQ water. • Filter sterilised through 0.20 µm pore size, aliquoted and stored at -20°C.
	$MnCl_2$		• 1 g was dissolved in 1000 mL of milliQ water. • Filter sterilised through 0.20 µm pore size, aliquoted and stored at room temperature.
	$ZnSO_4$		• 25 g was dissolved in 1000 mL of milliQ water. • Filter sterilised through 0.20 µm pore size, aliquoted and stored at room temperature.

Appendix 3 continued.

Nucleic acid and lipids	1000	• 6.25 g of choline chloride, 0.63 g of myo-inositol, 8.13 g of inosine and 7.50 g of uridine were dissolved in 1000 mL of milliQ water. • Filter sterilised through 0.20 μm pore size, aliquot and store at 4°C in the dark.
Vitamin	1000	• 0.067 g of thiamine, 0.033 g of riboflavin, 0.399 g of nicotinic acid, 0.516 g of calcium panthothenate, 0.083 g of pyridoxine and 0.007 g of biotin were dissolved in 1000 mL of milliQ water. • Filter sterilised through 0.20 μm pore size, aliquoted and stored at -20 °C in the dark.
Folic acid	1000	• 0.5 g of folic acid was dissolved in milliQ water by dropwise addition of sodium hydroxide (2M). • Filter sterilised through 0.20μm pore size, aliquoted and stored at -20 °C in the dark.

Appendix 3 continued.

Amino acid solutions (added after the media is autoclaved)

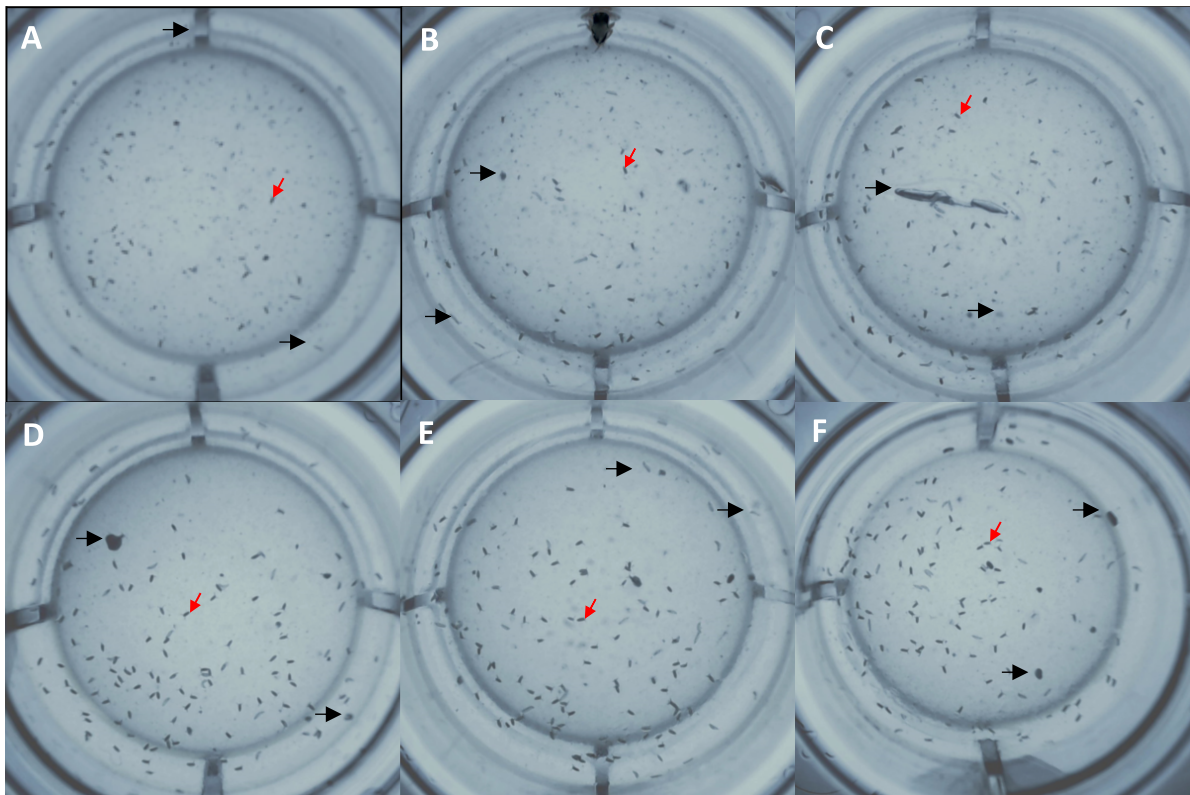
	Amino acid	Amount (g/200 mL)	
		FLYaa ratio	MMaa ratio
Essentials	Phenylalanine	3.33	2.60
	Histidine	2.16	2.00
	Isoleucine*	0.00	0.00
	Lysine	4.51	3.80
	Leucine*	0.00	0.00
	Methionine	1.99	1.60
	Arginine	5.38	1.60
	Threonine	3.65	4.00
	Valine	3.96	5.60
	Tryptophan	1.06	1.00
Non-essentials	Alanine	3.64	7.00
	Cysteine**	0.34	0.10
	Aspartic acid	3.87	3.40
	Glutamic acid***	0.00	0.00
	Glycine	2.53	6.40
	Asparagine	3.40	3.40
	Proline	3.23	3.00
	Glutamine	3.70	5.00
	Serine	4.55	3.80
	Tyrosine*	0.00	0.00

* Isoleucine, leucine and tyrosine are excluded from the solutions as their solubility is too low.

** Added to medium as a separate (10 g/200 mL) solution since cysteine precipitates out over time.

*** Added separately from a glutamic acid stock solution (20 g/200 mL)

Appendix 4. Images of wells used to train the model in QuanitFly. The images selected for the model were taken on experimental day 15 containing the diet of FLYaa ratio. (A-C) wells selected from 37S:37N diet, the lowest nutrient dense diet and (D-F) wells selected from 112S:112N diet, the highest nutrient dense diet. All diets contained the P:C equivalent of 1:3.6. The red arrow highlights an example of an egg in the well. The black arrow highlights examples of non-eggs in the well.



Appendix 5. The macros used for cropping batches of the well images to a size suitable for QuanitFly.

```
//Macro written by Dominic Waithe for Matthew Piper. (c) 2017.
//This macro uses the Hough circular transform to identify the sides of the chamber.
//Once identified the vial the image is down-sampled and cropped.

///Parameters
edge_mag = 70; //Threshold for edge magnitude, the lower this value the more of the edges
will be captured.

////// Start of script.
gtitle =getTitle();
run("Colors...", "foreground=black background=white selection=yellow");
//Batch mode for stopping windows from opening.
run("Bin...", "x=4 y=4 bin=Average");
setBatchMode(false);
run("Select None");
run("Duplicate...", " ");
run("8-bit");
setAutoThreshold("Default dark");
run("Convert to Mask");
run("Create Selection");
close();

run("Duplicate...", " ");
//run("8-bit");
run("Restore Selection");
run("Enlarge...", "enlarge=-40");
run("Crop");
run("Specify...", "width=600 height=600 x=323 y=322 oval centered");
run("Clear Outside");
```

Appendix 5 continued.

```
//run("Find Edges");
run("Crop");
run("Canvas Size...", "width=600 height=600 position=Center");
```